

Effects of Olive Leaf and Marigold Extracts on the Utilization of Nutrients and on Bone Mineralization using Two Different Oil Sources in Broilers

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The aim of this study was to investigate the effects of olive leaf and marigold extracts on the apparent total tract digestibility (ATTD) of the principal nutrients and energy, as well as on mineral utilization (Ca, P, Mg, Mn, Fe, Cu and Zn) in relation to bone characteristics in broilers fed walnut- or linseed oil-supplemented diets. Thirty-six 12-day-old commercial broilers Ross 308 were reared in metabolic cages, assigned to one of the six dietary treatments (3×2 factorial design): three supplements (not supplemented, olive leaf extract, or marigold extract), and two oils (walnut or linseed oil). The results showed that the marigold extract reduced Zn and P balances and tended to lower the balance of ash and Mg, and the ATTD of Zn and Mg. Diets with linseed oil increased the ATTD of acid detergent fiber and reduced the ATTD of the organic residue and Cu. No differences in the bone characteristics of *tibia* were observed between treatments. These results indicated that the inclusion of marigold extract had a negative effect on the Zn and P balance, and that neither extract had any major effect on the digestion and utilization of energy and other investigated nutrients, or on bone mineralization, irrespective of the oil source included in the diet.

Key words: bone mineralization, broiler, marigold, nutrient utilization, olive leaf

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Introduction

Surveys have shown that plant secondary metabolites not only improve various physiological parameters but also enhance the health, productivity, and quality of animal products (Surai, 2014). A wide range of plants, including olive leaves and marigold, contain different bioactive molecules.

Olive (*Olea europaea* L.) fruits and leaves have antimicrobial, antioxidant, anti-inflammatory, antithrombotic, antiatherogenic, and antihypertensive actions. These are used to prevent or treat cardiovascular disease, hypertension, bacterial infections, inflammation, cancer, and diabetes (Obied *et al.*, 2005).

Due to its antimicrobial, antiparasitic, antioxidant, anti-inflammatory, and immunomodulatory properties, marigold (*Calendula officinalis* L.) has been used for centuries to treat internal and external inflammatory conditions, such as gastrointestinal inflammatory disorders, gastric and duodenal ulcers, wounds, and burns. (Braun and Cohen, 2015).

The effects of olive oil are exerted by polyphenols, such as hydroxytyrosol, oleuropein, tyrosol, elenolic acid, catechol, rutin, and tocopherols (Ghanbari *et al.*, 2012), while those of marigold are attributed to secondary metabolites, such as triterpene saponins, flavonols, quercetin, carotenoids, flavoxanthin, and auroxanthin (Korakhashvili *et al.*, 2007; Braun and Cohen, 2015). The European Food Safety Authority (EFSA, 2011) permits health claims regarding the contribution of olive oil polyphenols to the protection of blood lipids from oxidative stress.

Despite a considerable amount of research conducted in humans, only a few studies have investigated the use of these extracts as natural feed supplements in farm animals (Frankič *et al.*, 2008), including poultry (Sarica and Ürkmez, 2016). Although olive leaf extract is used in traditional medicine, the utilization of whole leaves has only been studied in animal nutrition (Paiva-Martins *et al.*, 2009; Botsoglou *et al.*, 2010; Christaki *et al.*, 2011; Botsoglou *et al.*, 2012a). A study on the use of olive leaves in growing turkeys fed diets supplemented with 1% olive leaves showed no effect on body weight, but the oxidative and bacterial stability of raw turkey meat was increased during refrigerated storage (Botsoglou *et al.*, 2010). An improvement in the oxidative stability of meat was also observed in pigs fed olive leaves as

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a dietary supplement compared to those fed a control diet (Paiva-Martins *et al.*, 2009; Botsoglou *et al.*, 2014). Dietary supplementation with olive leaves as a feed antioxidant in laying hens has been used to obtain n-3 FA-enriched eggs (Botsoglou *et al.*, 2012b, c, 2013a, b). However, since a high level of olive leaf inclusion in pig diets (Paiva-Martins *et al.*, 2009), and possibly poultry diets, seems to reduce the feed intake and growth performance, it is difficult to evaluate the effect of olive bioactive substances when using whole leaves. The use of oleuropein and hydroxytyrosol-rich olive oil (Güclü *et al.*, 2008), or olive leaves extracts, is a more promising but expensive approach.

