Effect of Olive Leaves or Marigold Petal Extract on Oxidative Stress, Gut Fermentative Activity, and Mucosa Morphology in Broiler Chickens Fed a Diet Rich in n-3 Polyunsaturated Fats

Tatjana Pirman¹, Vida Rezar¹, Milka Vrecl², Janez Salobir¹ and Alenka Levart¹

¹ Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia
² Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

An experiment in broilers was conducted to investigate the effect of olive (Olea europea) leaves and marigold (Calendula officinalis) petal extract supplementation on oxidative stress, characteristics of intestinal contents, and on the morphology of the small intestine. Oxidative stress was induced by a n-3 polyunsaturated fatty acids rich diet. 1-day-old male broiler chickens, Ross 308, were housed in a deep litter system. After the first 21 days, animals were randomly divided into three groups of 16 animals in two replicates and fed, until slaughter on day 39, a diet that contained 7% linseed oil. Control diet (Cont) remained unsupplemented, while both experimental diets were supplemented with olive leaves (OliveEx) or marigold petal (MarigEx) extracts. Oxidative stress was evaluated in blood and liver by measuring markers of lipid peroxidation (malondialdehyde (MDA), isoprostanes), rate of DNA damage in lymphocytes and in blood (comet assay, 8-hydroxy-2’deoxyguanosine (8-OHdG)), and activity of antioxidant and liver enzymes in blood. In different parts of the intestine, levels of short chain fatty acids (SCFA), and viscosity of intestinal contents were measured, and the health of the gastrointestinal tract was assessed using histological measurements. OliveEx significantly \( p<0.05 \) decreased the MDA and 8-OHdG concentration in plasma, and the level of ethanoic acid in small intestinal contents and total SCFA in caecum, indicating improved oxidative status and increased microbial activity in the intestine. MarigEx significantly \( p<0.05 \) decreased the rate of lymphocyte DNA damage and the crypt depth in duodenum, indicating potentially beneficial effects on the immune system and the health of the small intestine. In conclusion, dietary OliveEx and MarigEx supplementation improved some markers of oxidative stress and intestinal health. However, positive effects could be more pronounced in more unfavorable environmental conditions or in cases of diseases, but further studies are needed.

Key words: broiler chicken, intestine morphology, marigold, olive leaves, oxidative stress, short chain fatty acids

J. Poult. Sci., 58: 119–130, 2021

Introduction

Polyunsaturated fatty acids (PUFA) play an important role in human and animal nutrition. The beneficial effects of n-3 PUFA on human health have been documented in numerous studies (Vannice and Rasmussen, 2014; Ludwig et al., 2018; Stupin et al., 2019). Diets enriched in n-3 PUFA are used in poultry to increase their contents in meat and eggs and to produce products with higher nutritive value, i.e. functional foods (Givens and Gibbs, 2008; Fraeye et al., 2012). Unfortunately, this diet is often neglected because of the higher susceptibility of PUFA to lipid oxidation, meaning that such improvement in nutritive value could also have negative consequences. It is well described that supplementing poultry’s diets with more than 5% of high PUFA-containing oils like linseed oil, soybean oil etc., results in the development of oxidative stress, which is reflected in the excessive formation of free radicals and aldehydes, such as malondialdehyde (MDA), isoprostanes, that cause increased DNA damage, altered formation of various antioxidant enzymes etc. (Eder et al., 2005; Lykkesfeldt and Svendsen, 2007; Voljč et al., 2011; Voljč et al., 2013). Consequently, impaired animal health, and reduced productivity and meat quality are observed (Estève, 2015). In such situations, the requirements for antioxidants are increased, therefore, additional antioxi-
dant supplementation should be considered (Leskovec et al., 2019). There is an increased interest in the use of natural plant supplements that are especially rich in various plant phenolic compounds.

Olive (Olea europaea) leaves possess antimicrobial, anti-inflammatory, antithrombotic, antiatherogenic, antihypertensive, and antioxidative action. In traditional medicine, they can be used as a preventive measure for the treatment of cardiovascular diseases, hypertension, inflammation, cancer, and diabetes (Obied et al., 2005). The antioxidant activity of olive polyphenols, e.g. tyrosol, hydroxytyrosol, oleuropein, and pinoresinol is well known (Visioli et al., 2002; Silva et al., 2006; Servili et al., 2009). Furthermore, these compounds have also been acknowledged as antioxidants in humans by the European Food Safety Authority (EFSA) through a health claim, which states that hydroxytyrosol can protect low-density lipoproteins (LDL) from oxidation (EFSA, 2011). The potential of olive polyphenols to reduce oxidative stress and to influence the health of farm animals has not been extensively studied. No studies have been performed on poultry, and the scarce research on pigs shows that olive leaves or extract supplementation has some positive effects on oxidative stress (Paiva-Martins et al., 2014; Leskovec et al., 2019). Additionally, diet supplementation with olive leaves decreased lipid oxidation in n-3 enriched-pork, and reduced bacterial growth in turkey breast fillets during refrigerated storage (Botsoglou et al., 2010; Botsoglou et al., 2012).

Marigold (Calendula officinalis) has been used for centuries in the treatment of different inflammations, as well as medical conditions in the gastrointestinal tract such as gastric and duodenal ulcers, gastrointestinal inflammatory disorders, and wounds (Braun and Cohen, 2015). In addition, marigold might also possess antibacterial, antifungal, antiviral, antimutagenic, hepatoprotective, renoprotective, and free radical scavenging properties (Preethi et al., 2006; Chandran and Kutten, 2008). The antioxidant properties of marigold (Ukiya et al., 2006), originate from its polyphenols and carotenoids (Četković et al., 2003; Miliauskas et al., 2004), and from its essential oils, flavonoids, sterols, carotenoids, tannins, sapo-nins, triterpene alcohols, polysaccharides, a bitter principle, mucilage, and resin (Vidal-Ollivier et al., 1989; Khalil et al., 2007). In vitro studies showed the effective radical scavenging capacity of several differently prepared extracts of marigold flowers (Četković et al., 2003; Miliauskas et al., 2004). This was also confirmed in in vivo studies in rodents, which showed the significant antioxidant activity of marigold extracts, manifested as the reduction of lipid oxidation and free radical formation, and the activity of some antioxidant enzymes (Preethi et al., 2006; Gladine et al., 2007). In animal nutrition, marigold flower extract is known as a natural alternative for increasing the xanthophyll contents and color intensity in eggs and skin of chicks (Pérez-Vendrell et al., 2001; Skrivan et al., 2015; Wang et al., 2017); however, not a lot of interest has been given to the antioxidant potential of marigold in farm animals. One study conducted by our group in pigs showed that marigold extracts, at a level proposed for internal use by traditional medicine, have a promising protective effect against lipid oxidation and DNA damage induced by high dietary PUFA intake (Frankič et al., 2009).

