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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

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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

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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 (Estévez, 2015). In such situations, the requirements for antioxidants are increased, therefore, additional antioxi-

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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, saponins, 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 PUFA oxidation, can induce damage to intestinal epithelial cells, depending on the intestinal segment, which may negatively affect gut morphology (Konieczka *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 cm[2], 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 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℃ at the beginning (one day old chick), subsequently the temperature in the facility was gradually decreased to 21℃ 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 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 polyphenols/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 μmol 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[1]	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 D_3, 5,000 IU, vitamin E 12 IU, vitamin K 3 mg, thiamine (B_1) 2.0 mg, riboflavin (B_2) 6 mg, niacin 57 mg, pantothenic acid 14 mg, pyridoxine (B_6) 3.5 mg, biotin 0.15 mg, folic acid 1.75 mg, vitamin B_1 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)[1]	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 SFA[2]	11.82	11.65
Σ MUFA[3]	18.00	17.76
Σ PUFA[4]	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℃), transferred into 1.5 mL Eppendorf tubes, and stored at −80℃ until analysis. Blood samples for measuring F_2-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℃), transferred into 1.5 mL Eppendorf tubes and stored at −80℃ until analysis, except for liver enzymes, which were analyzed in fresh serum. Whole blood was collected for analysis of glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities. Blood samples were collected into tubes containing anticoagulant lithium heparin. Whole blood was transferred into 1.5 mL Eppendorf tubes and immediately frozen at −80℃ until analysis.

Liver and intestinal tract were removed. Liver was weighed, stored at −80℃ 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℃ 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 KH_2PO_4 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 (C_{18}) 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℃.

Determination of F_2-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 Intestine 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 ($30\,m \times 0.25\,mm \times 0.25\,\mu m$) (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 to the crypt-villus junction and the crypt depth measured 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 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 $p < 0.05$.

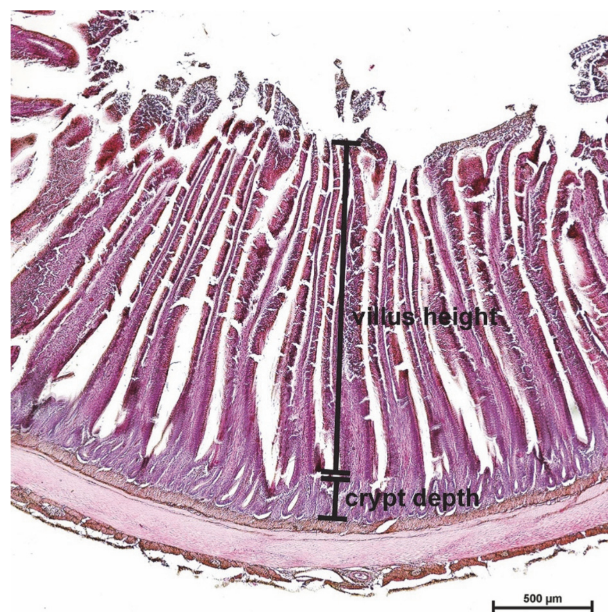

Fig. 1. **A representative histological cross-section of jejunum.** Villus height was measured from the tip of the villus to the crypt-villus junction and the crypt depth from the crypt-villus junction to the crypt base. H&E staining. - change the sentence.

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 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).

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) *	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**

	Cont	OliveEx	MarigEx	SEM	p-value
Blood parameters					
DNA damage					
Comet assay					
- Tail DNA (%)	17.46[a]	16.17[ab]	13.18[b]	1.02	0.022
- OTM[1]	7.99[a]	6.46[ab]	4.07[b]	0.91	0.022
8-OHdG[2] (ng/mL)	24.74	40.60	39.03	9.79	0.446
Lipid oxidation					
MDA[3] (nmol/mL)	0.72[a]	0.54[b]	0.59[ab]	0.04	0.007
F_2-isoprostanes (pg/mL)	48.58	35.98	43.90	9.61	0.665
Antioxidant enzymes					
SOD[4] (U/g of haemoglobin)	1182	1164	1230	78	0.831
GPx[5] (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					
- AST[6] (U/L)	304.5	349.2	338.8	16.94	0.165
- ALT[7] (U/L)	2.44	2.50	2.30	0.26	0.855
- GGT[8] (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 $p<0.05$.