There are fewer studies on the use of marigold extracts as a feed supplement compared to those on the use of olive leaves. Rajput *et al.* (2012) found that the body and thymus weights of broilers fed a marigold flower extract-supplemented diet improved compared to those of animals fed a control diet. Results from pigs were also promising, as Frankič *et al.* (2008) reported antioxidant effects of marigold petals and flower-top extracts *in vivo*, comparable to those of vitamin E.

Owing to these properties of polyphenols, positive effects on nutrient digestion and metabolism can be expected from their use; such positive effects were observed in broilers with regard to dry matter and carbohydrates (Hernández *et al.*, 2004), nutrient and energy retention (Brenes *et al.*, 2010), gastrointestinal tract health (Wang *et al.*, 2008), and excreta dry matter content (Rezar and Salobir, 2014). Plant polyphenols can also interfere with some micro and macronutrients (for instance by binding Zn, Fe, and Cu.), which can reduce the digestibility of minerals, and thus, their availability (Surai, 2014). Studies have shown that different polyphenols exert positive effects on the prevention of bone loss in ovariectomized mice (Hagiwara *et al.*, 2011) and in the loss of bone mineral density and strength in obese rats (Shen *et al.*, 2012).

No data are available on the effects of marigold and olive leaf extracts on digestion in broilers. This is especially important since plant bioactive molecules may also exert negative effects on the digestion of some nutrients, especially microminerals (Surai, 2014). This may lead to leg disorders, which have been recognized as a major cause of poor animal welfare in commercial broiler production (EU, 2000).

The lack of scientific research on the effects of olive and marigold extracts on digestion shows there is a need to assess their effects on the balance and utilization of principal nutrients and energy, and on the mineral utilization in relation to bone mineralization in broilers. Substances with antioxidative, antimicrobial, and anti-inflammatory activity that are found in olive leaves and marigold extracts may have different effects in the gastro-intestinal tract under different conditions. Because feed can be susceptible to oxidation, the level of oxidative stress in the intestine might be one such effect; therefore, the production of experimental diets has been based on walnut and linseed oils, which are rich in polyunsaturated fatty acids (PUFA). Diets enriched in n-3 PUFA are used in the production of meat with functional

properties (Voljč *et al.*, 2013). However, linseed oil has a higher concentration of n-3 PUFA than walnut oil (Table 2), and thus, has higher susceptibility to oxidation. Different fatty acid (FA) compositions also affect the absorption and utilization of fats and other nutrients. The absorption of fats is lower when FAs are saturated (SFA) and have a long chain (Ward and Marquardt, 1983), and fat utilization is normally higher in diets containing blends of fats and/or combinations of SFAs and unsaturated FAs (UFA) (Ketels and De Groote, 1989; Wang *et al.*, 2013). Lin and Chiang (2010) and Tanchaonrat and Ravindran (2014) reported a greater formation of insoluble soaps (especially with Ca) with SFA compared to UFA. Consequently, feeding sources rich in UFA or blends of different fat sources can improve digestion, especially since the digestive tract of young birds is not fully developed (Carew *et al.*, 1972; Wiseman and Salvador, 1991). Therefore, both extracts may exhibit diverse effects when animals are fed selected oils.

Thus, in the present study, a balance trial experiment was conducted to measure the apparent total tract digestibility (ATTD) and the balance of energy, major nutrients, some minerals, and dry matter content of the excreta, as well as various bone characteristics.