As mentioned, besides antioxidant activity, both plant extracts are known to possess other activities that might be important for animal health and productivity. One of the most relevant is intestinal health, which includes morphological integrity, physiological functions, tissue metabolism, developed barrier functions, efficient immune response, sustained inflammatory balance, and adequate microbiota (Diaz Carrasco et al., 2019). Modern fast-growing chicken breeds’ with high feed intake, imperative for maintaining high productivity, can make the digestive tract vulnerable to impaired functionality, which can be assessed using histological approaches (Svihus, 2014). Both villus height and crypt depth in the small intestine are related to the absorption capacity of nutrients and are important indicators of intestinal health (Yazdani et al., 2013). As the intestine is the major site for lipid absorption, secondary products of lipid oxidation formed during PUFAs oxidation, can induce damage to intestinal epithelial cells, depending on the intestinal segment, which may negatively affect gut morphology (Koniecza et al., 2018). In general, plant extracts are known to interfere with the health of the intestine directly and through an effect on gut microbiota (Diaz Carrasco et al., 2019; Oviedo-Rondón, 2019). To the best of our knowledge, there have been no studies published on the effects of marigold and olive extracts on the health of the intestine. There are studies researching the influence of olive leaves or olive leaves extract-supplemented diets on the nutrient digestibility of the chicken (Leskovec et al., 2018), performance, intestinal, and carcass characteristics (Shafey et al., 2013); however, no data about the effects of olive polyphenols on the conditions in the intestine and health status of broiler chickens exist.

The above-mentioned facts encouraged us to evaluate if olive leaves or marigold petal extract, supplemented primarily as dietary antioxidants to ameliorate the negative effects of oxidative stress induced by high n-3 PUFA intake, might also have effects on the characteristics of intestinal contents, and on the histological parameters of different parts of the intestine.

Materials and Methods

The experiment was performed in the research facility of the Department of Animal Science in Biotechnical Faculty of the University of Ljubljana. The study was conducted in accordance with the principles and specific guidelines presented Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010) and the protocol was approved by the Animal Ethics Committee of the Veterinary Administration of the Republic of Slovenia (U34401-3/2014/8).

Animals and Dietary Treatments

A total of 84 1-day-old male broiler chickens Ross 308 (obtained commercially) were housed in six pens in a deep litter system. Size of the pen was 95 cm (width) × 126 cm
(length) = 11,970 cm², sawdust was used as litter, thickness of the litter was 10–15 cm. Diet and water were provided ad libitum and plastic poultry feeder per each pen was used, with a circumference of 120 cm. Nipple watering system with five nipple drinkers in each pen was installed to water the birds. For the first six days of the experiment, a platter with a diameter 50 cm for feed and a hand water drinker, as well as nipple system, in each pen was applied. Rearing conditions were regulated according to the recommendations for Ross 308 broilers (Aviagen, 2014), the ambient temperature was around 32°C at the beginning (one day old chick), subsequently the temperature in the facility was gradually decreased to 21°C on day 27 and remained constant until the end of experiment. For the first three days, the temperature was also maintained using heat lamps. The level of humidity was never below 50%. The intensity of light was around 40 lux, and the light regime was as follows: first seven days with 23 hours of light and 1 hour of dark; from day 8 till three days before slaughtering, animals had six hours of dark (from 22.00 till 00:00 and from 2:00 a.m. till 6:00 a.m.); and on the last three days two hours of dark, from 22:00 till 00:00. Animals were fed twice per day (the feed was weighted). The leftover feed was weighed once per week, except at the last three days two hours of dark, from 22:00 till 00:00. Animals were fed twice per day (the feed was weighted). The leftover feed was weighed once per week, except at the beginning of the experiment, when animals received weighed feed each day in a platter and the leftover feed was weighed and discarded the next morning.

Chickens were weighed individually each week. Until day 21 of the experiment, the average weight was recorded per group. On day 21, chickens were individually labelled, weighed, and randomly divided into three groups with two replicate pens each comprising 16 birds. Diet consumption per group was recorded weekly.

Chickens were fed wheat and soybean meal-based diets in a mashed form (Table 1), formulated according to broiler nutrition specifications for Ross 308 (Aviagen, 2016). During the first 21 days, the animals were fed starter diet. From day 21 to the end of the experiment on day 39, finisher diets were enriched with 7% of linseed oil and supplemented according to different dietary treatments: Cont, without additional supplementation; OliveEx, supplemented with olive leaves extract; and MarigEx supplemented with marigold petal extract.

Olive leaves and marigold flower leaves were collected locally. Ethanol olive leaves extract and marigold propylene glycol-water extract (1:1) were prepared (Isaac, 1992; Müller and Hildebrand, 1998). The olive leaves extract was supplemented at 6 mL (equivalent to 10 g of olive leaves) per kg of diet and marigold extract at 5 mL (equivalent to 600 mg of fresh flowers/petals) per kg of diet, as proposed for internal use in traditional medicinal literature (Barnes et al., 2002). The preparation and chemical composition of both extracts were described and explained by Leskovec et al. (2018). Olive leaves extract contained 420 ± 20 μmol total phenols/mL (gallic acid equivalents), 70 ± 1.0 nmol carotenoids/mL, 1.48 ± 0.02 μmol chlorophylls/mL, 58.5 ± 4.5 nmol catechin equivalents/mL (flavon-3-ols), 0.79 ± 0.02 nmol rutin equivalents/mL (flavonoids), and extract 53.0 ± 4.2 mg oleuropein/mL. Marigold petal extract contained 2.84 ± 0.03 μmol total polyphenols/mL (gallic acid equivalents), 10.8 ± 1.0 nmol carotenoids/mL, 12.4 ± 0.07 nmol chlorophyll/mL, 0.71 ± 0.11 nmol catechin equivalent/mL (flavon-3-ols), and 12.2 ± 0.3 nmol rutin equivalents/mL (flavonoids).

During the experiment, samples of the diets were taken for proximate analysis, determination of fatty acid composition,

Table 1. Composition of the basal starter and finisher diets

| Composition of diets (g/kg)   | Starter | Finisher |
|-------------------------------|---------|----------|
| Wheat                         | 500     | 570      |
| Soybean meal                  | 380     | 322      |
| Linseed oil                   | 70      | 70       |
| Limestone                     | 16.5    | 13.3     |
| Salt                          | 4.3     | 4.3      |
| Monocalcium phosphate         | 17.6    | 13.4     |
| L-lysine                      | 2.2     | 0.07     |
| DL-methionine                 | 3.3     | 1.9      |
| L-threonine                   | 1.1     | 0.03     |
| Vitamin-mineral mix           | 5       | 5        |

1 Calculated to meet mineral and vitamin requirements for Ross 308 finisher, except vitamin E, and provided per kilogram of the diet: Cu 16 mg, I 1.25 mg, Fe 20 mg, Mn 120 mg, Se 0.3 mg, Zn 100 mg, vitamin A 10,000 IU, vitamin D₃, 5,000 IU, vitamin E 12 IU, vitamin K 3 mg, thiamine (B₁) 2.0 mg, riboflavin (B₂) 6 mg, niacin 57 mg, pantothenic acid 14 mg, pyridoxine (B₆) 3.5 mg, biotin 0.15 mg, folic acid 1.75 mg, vitamin B₁₂ 0.016 mg.