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 5. **Mass of different parts of intestine, viscosity of small intestine contents, and concentration of short chain fatty acid (SCFA) in contents of small intestine and caecum**

	Cont	OliveEx	MarigEx	SEM	p-value
Mass (g)					
Small intestine	44.79	49.54	48.35	1.88	0.195
Caecum	8.85	9.41	9.85	0.61	0.517
Colon	2.96	2.94	3.52	0.16	0.054
Viscosity (cP)					
Small intestine contents	2.37	2.44	2.94	0.21	0.145
SCFA in small intestine (μmol/g)					
Ethanoic acid	1.45[b]	4.09[a]	1.86[b]	0.75	0.041
SCFA in caecum (μmol/g)					
Ethanoic acid	45.03[b]	67.03[a]	53.76[ab]	5.47	0.026
Propanoic acid	3.87	5.12	3.86	0.50	0.139
2-methylpropanoic acid	0.65	0.65	0.59	0.07	0.784
Butanoic acid	9.47[b]	12.93[a]	9.27[b]	1.12	0.046
3-methylbutanoic acid	0.84	1.03	0.91	0.08	0.089
Pentanoic acid	1.03	0.91	0.84	0.08	0.274
Sum of SCFA	60.65[b]	87.24[a]	69.09[ab]	6.68	0.025
Proportion of SCFA in caecum (%)					
Ethanoic acid	73.97	76.15	77.66	1.11	0.077
Propanoic acid	6.25	5.85	5.58	0.35	0.399
Butanoic acid	15.51	15.39	13.42	1.01	0.277

SCFA=short chain fatty acid. Different small letters within a line indicate 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 F_2-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

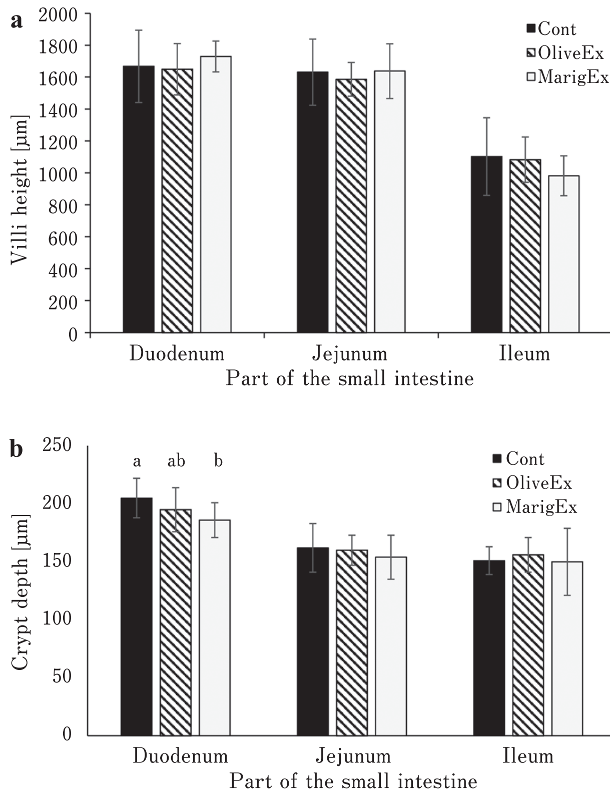

Fig. 2. **Height of the villi (a) and depth of the crypts (b) in individual parts of the small intestine.** On the abscissa are different parts of the small intestine: duodenum, jejunum and ileum, on the ordinate is the length/depth in μm. Different small letters show significant differences at $p < 0.05$ within the same part of the small intestine (duodenum crypt depth, F value 3.58 and p value 0.0406).

induce alterations in T-cell function, and thus may subsequently impair immune response (Barnett and Barnett, 1998). Thus, increased DNA integrity indicates potentially beneficial effects of marigold extract on the immune system and helps to explain the enhanced antibody titers against Newcastle and influenza viruses in broiler chickens supplemented with marigold flower extract (Rajput *et al*., 2012).

The results in the olive leaves extract-supplemented group showed a different picture. A reduction in lipid oxidation due to significantly ($p < 0.05$) reduced concentration of MDA was observed, and a numerical reduced concentration of F_2-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 (Choct *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 (Choct *et al*., 1996; Choct *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 propanoate 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 *Lactobacilus 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.

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Conflict of Interest

The authors declare no conflict of interest.

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