Materials and Methods

Animals and Feed Mixtures

Thirty-six 12-day-old male broiler chicks (Ross 308) were individually caged in metabolic cages (a wire floor cage measuring 450 × 380 × 480 mm, equipped with a nipple drinker), which allowed us to collect excreta quantitatively. Experimental animals were selected from a flock of 80 animals reared on deep litter based on a similar body weight at 12-days of age.

The experiment began after a 7-day adaptation period to the individual rearing system (day 1; age, 19 days) by randomly assigning animals to one of six experimental groups (six animals per treatment), each receiving a different dietary treatment (Table 1). The six dietary treatments were provided in a 3 × 2 factorial design: three supplement treatments (not supplemented, olive leaf extract, or marigold extract) and two different oils (walnut or linseed oil). On day 8 (age, 26 days), the excreta collection period began. The excreta were collected daily for 5 days, and frozen at -20°C for further analyses. Prior to excreta collection, feed was withdrawn for 18 h in order to empty the digestive tract. After the last excreta collection, animals were weighed and fed the experimental diets *ad libitum* for 5 days. The dry matter content of the excreta was then measured by individual collection from 8.00 to 14.00 h on the first and the third day after excreta collection (age, 32 and 34 days, respectively). Finally, the animals were slaughtered at 36-days of age and *tibia* bones were removed, packed in plastic bags to avoid dehydration, and stored at 2°C for further measurements. Animals were weighed at the beginning (1037 ± 75 g) and end (1451 ± 19 g) of the excreta collection period.

The animals had free access to feed and water throughout the trial, and recommended temperature and humidity levels

Table 1. **Composition and analyses of the experimental diets**

Extract	Walnut oil			Linseed oil		
	Control	Olive	Marigold	Control	Olive	Marigold
Wheat, g/kg	660	660	660	660	660	660
Soybean meal, g/kg	239	239	239	239	239	239
Limestone, g/kg	11.2	11.2	11.2	11.2	11.2	11.2
Salt, g/kg	4.41	4.41	4.41	4.41	4.41	4.41
Monocalcium phosphate, g/kg	9.41	9.41	9.41	9.41	9.41	9.41
Methionine, g/kg	0.98	0.98	0.98	0.98	0.98	0.98
Premix, g/kg ¹	5.0	5.0	5.0	5.0	5.0	5.0
Walnut oil, g/kg	70.0	70.0	70.0			
Linseed oil, g/kg				70.0	70.0	70.0
Olive leaves extract		+			+	
Marigold petals extract			+			+
Feed analyses						
ME, MJ/kg ²	13.4	13.4	13.4	13.4	13.4	13.4
Dry matter, g/kg	890	883	886	890	882	887
Gross energy, MJ/kg DM ³	19.9	19.9	19.9	19.9	20.1	19.8
Ash, g/kg DM ³	51.3	53.2	52.6	50.8	50.6	50.3
Crude protein, g/kg DM ³	206	204	204	200	207	200
Crude fat, g/kg DM ³	91.2	90.6	90.7	90.3	91.6	89.2
NDF, g/kg DM ³	128	131	126	126	129	126
ADF, g/kg DM ³	49.7	46.9	46.0	47.9	49.5	49.3
P, g/kg DM ³	6.45	6.57	6.51	6.39	6.43	6.35
Ca, g/kg DM ³	7.97	8.38	8.00	7.79	8.36	8.09
Mg, g/kg DM ³	1.43	1.48	1.51	1.46	1.37	1.43
Fe, mg/kg DM ³	175	199	166	175	187	180
Cu, mg/kg DM ³	26.3	28.1	24.5	28.0	25.5	28.2
Zn, mg/kg DM ³	157	172	158	163	171	170
Mn, mg/kg DM ³	351	377	347	335	372	351