Table 2. Chemical and fatty acid composition and vitamin E content of the diets

| Composition of diets | Starter | Finisher |
|----------------------|---------|----------|
| Chemical composition (g/kg) |         |          |
| Dry matter           | 898     | 892      |
| Crude protein        | 236     | 213      |
| Ether extract        | 79      | 82       |
| Crude fiber          | 41      | 35       |
| Ash                  | 64      | 57       |
| Digestible energy (MJ/kg) | 12.78   | 13.05    |

Main fatty acids (g of fatty acids/100 g of total fatty acids)

| C16:0 | 7.99 | 7.90 |
| C18:0 | 3.27 | 3.23 |
| C18:2 n-6 | 23.83 | 24.08 |
| C18:3 n-3 | 46.32 | 46.51 |
| Σ SFA² | 11.82 | 11.65 |
| Σ MUFA³ | 18.00 | 17.76 |
| Σ PUFA⁴ | 70.18 | 70.59 |
| Σ n-3 PUFA | 46.32 | 46.51 |
| Σ n-6 PUFA | 23.86 | 24.08 |
| n-6 : n-3 PUFA | 0.51 : 1 | 0.52 : 1 |

Vitamin E (mg/kg)

| α - tocopherol | 22.42 | 21.44 |
| γ - tocopherol | 23.25 | 22.96 |

¹ Digestible energy was calculated according to GfE (GfE, 1999). ² Saturated fatty acids. ³ Monounsaturated fatty acids. ⁴ Polyunsaturated fatty acids.
and concentration of vitamin E (Table 2). Proximate analysis was determined using standard procedures (AOAC, 2000). Methyl esters of fatty acids were prepared according to the procedure of Park and Goins (1994). Concentrations of vitamin E in samples of diets were measured according to the methodologies of Abidi and Mounts (1997) and Rupérez et al. (2001) using Agilent 1260 Infinity HPLC.

**Blood, Liver, and Intestinal Tissue Sampling**

At the end of the experiment, 2 mL of blood of the chickens was collected from the wing vein into tubes containing anticoagulant (K2 EDTA) for the determination of DNA fragmentation of blood lymphocytes. A total of 16 animals per group (equal number from each pen) were slaughtered, and blood samples were collected. Blood samples for the purpose of measuring MDA in plasma were collected into K2 EDTA tubes. Plasma was separated by centrifugation (1,000 × g for 15 min at 4°C), transferred into 1.5 mL Eppendorf tubes, and stored at −80°C until analysis. Blood samples for measuring F2-isoprostanes, 8-hydroxy-2’-deoxyguanosine (8-OHdG), and liver enzymes, were collected into tubes with no added anticoagulant. Serum was separated by centrifugation (3,500 × g for 10 min at 4°C), transferred into 1.5 mL Eppendorf tubes and stored at −80°C until analysis, except for liver enzymes, which were analyzed in fresh serum. Whole blood was collected from the wing vein into tubes containing anticoagulant lithium heparin. Whole blood was transferred into 1.5 mL Eppendorf tubes and immediately frozen at −80°C until analysis.

Liver and intestinal tract were removed. Liver was weighed, stored at −80°C and homogenized before analysis. Small intestine was weighed and divided into duodenum, jejunum and ileum. Both caecums were weighed together and colon was weighed. Tissue samples for histological measurements were taken on the following parts of the small intestine: duodenum, the final part of descending duodenum (pars descendens duodeni), before the caudal duodenal flexure (flexura duodeni caudalis); jejunum, middle part of the jejunum; and ileum, part of ileum before its extended part (ampulla ilei). Samples were fixed in 5% buffered formalin solution until the analyses were performed.

The contents of small intestine and caecum were squeezed out by finger pressure, collected in the Eppendorf tubes, and stored at −20°C until an analysis of short chain fatty acid (SCFA) was performed. Viscosity of small intestine contents was determined on the same day using a separate aliquot of small intestinal contents.

**Malondialdehyde (MDA) Determination in Plasma and Liver**

Concentration of MDA in plasma and liver was measured using HPLC, according to the methodology of Wong et al. (1987) modified by Fukunaga et al. (1995), and the quantification of MDA was performed using external standard (TEP, 5-point calibration curve). The procedure for the MDA determination in the liver was already described in Trebušak et al. (2014) and Voljč et al. (2011). Agilent HPLC equipped with a 1260 Infinity FLD fluorescence detector was used. The mobile phase consisted of 50 mmol/L KH2PO4 buffer (pH 6.8) and methanol in a gradient mode. 10 μL aliquot was injected into a reversed-phase C18 HPLC chromatographic column (HyperClone 5u ODS (C18) 120A, 4.6 × 150 mm; Phenomenex Inc., USA). Flow rate of the mobile phase was 1 mL/min and column temperature was set at 25°C.

**Determination of F2-Isoprostanes Level in Serum**

Specific enzyme linked immunosorbent assay (ELISA) kits were used for measuring serum levels of 8-isoprostane (kit iPF2a-VI EIA; Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer’s protocol. Each sample was tested in duplicate. The amount of iPF2a-VI was determined by using competitive ELISA. The reaction between acetylcholinesterase (AChE) and Ellmans reagent was measured at 412 nm with ELISA reader (EL 808, BIO-TEK).

**Lymphocyte Isolation and DNA Integrity Analysis by Comet Assay**

The Comet Assay was performed in line with Singh et al. (1988), with slight modifications as described by Rezar et al. (2003). Lymphocytes were isolated from the fresh blood samples in accordance with a modified procedure described by Singh (1997). An Olympus CH 50 epifluorescent microscope (Olympus, Tokyo, Japan) was used to examine the lymphocyte nuclei (100-W Hg lamp, excitation filter of 480 to 550 nm, and barrier filter of 590 nm). The images were captured using an Andor Luca-R EMCCD charge-coupled device camera and analyzed. The nuclear DNA damage was estimated using Comet 7 computer software (Andor technology). The results are presented as the percentage of DNA in the tail of the comet and as the Olive tail moment (OTM). OTM is calculated as the product of the tail length and the fraction of total DNA in the tail (Olive et al., 1992).

**DNA Integrity Analysis by determination of 8-hydroxy-2’-deoxyguanosine (8-OHdG) Level in Serum**

Serum 8-OHdG level was determined using sensitive competitive ELISA (kit Stress-Xpress, EKS 350; Stressgen, Victoria, Canada) according to the instructions of the manufacturer. Each sample was tested in duplicate. The reaction between horseradish peroxidase (HRP) and tetramethylbenzidine (TMB) was measured at 450 nm with an ELISA reader (EL 808, BIO-TEK, Winooski, VT).

**Determination of Liver Enzymes**

The serum levels of liver enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transpeptidase (GGT), were determined using an automated biochemistry analyzer RX-Daytona (Randox, Crumlin, UK) (Nemec Svete et al., 2012).