¹ copper 3,200 mg/kg, iodine 250 mg/kg, iron 8,000 mg/kg, manganese 24,000 mg/kg, selenium 60 mg/kg, zinc 20,000 mg/kg, vitamin A 2,000,000 IU/kg, vitamin D₃ 1,000,000 IU/kg, vitamin E 1,820 IU/kg, vitamin K 600 mg/kg, thiamine 400 mg/kg, riboflavin 1,200 mg/kg, niacin 11,400 mg/kg, pantothenic acid 2,800 mg/kg, pyridoxine 700 mg/kg, biotin 30 mg/kg, folic acid 350 mg/kg, cyanocobalamin 3.2 mg/kg, choline 150,000 µg/kg; ² calculated value (GFE, 2006); ³ dry matter

Table 2. **Fatty acid composition of walnut and linseed oils, and of experimental diets**

	Walnut oil	Linseed oil	Walnut oil based diets	Linseed oil based diets
SFA, g/kg ¹	9.94	9.13	9.62	9.00
MUFA, g/kg ²	15.5	17.3	12.2	13.5
PUFA, g/kg ³	70.2	69.1	55.1	54.1
n-3 PUFA, g/kg ⁴	10.9	54.0	7.93	35.5
n-6 PUFA, g/kg ⁵	59.3	15.2	47.2	18.6
n-6/n-3	5.44	0.28	5.94	0.53

¹ saturated fatty acids; ² monounsaturated fatty acids; ³ polyunsaturated fatty acids; ⁴ mainly C18:3 n-3; ⁵ mainly C18:2 n-6

for growing chickens were maintained throughout the whole period (Aviagen, 2014). Animals were fed mash diets formulated according to the broiler nutrition specifications for Ross 308 (Aviagen, 2014), except for the content of crude protein, essential amino acids, Ca, P, Mg, and K, which were 20% lower than recommended. This enabled differences in the ATTD and balance of nutrients to be measured

(Table 1). The fatty acid profile of walnut and linseed oil, and of the feed mixtures, is presented in Table 2.

The study protocol was approved by the Animal Ethics Committee of the Veterinary Administration of the Republic of Slovenia.

Preparation of Extracts

Olive leaves were collected manually from olive trees in

March, immediately after pruning (variety Istrian white, "istrska belica"). The leaves were collected from an organic olive plantation located in the Slovenian Istria region and were air dried (45–55°C, protected from light) and milled using a hammer mill (particle size <2 mm). The olive leaf extract was produced by immersing dried olive leaves in 70% ethanol (1:4 proportion=250 g of olive leaves per 1000 mL ethanol) and shaking the mixture on an orbital platform shaker in brown glass bottles for 4 days. After extraction, the liquid was filtered through a gauze and concentrated by solvent evaporation at 40°C to 15% of the initial weight, protected from light (Müller and Hildebrand, 1998). Chlorophyll was not removed from the extract. Concentrated extract from olive leaves was added at a rate of 6 mL per kg of feed (equivalent to 1% of the weight of olive-leaf supplementation in the diet). Concentrated olive leaf extract contained $420 \pm 20 \mu\text{mol}$ gallic acid equivalents/mL (total polyphenols), $70 \pm 1.0 \text{ nmol/mL}$ carotenoids, $1.48 \pm 0.02 \mu\text{mol/mL}$ chlorophylls, $58.5 \pm 4.5 \text{ nmol}$ catechin equivalents/mL (flavan-3-ols), and $0.79 \pm 0.02 \mu\text{mol/mL}$ rutin equivalents (flavonoids). The evaporated extract contained $53.0 \pm 4.2 \text{ mg}$ oleuropein/mL. Analytical methods are described below. Conversely, marigold extract was produced to form a tincture. Using a 1:4 proportion, ground petal leaves (200 g) were mixed with propylene glycol: water (1:1; 800 g). The extraction lasted for 42 days and was performed in a place protected from light. Isaac (1992), and Müller and Hildebrand (1998) stated that these extracts are stable, concentrated in flavonoids and other phenolic derivatives, and in lipophilic substances. Marigold extract contained $2.84 \pm 0.03 \mu\text{mol}$ gallic acid equivalents/mL (total polyphenols), $10.8 \pm 1.0 \text{ nmol/mL}$ carotenoids, $12.4 \pm 0.07 \text{ nmol/mL}$ chlorophyll, $0.71 \pm 0.11 \text{ nmol}$ catechin equivalents/mL (flavan-3-ols), and $12.2 \pm 0.3 \text{ nmol/mL}$ rutin equivalents (flavonoids). Analytical methods are described below. The extract was administered at a rate of 5 mL per kg of feed (equivalent to 1 g of fresh petals), as proposed for internal use in traditional medicine (Barnes *et al.*, 2002). Both plant extracts were added to the diet by mixing into the oil (linseed or walnut).

Extract Analysis

Both extracts were analyzed spectrophotometrically to determine total phenols, flavonoids, chlorophylls, carotenoids, and flavan-3-ols. Total phenol content was determined using modified the Folin-Ciocalteu polyphenolic assay (Agbor *et al.*, 2014; Singleton and Rossi., 1965), flavonoids by the AlCl_3 colorimetric method (Lin and Tang, 2007), chlorophylls by the method described by Vernon (1960), carotenoid as described by Biehler *et al.* (2010), and flavanols using the 4-dimethylaminocinnamaldehyde assay as proposed by Wallace and Giusti (2010). The content of oleuropein was analyzed by HPLC following method COI/T. 20/Doc no. 29, with modifications.

Analytical Methods

Excrements were defrosted, homogenized, and freeze-dried prior to the analyses. The content of crude nutrients, fiber, energy, and minerals in diets, excreta, and bones were determined by standard procedures (Naumann and Bassler,

1997). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined in an Ankom Fibre Analyser (Ankom, USA) using the producer's instructions. Gross energy was determined in a calorimeter IKA C 200 (IKA, Germany). The dietary apparent metabolizable energy (N corrected; AME_N) values were calculated with correction for N retention, using a value of 34.42 kJ/g retained N (Hill and Anderson, 1958). Ca, Mg, Fe, Cu, Zn, and Mn were analyzed by flame atomic absorption spectrometry (Perkin Elmer, Analyst 200), while P was analyzed by spectrometry (Varian Cary 50, Probe UV-Visible Spectrophotometer).

Prior to measurement of bone strength, the *tibia* was cleaned of muscle tissue and the weight and length of the fresh bone were determined. The bone strength was measured using an Instron universal testing machine (Model 3345, 50-kg load cell, crosshead speed 50 mm/min, bone supported on a 30-mm span). Thereafter, the bones were frozen at -20°C for further analyses.

Statistical Analyses

Data were analyzed by the General Linear Models (GLM) procedure from SAS software (SAS Institute Inc., 2002–2010). Least square means (LSM) are presented, differences were determined by a Tukey–Kramer multiple comparison test. The dispersion is expressed as the standard error of the mean (SEM). In the statistical model, the fixed effects of the oil, the supplement, and their interaction were included. To reduce the effect of variability in the initial and final body weights, and in the bone weight, these factors were included as a linear regression when appropriate. Statistical significance was considered when $p < 0.05$.

Results

The animals adapted well to the experimental conditions, no health-related problems or diet rejection were observed. No differences in feed intake were observed among groups, although the final body weight of the animals fed the two diets supplemented with plant extracts was reduced by 3% compared to that of the animals fed the control diets (Table 3).

No differences in the ATTD of dry matter, organic matter, crude protein, crude fat, NDF, gross energy, AME_N coefficient, and excreta dry matter were observed among groups, regardless of the presence of a supplemented extract, or the oil. However, a significantly increased ATTD of ADF (5%) and a significantly reduced ATTD of organic residue (2%) were observed in the linseed oil group in comparison to the walnut oil group. Supplementation with the marigold extract reduced the nitrogen balance (6%; $p=0.085$) (Table 4).