**Measurement of Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) Activity**

Activity of GPx in whole blood hemolysates was determined spectrophotometrically with an automated biochemical analyzer RX-Daytona (Randox, Crumlin, UK) using the commercial Ransel kit (Randox Laboratories, Crumlin, UK), which is based on the method of Paglia and Valentine (1967). Blood hemolysates were diluted 41-fold before analyses with Ransel Diluent (Diluting agent, Randox Laboratories, Crumlin, UK).
Activity of GPx was expressed as units per gram of hemoglobin (U/g Hgb). Hemoglobin concentration was determined by the cyanmethemoglobin method using automated hematological analyzer ADVIA 120 (Siemens, Munich, Germany) (Paglia and Valentine, 1967). SOD activity in whole blood hemolysates was determined spectrophotometrically with an automatic biochemical analyzer RX Daytona (Randox Laboratories), using commercially available Ransod kit (Randox Laboratories), which is based on the original method of McCord and Fridovich (1969). Before analyses samples of hemolysates were diluted 1:200 with Ransod Sample Diluent (0.01 mmol/L phosphate buffer, pH 7.0; Randox Laboratories). Activity was expressed as U/g Hgb. Hemoglobin concentration was determined by the cyanmethemoglobin method using automated hematological analyzer ADVIA 120 (Siemens, Munich, Germany) (McCord and Fridovich, 1969).

Viscosity of Small Intestinal Contents
The collected samples of small intestinal contents were centrifuged at 9500 \( \times g \) for 10 minutes. The intestinal viscosity analysis was carried out according to Bedford and Classen (1992).

Short Chain Fatty Acids (SCFA) Analyses
The concentrations of SCFA in the contents of the small intestine and caecum were determined by gas chromatography using the Agilent 6890A GC system equipped with FID detector (Agilent, Santa Clara, CA, USA) and DB-FASTWAX UI capillary column (30 m \( \times 0.25 \text{ mm} \times 0.25 \text{ \mu m} \)) (Agilent). Prior to the injection (2 \( \mu L \) diethyl ether extract, split 10:1), diethyl ether extracts were prepared using the method reported in Holdeman and Moore (1975), with some modifications, described by Pirman et al. (2007).

Histologic Measurements
Parts of the small intestine (duodenum, jejunum and ileum), which were fixed in 5% buffered formalin solution, were embedded in paraffin using a standard procedure. Subsequently, an evenly spaced series of histologic sections (50 \( \mu m \) intersection interval) were cut at 5 \( \mu m \) and stained with hematoxylin and eosin (H&E). Histomorphometric analysis was performed on H&E-stained tissue sections using a Nikon Ni/U light microscope equipped with a DS-Fi1 camera and analyzed with NIS-Elements imaging software, NIS-Elements Basic Research (Nikon instruments Europe B.V., Badhoevedorp, The Netherlands). Villus height was measured from the tip of the villus to the crypt-villus junction and the crypt depth from the crypt-villus junction to the crypt base as illustrated in Fig. 1.

Statistical Analyses
Data were analyzed using the General Linear Models (GLM) procedure of the SAS/STAT module (SAS Institute Inc., Cary, NC, 2002-2010). Least square means (LSM) are shown in the results, the differences being means determined by a Tukey-Kramer multiple comparison test. The dispersion was expressed as the standard error of the mean (SEM). In the statistical model, the fixed effects of diet were included. Statistical significance was considered when \( p < 0.05 \).

Results
Growth Performance
Animals adapted well to the experimental conditions, and no rejection of the diets or health-problems were observed. Upon completion of the study, the average body weight gain from day 21 to slaughter on day 39 did not differ significantly among groups.

Body weight at the end of experiment on day 39 (Table 3) was 2175 g, 2356 g, and 2416 g, in Cont, OliveEx and MarigEx, respectively, indicating that the diet had no significant effect on the weight gain of chickens. Similar results for all three groups were obtained in average feed intake and average feed conversion (g/g), 1.75, 1.57, and 1.63, in Cont, OliveEx and MarigEx, respectively.

Parameters of Oxidative Stress
In the blood plasma, the tail of the comet and OTM in MarigEx group were significantly \( (p < 0.05) \) lower as compared to that of the Cont, while the MDA concentration was significantly \( (p < 0.05) \) lower in the OliveEx group as compared to that of the Cont group. The differences in \( \text{F}_2 \)-isoprostanes, 8-OHdG, and antioxidant enzymes were not significant, due to a rather high variability inside the groups. Biochemical analysis of blood serum showed that AST, ALT, and GGT activities were not different among the groups (Table 4). Only numerical higher value in mass of liver and MDA concentration in the MarigEx group was obtained as compared to the other groups (Table 4).

Fig. 1. A representative histological cross-section of jejunum. Villus height was measured from the tip of the villus to the crypt-villus junction and the crypt depth from the crypt-villus junction to the crypt base. H&E staining.
Intestinal Characteristics and Contents

There were no significant differences in the mass of different parts of the intestine (Table 5) and in viscosity of small intestine contents. The concentration of ethanoic acid in the small intestine contents was significantly \((p < 0.05)\) higher in the OliveEx group as compared to that in Cont. In the caecum, significant \((p < 0.05)\) higher levels of ethanoic and butanoic acid were detected in the OliveEx group (Table 5), and consecutively the sum of SCFA increased in comparison to those in Cont. 2-metilpropanoic, 2-metilbutanoic, and pentanoic acids were also detected, but the levels of each acid were less than 2% in the sum of SCFA and were, therefore, not reported. Relative proportions of SCFA in the caecum were not significantly different among the groups (Table 5).

Histology of the Small Intestine

Our results also showed that none of the supplemented extracts had an effect on the villus height in different parts of the small intestine. However, marigold petal extract significantly \((p < 0.05)\) decreased the crypt depth in the duodenum, but not in the other parts of the small intestine (Fig. 2). The ratio between the height of the villi and depth of the crypt was the highest in the jejunum (10.19, 9.96, and 10.62, in Cont, OliveEx and MarigEx, respectively) and the lowest in the ileum (7.34, 6.94, and 6.60, in Cont, OliveEx and MarigEx, respectively). In the duodenum, the ratios were 8.13, 8.46, and 9.31, in Cont, OliveEx and MarigEx, respectively, however, differences among the groups in the

Table 3. Performance of broilers fed different dietary lipid and vitamin E sources, and their amounts from day 21 to 35

| Performance          | Cont   | OliveEx | MarigEx | SEM   | p-value |
|----------------------|--------|---------|---------|-------|---------|
| Body weight 21st day (g) | 889    | 952     | 992     | 33.3  | 0.111   |
| Body weight 28th day (g)  | 1456   | 1584    | 1600    | 63.8  | 0.237   |
| Body weight 35th day (g)   | 2121   | 2285    | 2362    | 80.7  | 0.119   |
| Body weight 39th day (g)    | 2175   | 2356    | 2416    | 80.3  | 0.101   |
| Feed consumption (g/g) *1  | 3480   | 3486    | 3710    |       |         |
| Feed conversion (g/g) *1   | 1.75   | 1.57    | 1.63    |       |         |

*1 Calculated as feed consumption/growth rate. * Average value in group, since animals were group housed and individually fed, consumption data could not be obtained.