Mineral utilization (Table 5) revealed that supplementation with marigold extract significantly reduced the Zn and P balance (24 and 8%, respectively), the ATTD of Zn (21%; $p=0.059$), and the balances of ash (7%; $p=0.055$) and Mg (13%; $p=0.097$) compared to the control groups. In addition, the ATTD of Mg was negatively affected by the marigold extract (12%; $p=0.072$) compared to the olive leaf extract. Comparison of oils revealed reduced Cu ATTD (6%) in the linseed oil group compare to the walnut oil group.

Table 3. Final body weight at 31 days of age, and average feed intake in the excreta collection period (from 26 to 31 days of age)

Extract	Walnut oil			Linseed oil			SEM	P-oil	P-supplement	P-interaction
	Control	Olive	Marigold	Control	Olive	Marigold				
Final body weight, g	1497	1450	1428	1463	1421	1448	19.4	0.36	0.05	0.39
Feed intake, g/day	139	131	125	134	129	134	3.70	0.86	0.11	0.20

Table 4. ATTD of crude nutrients, fiber fractions, nitrogen balance, energy content of feed, and excreta dry matter content

Extract	Walnut oil			Linseed oil			SEM	P-oil	P-supplement	P-interaction
	Control	Olive	Marigold	Control	Olive	Marigold				
Dry matter ATTD ¹	0.778	0.769	0.769	0.767	0.769	0.750	0.0009	0.16	0.31	0.59
Organic matter ATTD ¹	0.792	0.791	0.782	0.781	0.780	0.764	0.0009	0.17	0.31	0.65
Crude protein N balance, g/day	2.67	2.53	2.38	2.59	2.54	2.51	0.078	0.75	0.09	0.33
ATTD ¹	0.667	0.671	0.662	0.670	0.677	0.658	0.008	0.69	0.17	0.83
Crude fat ATTD ¹	0.940	0.938	0.938	0.939	0.948	0.935	0.005	0.65	0.35	0.38
NDF ATTD ¹	0.443	0.420	0.396	0.444	0.433	0.412	0.026	0.63	0.30	0.95
ADF ATTD ¹	0.405	0.403	0.371	0.415	0.414	0.416	0.001	0.01	0.19	0.21
Organic residue ² ATTD ¹	0.897	0.885	0.895	0.875	0.877	0.859	0.012	0.04	0.76	0.59
Gross Energy ATTD ¹	0.817	0.810	0.810	0.810	0.812	0.791	0.008	0.22	0.19	0.44
AME _N coefficient (AME _N /GE) ³	0.775	0.768	0.767	0.767	0.769	0.767	0.006	0.36	0.49	0.76
AME _N MJ/kg DM	13.7	13.5	13.6	13.6	13.6	13.4	0.13	0.39	0.37	0.54
Excreta dry matter Excreta dry matter, g/kg	212	223	206	213	220	231	11.7	0.45	0.73	0.50

¹ Apparent total tract digestibility coefficient;

² Organic residue is organic matter – crude protein – crude fat – NDF;

³ AME_N – nitrogen-corrected apparent metabolic energy; GE – gross energy

No other macro- or micronutrients were affected by the dietary treatment.

The bone characteristics are presented in Table 6. There were no statistical differences among treatments, except for a negative effect of walnut oil ($p=0.083$) and olive leaf extract ($p=0.056$) on the length of the *tibia* and the lower ratio between *tibia* ash weight and dry defatted weight on walnut oil ($p=0.061$), compared to linseed oil diets.

Discussion

Several studies have shown that natural supplements containing polyphenols can improve productivity in broilers, despite inconsistencies in the results obtained. For instance,

the results obtained for grape and olive pomace, olive leaves, grape seeds, marigold, cinnamon, sage, green tea, tannin, thyme, and rosemary extracts, as well as different mixtures of polyphenol sources, can either increase (Eid *et al.*, 2003; Tavarez *et al.*, 2011; Lu *et al.*, 2014; Bravo *et al.*, 2014), decrease, or have no effect on productivity (Goni *et al.*, 2007; Gurbuz *et al.*, 2010; Brenes *et al.*, 2010; Rezar and Salobir, 2014).