Table 4. Parameters of oxidative stress measured in blood and liver

| Blood parameters                  | Cont   | OliveEx | MarigEx | SEM   | p-value |
|-----------------------------------|--------|---------|---------|-------|---------|
| DNA damage                         |        |         |         |       |         |
| Comet assay                        |        |         |         |       |         |
| - Tail DNA (%)                    | 17.46a | 16.17ab | 13.18b  | 1.02  | 0.022   |
| - OTM1                            | 7.99a  | 6.46ab  | 4.07b   | 0.91  | 0.022   |
| 8-OHdG2 (ng/mL)                   | 24.74  | 40.60   | 39.03   | 9.79  | 0.446   |
| Lipid oxidation                   |        |         |         |       |         |
| MDA3 (nmol/mL)                    | 0.72a  | 0.54b   | 0.59ab  | 0.04  | 0.007   |
| F2-isoprostanes (pg/mL)           | 48.58  | 35.98   | 43.90   | 9.61  | 0.665   |
| Antioxidant enzymes               |        |         |         |       |         |
| SOD4 (U/g of haemoglobin)         | 1182   | 1164    | 1230    | 78    | 0.831   |
| GPx5 (U/g of haemoglobin)         | 486    | 501     | 477     | 17    | 0.586   |
| Liver                             |        |         |         |       |         |
| Mass (g)                          | 34.9   | 36.1    | 38.6    | 1.41  | 0.184   |
| % of body mass                    | 1.60   | 1.53    | 1.60    | 0.03  | 0.140   |
| MDA (nmol/g)                      | 1.48   | 1.59    | 1.62    | 0.11  | 0.670   |
| Liver enzymes                     |        |         |         |       |         |
| - AST6 (U/L)                      | 304.5  | 349.2   | 338.8   | 16.94 | 0.165   |
| - ALT7 (U/L)                      | 2.44   | 2.50    | 2.30    | 0.26  | 0.855   |
| - GGT8 (U/L)                      | 29.37  | 24.87   | 26.86   | 1.62  | 0.189   |

1 OTM=Olive Tail Moment. 2 8-OHdG=8-hydroxy-2’deoxyguanosine. 3 MDA=malondialdehyde. 4 SOD=superoxide dismutase. 5 GPx=glutathione peroxidase. 6 ALT=alanine aminotransferase. 7 AST=aspartate aminotransferase. 8 GGT=gamma glutamyl transpeptidase. Different small letters within a line show significant differences at \(p<0.05\).
same part of the small intestine were not significantly different.

**Discussion**

Dietary oxidative stress causes an imbalance in the physiological status of animals, which leads to a general reduction in efficiency of the antioxidant network defense system that results in impaired health and reduced performance. Plant extracts contain polyphenols and other substances that exert antioxidative and other health benefits such as antibacterial, antiviral, anti-inflammatory, and anticancer effects (Duthie et al., 2000; Surai et al., 2014). Although olive and marigold extracts have some known beneficial effects to human health, only a few trials have been conducted in farm animals.

In the presented experiment, the effect of supplementation with both plant extracts was measured under controlled conditions in which dietary oxidative stress was induced by high PUFA intake. All diets were prepared with 7% linseed oil, which contains over 65% PUFA, and is known to increase dietary oxidative stress, which was confirmed in our previous studies on chickens (Voljč et al., 2011; Voljč et al., 2013), pigs (Leskovec et al., 2019) and rabbits (Trebušak et al., 2014). It is known that a high intake of PUFA increases the nutritive requirements for antioxidative vitamins (Raederstorff et al., 2015). Thus, dietary oxidative stress in those experiments was additionally increased by the fact that the supply of supplemented vitamin E was at the minimal requirement level recommended by NRC (1994).

The results of growth performance showed no effects in both extracts. Since the number of animals in our experiment was sufficient only to closely examine the physiological effects, the importance of growth performance result should not be overestimated. Additionally, two larger growth trials in broilers with marigold extract supplementation resulted in no effect or in improved growth performance (Rajput et al., 2012; Wang et al., 2017). This might mean that, at least, no negative effect on animal performance is expected.

To assess the oxidative status of the birds, different markers were analyzed in blood plasma and in the liver. The results indicated that the effects of both extracts are different and that both supplements were only partially able to prevent the consequences of dietary oxidative stress. As in the study of Frankič et al. (2009) in growing pigs, marigold extract was not efficient in reducing the extent of PUFA oxidation and thus inhibiting plasma MDA and F₂-isoprostane formation. However, it exhibited a protective effect on the DNA of lymphocytes, as the percentage of fragmented DNA in the tail of the comet and Olive tail moment were lower in comparison to non-supplemented group. Interestingly, the level of 8-OHdG in plasma, which is influenced by both, the extent of DNA damage, and the rate of DNA repair, was not decreased in marigold supplemented chickens. The same effect has previously been observed also in growing pigs supplemented with marigold petals or flower tops extracts, where the authors concluded that the amount of marigold extracts proposed for internal use by traditional medicine protects the organism against DNA damage induced by high PUFA intake (Frankič et al., 2009). Genetic damage can
due to significantly showed a different picture. A reduction in lipid oxidation was observed, and a numerical reduced concentration of F2-isoprostanes that are markers of arachidonic acid peroxidation. A similar outcome was reported by Oke et al. (2017) researching the supplementation of olive leaves extract in the drinking water as a measure for alleviation of adverse effects of heat stress in broilers. On the other hand, the potential of olive leaves extract to reduce lipid oxidation and MDA levels in plasma was not observed in growing pigs (Leskovec et al., 2018) and laying hens (Rezar et al., 2015) where oxidative stress was also induced by high dietary PUFA. The markers of DNA integrity showed that the rate of damaged DNA presented as % of tail DNA was only numerically reduced, but the level of plasmatic 8-OHdG was significantly (p<0.05) reduced. This might be a function of lower DNA fragmentation or reduced rate of DNA repair.

An influence of both extracts on the activity of antioxidative enzymes GPx and SOD was not observed. This is in accordance with our previous results with marigold and olive leaves extracts in pigs (Frankič et al., 2009; Leskovec et al., 2019), and in contrast to the results of Liu et al. (2014) who observed a support of antioxidant protection by increased activity of endogenous antioxidant enzymes such as GPx, SOD, and catalase, as a result of resveratrol supplementation in black-boned chickens.

Another aim of this study was to investigate the effects of both supplements on the characteristics of intestinal contents and on the histological parameters of different parts of the intestine. At least in healthy birds, viscosity of the intestinal contents depends largely on the fiber content and ratio among soluble and insoluble fiber in the diet (Chocht et al., 1999). Moreover, it has an influence on the digestibility of nutrients, not only directly, but also through the changes in the activity of the host-associated microbiota. High viscosity in the small intestine reduces the speed and the amount of nutrients digested with endogenous enzymes, delivering more substrate for microbiota. Viscosity reduction by non-starch polysaccharide enzyme supplementation has been shown in the past to influence the intestinal fermentation along the gut by reducing fermentation in the small intestine and increasing it in the caeca (Chocht et al., 1996; Chocht et al., 1999). In our study, viscosity of small intestinal contents did not change with either supplementation of OliveEx or MarigEx. This suggests that the fermentation in all three groups was similar, with mostly enzymatic degradation of carbohydrates before microbial degradation. Moreover, since extracts, which do not contain significant amounts of fibers, and not plants were used for supplementation, no direct effects regarding changes in the viscosity of the intestinal contents were anticipated.