Due to the small number of animals included in our trial, and the specificity of rearing, the production parameters obtained in the present study have an illustrative value. Animals in our trial showed comparable feed intake and growth in all groups with a tendency of the extracts to impair the

Table 5. Balance and ATTD of macro and micro minerals

Extract	Walnut oil			Linseed oil			SEM	P-oil	P-supplement	P-interaction
	Control	Olive	Marigold	Control	Olive	Marigold				
Ash										
Balance, g/day	3.38	3.18	3.02	3.23	3.17	3.13	0.098	0.84	0.07	0.44
ATTD ¹	0.535	0.533	0.531	0.529	0.540	0.511	0.008	0.30	0.11	0.30
Ca										
Balance, g/day	0.557	0.519	0.517	0.554	0.545	0.520	0.020	0.59	0.16	0.75
ATTD ¹	0.563	0.557	0.581	0.576	0.589	0.541	0.016	0.88	0.76	0.15
P										
Balance, g/day	0.440	0.414	0.385	0.424	0.412	0.413	0.013	0.78	0.05	0.31
ATTD ¹	0.555	0.554	0.541	0.553	0.559	0.558	0.008	0.99	0.09	0.89
Mg										
Balance, mg/day	66.9	63.6	56.2	66.3	67.2	59.2	4.21	0.56	0.08	0.86
ATTD ¹	0.372	0.380	0.350	0.383	0.404	0.341	0.021	0.60	0.08	0.78
Mn										
Balance, mg/day	11.4	11.9	12.5	12.1	13.6	11.3	1.55	0.78	0.79	0.70
ATTD ¹	0.262	0.293	0.311	0.281	0.338	0.264	0.035	0.85	0.43	0.49
Fe										
Balance, mg/day	5.42	5.86	4.99	4.93	5.55	4.79	0.481	0.35	0.27	0.96
ATTD ¹	0.243	0.286	0.257	0.232	0.262	0.220	0.024	0.22	0.23	0.88
Cu										
Balance, mg/day	1.39	1.38	1.29	1.32	1.28	1.24	0.062	0.13	0.29	0.93
ATTD ¹	0.422	0.447	0.435	0.417	0.418	0.386	0.014	0.02	0.29	0.34
Zn										
Balance, mg/day	7.01	5.14	4.91	5.48	5.79	4.59	0.544	0.36	0.03	0.14
ATTD ¹	0.352	0.269	0.269	0.281	0.308	0.232	0.028	0.32	0.07	0.15

¹ Apparent total tract digestibility coefficient.

Table 6. The different physical and chemical aspects studied in tibia bones

Extract	Walnut oil			Linseed oil			SEM	P-oil	P-supplement	P-interaction
	Control	Olive	Marigold	Control	Olive	Marigold				
Weight, g	14.4	14.1	15.0	15.1	14.5	14.6	0.34	0.41	0.28	0.31
Length, mm	99.1	97.2	98.4	100.0	98.4	99.3	0.70	0.08	0.06	0.98
Max force, N	316	304	330	281	292	309	23.7	0.24	0.58	0.88
Max bend, mm	2.61	2.07	2.11	2.19	2.16	2.39	0.163	0.89	0.25	0.11
Dry defatted / fresh weight	36.4	35.9	36.9	36.2	36.2	36.0	0.47	0.52	0.63	0.52
Ash weight / dry defatted weight	39.4	38.8	38.0	39.7	39.1	39.9	0.55	0.06	0.46	0.28

growth parameters of broilers compared to broilers fed control diets.