SCFA are among the key products of microbial fermentation in the gut and include butanoic, ethanoic, lactic, propanoic, valeric, and iso-valeric acids (Jamroz et al., 2002). SCFA have specific roles in the gastrointestinal tract, namely modulation of the gut microflora, stimulation of the gut epithelial cell proliferation and differentiation, thus increasing the gastrointestinal tract absorptive surface area. Moreover, acetate and propionate also act as an energy substrate for tissues (Yadav and Jha, 2019).

In the present study, the ethanoic acid concentration in the small intestine contents was higher in OliveEx group in comparison to MarigEx and Cont groups. Ethanoic acid was the major acid in all groups, and it is considered the main product of the fermentation by the heterofermentative bacteria in the intestine (Immersell et al., 2003). The levels of other analyzed SCFA in the small intestine were very small and variable (data not presented). In chickens, the caecum is the most important site of fermentation in the gastrointestinal tract, where unabsorbed fermentable material (mainly carbohydrates), delivered from the upper digestive tract, are
transformed into SCFA and gasses (Jamroz et al., 2002). In our study, the levels of propanoic acid, which has been suggested as the end product of carbohydrate fermentation (Rodriguez-Colinas et al., 2013), was at a similar level in all groups and comparable to Masey-O’Neill et al. (2014). Supplements increased the SCFA levels in the small intestinal and caecum contents, especially ethanoic acid and consequently total SCFA levels (Table 5), but only ethanoic acid and total SCFA levels in the OliveEx group were significantly ($p<0.05$) higher in comparison to the Cont group. The ratio among acids in caecum increased from $9.4 : 1 : 3.2$ to $10.6 : 1 : 3.5$ to $11.3 : 1 : 4.0$ in Cont, OliveEx, and MarigEx, respectively. The increase in SCFA levels and shift in proportion of SCFA may be explained by modulation of the intestinal microbial community structure, induced by supplemented plant extracts. The specific mechanisms by which plant extracts modulate intestinal microbial community have not been entirely elucidated. Recent studies indicate that plant extracts have the potential to alter the diversity and function of intestinal microbiota, through their antimicrobial action against pathogens (Diaz Carrasco et al., 2016), or through the improvement of microbial metabolic function, including protein digestion and absorption, amino acid metabolism, and lipid biosynthesis (Zhu et al., 2019). Additionally, plant extracts can have an effect on the digestibility of nutrients (Leskovec et al., 2018) thus altering the composition of substrate, which is later utilized by intestinal microorganisms. The availability of different substrates could enable the development of microbiota suitable for the production of higher-energy metabolites required by birds (Lei et al., 2012).

There were no differences in masses of the small intestine, caecum, and colon among the groups. In the study by Shafey et al. (2013), the mass of ileum was significantly ($p<0.05$) increased by the higher levels of olive leaves in a diet, but not that of duodenum and jejunum, which was attributed to higher fiber content in the supplemented diets. Duodenal histology measurements were performed to monitor the effects of olive leaves or marigold petal extract on villus height or crypt depth (Nousiainen, 1991). Our results showed no significant differences in the villi height in duodenum, nor in other parts of small intestine between the groups, which is in accordance with the results of Leskovec et al. (2018), which showed that high concentration of n-3 fatty acids or olive leaves and marigold petal extracts does not have an important influence on nutrient utilization. However, crypt depth was significantly ($p<0.05$) reduced in MarigEx group as compared to that in the Cont. A deeper crypt indicates increased turnover of enterocytes. Shallow crypt in MarigEx can be indication of protection of the gut against harmful compounds produced by microflora (Hampson, 1986) and metabolites produced by Lactobacillus plantarum and against tannin (Levkut et al., 2019). The increase in villus height of different segments of the small intestine may also be attributed to the role of the intestine epithelium as a natural barrier against pathogenic bacteria and toxic substances that are present in the intestinal lumen (Paul et al., 2007).

The results of this study show that in broilers fed high PUFA diets, supplementation with olive leaves extract has an effect on the MDA and 8-OHdG concentration in plasma, and on the level of SCFA in intestine, indicating improved oxidative status and an increased microbial activity in the intestine. Marigold petal extract reduced lymphocyte DNA damage and decreased the crypt depth in duodenum, indicating potentially beneficial effects on the immune system and on the health of the small intestine.

Plant extracts, used in the experiments, exerted limited beneficial effects regarding the amelioration of oxidative stress and the health status of the gastrointestinal tract. The observed effects could be of more importance in different, more unfavorable environmental conditions (heat stress, poor management, toxins in feed) or during impaired health status (infections, diseases, etc.). In order to fully elucidate the potential beneficial effects of olive leaves and marigold petal extract in broiler diets, further studies under various conditions are recommended.

Acknowledgments

This study was financially supported by the Slovenian Research Agency, grant number P4-0097.

Conflict of Interest

The authors declare no conflict of interest.

References

Abidi SL and Mounts TL. Reversed-phase high-performance liquid chromatographic separations of tocopherols. Journal of Chromatography A, 782: 25–32. 1997.

AOAC. Official methods of Analysis of AOAC International. In: AOAC International (Horwith W ed.) AOAC: Gaithersburg, MD. USA. 2000.

Aviagen. ROSS 308 broiler: Management Handbook. http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf. 2014. Accessed on September 18, 2014.

Aviagen. ROSS 308 broiler: Nutrition Specifications. http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross308BroilerNutritionSpecs2014-EN.pdf. 2016.

Barnes J, Anderson LA and Phillipson JD. Herbal Medicines. 2nd ed. Pharmaceutical Press. London. 2002.

Barnett YA and Barnett CR. DNA damage and mutation: contributors to the age-related alterations in T cell-mediated immune responses? Mechanisms of Ageing and Development, 102: 165–176. 1998.

Bedford MR and Classen HL. Reduction in intestinal viscosity through manipulation of dietary rye and pentosanose concentration is effected through changes in the carbohydrate: composition in the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. Journal of Nutrition, 122: 560–569. 1992.

Botsoglou E, Govaris A, Christaki E and Botsoglou N. Effect of dietary olive leaves and/or α-tocopherol acetate supplementa-
tion on microbial growth and lipid oxidation of turkey breast fillets during refrigerated storage. Food Chemistry, 121: 17–22. 2010.

Braun L and Cohen M. Herbs and natural supplements. 3rd ed. Churchill Livingstone-Elsevier. Australia. 2015.

Chandran PK and Kuttan R. Effect of *Calendula officinalis* flower extract on acute phase proteins, antioxidant defence mechanism and granuloma formation during thermal burns. Journal of Clinical and Biochemical Nutrition, 43: 58–64. 2008.

Choc M, Hughes RJ and Bedford MR. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chicken fed wheat. British Poultry Science, 40: 419–422. 1999.

Choc M, Hughes RJ, Wang J, Bedford MR, Morgan AJ and Annison G. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. British Poultry Science, 37: 609–621. 1996.

Četkovič SG, Dilas SM, Čanadanović-Brunet JM and Tumbas VT. Thin-layer chromatography analysis and scavenging activity of marigold (*Calendula officinalis L.*) extracts. Acta Periodica Technologica, 34: 93–102. 2003.

Diaz Carrasco JM, Casanova NA and Fernández Miyakawa ME. Microbiota, gut health and chicken productivity: What is the connection? Microorganisms, 7, 374: doi: 10.3390/microorganisms 7100374. 2019.

Diaz Carrasco JM, Redondo LM, Redondo EA, Dominguez JE, Chacana AP and Fernández Miyakawa ME. Use of plant extract as an effective matter to control clostridium perfringens induced necrotic enteritis in poultry. Biomedical Research International, article ID 3278359, 15 p, 2016.

Duthie GG, Duthie SJ and Kyle JA. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutrition Research Reviews, 13: 79–106. 2000.

Eder K, Grünthal G, Kluge H, Hırche F, Spilke J and Brandsch C. Digestibility and energy value of non-starch polysaccharides in young chickens, ducks and geese, fed diets containing high amounts of barley. Comparative Biochemistry and Physiology, Part A, 131: 357–668. 2002.

Hampson DJ. Alterations in piglet small intestinal structure at weaning. Research in Veterinary Science, 40: 32–40. 1986.

Holdeman LV and Moore WEC. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University. University of Michigan. 1975.

Immervell VF, de Buck J, Pasmans F, Veelg P, Bottreau E, Fievez V, Hæsebrouck F und Ducatelatte R. Invasion of *Salmonella enteritidis* in avian intestinal epithelia l cells in vitro is influenced by short-chain fatty acids. International Journal of Food Microbiology, 85: 237–248. 2003.

Isaac O. Die Ringelblume: Botanic, Chemie, Pharmakologie, Toxikologie, Pharmazeutische und therapeutische Verwendung; Handbuch für Ärzte, Apotheker und andere Naturwissenschaftler. Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, Stuttgart. 1992.

Jamroz D, Jakobsen K, Knudsen KEB, Wilczekiewicz A and Orda J. Digestibility and energy value of non-starch polysaccharides in young chickens, ducks and geese, fed diets containing high amounts of barley. Comparative Biochemistry and Physiology, Part A, 131: 357–668. 2002.

Khalil MY, Moustafa AA and Naguib NY. Growth, phenolic compounds and antioxidant activity of some medicinal plants grown under organic farming conditions. World Journal of Agricultural Sciences, 3: 451–457. 2007.

Konieczka P, Barszcz M, Choct M and Smulikowska S. The interactive effect of dietary n-6: n-3 fatty acid ratio and vitamin E on tissue lipid peroxidation, DNA damage in intestinal epithelial cells, and gut morphology in chickens of different ages. Poultry Science, 97: 149–158. 2018.

Lei F, Yin Y, Wang Y, Deng B, Yu HD, Li L, Xiang C., Wang S, Zhu B and Wang X. Higher-level production of volatile fatty acids in vitro by chicken gut microbiotas than by human gut microorganisms as determined by functional analyses. Applied and Environmental Microbiology, 78: 5763–5772. 2012.

Leskovec J, Levart A, Žgur S, Jordan D, Pirman T, Salobir J and Rezar V. Effects of olive leaf and marigold extracts on the utilization of nutrients and on bone mineralization using two different oil sources in broilers. Journal of Poultry Science, 55: 17–27. 2018.

Leskovec J, Rezar V, Nemec Svete A, Salobir J and Levart A. Antioxidative effects of olive polyphenols compared to vitamin E in piglets fed a diet rich in n-3 PUFA. Animals, 9, 161: 1–11. 2019.

Lekvot MR, Revajová V, Levkutová M, Selecká E, Ševčíková Z, Karaffová V and Levkut MR. The influence of chestnut wood and flubendazole on morphology of small intestine and lymphocytes of peripheral blood, spleen and jejunum in broiler chickens. Helminthologia, 56: 273–281. 2019.

Liu LL, He JH, Xie HB, Yang YS, Li JC and Zou Y. Resveratrol induces antioxidant and heat shock protein mRNA expression
in response to heat stress in black-boned chickens. Poultry Science, 93: 54–62. 2014.

Ludwig DS, Willett WC, Volek JS and Neuhausler ML. Dietary fat: From foe to friend? Science, 362: 764–770. 2018.

Lykkesfeldt J and Svendsen O. Oxidants and antioxidants in disease: Oxidative stress in farm animals. Veterinary Journal, 173: 502–511. 2007.

Masey-O’Neill HV, Singh M and Cowieson AJ. Effects of exogenous xylanase on performance, nutrient digestibility, volatile fatty acids production and digestive tract thermal profiles of broilers fed on wheat- or maize-based diet. British Poultry Science, 55: 351–359. 2014.

McCord JM and Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry, 244, 22: 6049–6055. 1969.

Miliauskas G, Venskutonis PR and van Beek TA. Screening of radiant enzyme variables in horse blood sampled in a slaughterhouse and Patureau Mirand P. Dietary pectin stimulates protein metabolism in the digestive tract. Nutrition, 23, 1: 69–75. 2007.

Preethi KC, Kuttan G and Kuttan R. Antioxidant potential of an extract of Calendula officinalis flowers in vitro and in vivo. Pharmaceutical Biology, 44: 691–697. 2006.

Raeerdorff D, Wyss A, Calder PC, Weber P and Eggersdorfer M. Vitamin E function and requirements in relation to PUFA. British Journal of Nutrition, 114: 1113–1122. 2015.

Rajput N, Naem M, Ali S, Rui Y and Tian W. Effect of dietary supplementation of marigold pigment on immunity, skin and meat color, and growth performance of broiler chickens. Brazilian Journal of Poultry Science, 14: 233–304. 2012.

Rezar V, Pajt K, Marinsek Logar R, Ješe Janežic V, Salobir K, Orešnik A and Salobir J. Wheat bran and oat bran effectively reduce oxidative stress induced by high-fat diets in pigs. Annals Nutrition and Metabolism, 47: 78–84. 2003.

Rezar V, Levart A and Salobir J. The effect of olive by products and their extracts on antioxidative status of laying hens and oxidative stability of eggs enriched with n-3 fatty acids. Poljoprivreda, 21: 216–219. 2015.

Rodríguez-Collinas B, Kolda S, Baran M, Ballesteros AO, Rastall RA and Plou FJ. Analysis of fermentation selectivity of purified galacto-oligosaccharides by in vitro human faecal fermentation. Applied Microbiology and Biotechnology, 97, 5743–5752. 2013.

Rupérez FJ, Martin D, Herrera E and Barbas C. Chromatographic analysis of alpha-tocopherol and related compounds in various matrices. Journal of Chromatography A, 935: 45–69. 2001.

Servili M, Esposto S, Fabiani R, Urbani S, Taticchi A, Mariucci F, Selvaggini R and Montedoro GF. Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. Inflammopharmacology, 17: 76–84. 2009.

Shafey TM, Almufarrij SI and Abdulaziz Albatsah H. Effect of feeding olive leaves on the performance, intestinal and carcass characteristics of broiler chickens. International Journal of Agriculture and Biology, 15: 585–589. 2013.

Silva S, Gomes L, Leitao F, Coelho AV and Vilas Boas L. Phenolic compounds and antioxidant activity of Olea europaea L. fruits and leaves. Food Science and Technology International, 12: 385–395. 2006.

Singh NP, McCoy MT, Tice RR and Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 198: 259–267. 1992.

Oviedo-Rondón EO. Holistic view of intestinal health in poultry. Animal Feed Science and Technology, 250: 1–8. 2019.

Paglia DE and Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine, 70: 158–169. 1967.

Paiva-Martins F, Barbosa S, Silva M, Monteiro D, Pinheiro V, Mourão JL, Fernandes J, Rocha S, Belo L and Santos-Silva A. The effect of olive leaf supplementation on the constituents of blood and oxidative stability of red blood cells. Journal of Functional Foods, 9: 271–279. 2014.

Park PW and Goins RE. In situ preparation offatty acid methyl esters for analysis of fatty acid composition in foods. Journal of Food Science, 59: 1262–1266. 1994.

Paul SK, Halder G, Mondal MK and Samanta G. Effect of organic acid salt on the performance and gut health of broiler chicken. Journal of Poultry Science, 44: 389–395. 2007.

Pérez-Vendrell AM, Hernández JM, Llaudaro L, Schierle J and Bruñual J. Influence of source and ratio of xanthophyll pigments on broiler chicken pigmentation and performance. Poultry Science, 80: 320–326. 2001.

Pirman T, Ribeyre MC, Mosoni L, Remond D, Vrecl M, Salobir J and Patureau Mirand P. Dietary pectin stimulates protein metabolism in the digestive tract. Nutrition, 23, 1: 69–75. 2007.

Preethi KC, Kuttan G and Kuttan R. Antioxidant potential of an extract of Calendula officinalis flowers in vitro and in vivo. Pharmaceutical Biology, 44: 691–697. 2006.

Raeerdorff D, Wyss A, Calder PC, Weber P and Eggersdorfer M. Vitamin E function and requirements in relation to PUFA. British Journal of Nutrition, 114: 1113–1122. 2015.

Rajput N, Naem M, Ali S, Rui Y and Tian W. Effect of dietary supplementation of marigold pigment on immunity, skin and meat color, and growth performance of broiler chickens. Brazilian Journal of Poultry Science, 14: 233–304. 2012.

Rezar V, Pajt K, Marinsek Logar R, Ješe Janežic V, Salobir K, Orešnik A and Salobir J. Wheat bran and oat bran effectively reduce oxidative stress induced by high-fat diets in pigs. Annals Nutrition and Metabolism, 47: 78–84. 2003.

Rezar V, Levart A and Salobir J. The effect of olive by products and their extracts on antioxidative status of laying hens and oxidative stability of eggs enriched with n-3 fatty acids. Poljoprivreda, 21: 216–219. 2015.
there an interaction of exercise and dietary n-3 PUFA intake? 
Frontiers in Physiology, 1129: 1–19. 2019.
Surai PF. Polyphenol compounds in the chicken/animal diet: From 
the past to the future. Journal of Animal Physiology and Ani-
mal Nutrition, 98: 19–31. 2014.
Svilhus B. Function of the digestive system. Journal of Applied 
Poultry Research, 23: 306–314. 2014.
Trebušak T, Levart A, Frankič T and Pirman T. Effect of dietary 
linseed oil and Ganoderma lucidum or olive leaves supplementa-
tion on fatty acid composition and oxidative status of rabbits. 
World Rabbit Science, 22: 71–81. 2014.
Ukiya M, Akihisa T, Yasukawa K, Tokuda H, Suzuki T and Kimura 
Y. Anti-inflammatory, anti-tumor-promoting, and cytotoxic ac-
tivities of constituents of marigold (Calendula officinalis) 
flowers. Journal of Natural Products, 69: 1692–1696. 2006.
Vannice G and Rasmussen H. Position of the academy of nutrition 
and dietetics: dietary fatty acids for healthy adults. Journal of 
the Academy of Nutrition and Dietetics, 114: 136–153. 2014.
Vidal-Ollivier E, Elias R, Faure F, Babadjiamian A, Crespin F, 
Balansard G and Boudon G. Flavonal glycosides from Calen-
dula officinalis flowers. Planta Medica, 55: 73–74. 1989.
Visioli F, Poli A and Gall C. Antioxidant and other biological 
activities of phenols from olives and olive oil. Medical Re-
search Reviews, 22: 65–75. 2002.
Voljč M, Frankič T, Levart A, Nemec M and Salobir J. Evaluation of 
different vitamin E recommendations and bioactivity of α-
tocopherol isomers in broiler nutrition by measuring oxidative 
stress in vivo and the oxidative stability of meat. Poultry Sci-
ence, 90: 1478–1488. 2011.
Voljč M, Levart A, Žgur S and Salobir J. The effect of α-
tocopherol, sweet chestnut wood extract and their combination 
on oxidative stress in vivo and oxidative stability of meat in 
broilers. British Poultry Science, 54: 144–156. 2013.
Yadav S and Jha R. 2019. Strategies to modulate the intestinal 
microbiota and their effects on nutrient utilization, perform-
ance, and health of poultry. Journal of Animal Science and 
Biotechnology, 10, 2: https://doi.org/10.1186/s40104-018-0310-
9. 2019.
Yazdani A, Poorbaghi SL, Habibi H, Nazifi S, Rahmani F and 
Sepehrimanesh M. Dietary Berberis vulgaris extract enhances 
intestinal mucosa morphology in the broiler chicken (Gallus gallus). Comparative Clinical Pathology, 22: 611–615. 2013.
Wang S, Zhang L, Li J, Cong J, Gao F and Zhou G. Effects of 
dietary marigold extract supplementation on growth performance, 
pigmentation, antioxidant capacity and meat quality in 
broiler chickens. Asian-Australasian Journal of Animal Sci-
ence, 30: 71–77. 2017.
Wong SH, Knight JA, Hopfer SM, Zaharia O, Leach CN and 
Sunderman FW. Lipoperoxides in plasma as measured by liquid-
chromatographic separation of malondialdehyde-thiobarbituric 
acid adduct. Clinical Chemistry, 33: 214–220. 1987.
Zhu N, Wang J, Yu L, Zhang Q, Chen K and Liu B. Modulation of 
growth performance and intestinal microbiota in chickens fed 
plant extracts or virginiamycin. Frontiers in Microbiology, 10, 
1333: 1–16. 2019.