Natural polyphenol supplementation can influence the digestion of major nutrients, and consequently, the nutrient and energy utilization (Hernández *et al.*, 2004; Chamorro *et al.*, 2013, 2014). However, as for growth parameters, data on the effects of plant-extract supplementation on digestion in the literature are also variable. Negative effects on the digestion of starch, sugar, protein, amino acids, and fat have been found (Chamorro *et al.*, 2013; Surai, 2014), which could be due to the inhibition of amylases, proteases, and/or lipases in the small intestine, the binding of bile acids, and/or

a negative effect of polyphenols on the histological structure of the intestine (Surai, 2014). Negative effects on villus height and width in layers, and a shortened intestinal tract, have also been described when using marigold and grape seed extracts in broilers (Gurbuz *et al.*, 2010; Brenes *et al.*, 2010). This might differ when using combinations such as a mixture of phenol thymol and the essential oil carvacrol, which is known to increase the amount of pancreatic trypsin, lipases, and proteases in the intestine in broilers (Hashemipour *et al.*, 2013). Other studies have shown that intestinal absorption function (villus height, and villus height-to-crypt depth ratio) and protease and lipase activities

were improved with the inclusion of fermented *Ginkgo* leaves, which have been used in many experiments in chickens (Zhang *et al.*, 2013; Yu *et al.*, 2015; Zhang *et al.*, 2015). Further research is required to elucidate the effects of different supplements rich in polyphenols on the digestion of nutrients.

In the present study, dietary supplementation with both extracts, irrespective of the type of oil, had no major effect on the utilization of major nutrients and energy. The utilization of dry matter, organic matter, crude protein, crude fat, neutral detergent fiber, and energy was the same in all groups. Only supplementation with the marigold extract lowered the nitrogen balance by 6% ($p=0.085$).

Increased metabolic, and possibly intestinal oxidative stress occurs as a consequence of high n-3 PUFA intake (Voljč *et al.* (2013). Thus, a more pronounced effect of the extracts on the nutrient and energy utilization in the linseed oil group was expected, although it was not confirmed in the present study. The higher oxidative stress caused by high n-3 PUFA in the linseed oil group may be due to the significantly lower ATTD of the organic residue (2%) measured in this group, compared to the group fed diets with walnut oil rich in n-6 PUFA. Our results are not completely consistent with those of Dänicke *et al.* (1997), who showed that broilers fed a soya oil-supplemented diet exhibited higher ATTD of organic matter, crude fat, protein, and AMEN, and had higher ATTD of C18:2 n-6 and C18:3 n-3. Similarly, Poureslami *et al.* (2010) showed that broilers fed linseed oil (high C18:3 n-3) had higher apparent digestibility of C18:2 n-6 and C18:3 n-3 acid than broilers fed palm oil. Conversely, Crespo and Esteve-Garcia (2002) showed that there were no differences between sunflower oil (mainly C18:2 n-6) and linseed oil (mainly C18:3 n-3) in the digestibility of fat and nitrogen.

Evidence suggests that some plant extracts may have positive effects on the digesta dry matter (Rezar and Salobir, 2014), which might be a consequence of the lower digesta viscosity of polyphenols (Botsoglou *et al.*, 2010; Hashemipour *et al.*, 2014). Thus, they might have a positive influence on the digestion of nutrients. However, no effect on the digesta dry matter was observed in the present study.

The antimicrobial effect of both extracts is well known, and might be important in the intestine in relation to the degradation of non-starch polysaccharides (Taguri *et al.*, 2004; Efstratiou *et al.*, 2012; Surai, 2014). Because intestinal bacteria are responsible for fiber digestion, the FA composition of feeds might also have an influence on fiber ATTD. The results of the present study showed that neither extract had an effect on the digestibility of the fiber fractions or the organic residue. A different effect on fiber degradation was observed only between the two experimental oils used. The ATTD of ADF was significantly higher (5%) in the linseed group than in the walnut oil group. This was unexpected, since Zheng *et al.* (2005) and Dilika *et al.* (2000) reported bacterial inhibition by linoleic and oleic acid, which is in contradiction to our results.

Plant bioactive compounds, and especially polyphenols,

can interfere with the uptake and utilization of several micronutrients in the gastrointestinal tract, mostly by binding some trace minerals such as Zn, Fe, and Cu, and consequently forming indigestible complexes, which makes them unavailable for absorption in the intestine (Yang and Landau, 2000; Sandberg, 2002; Surai, 2014).

The present results revealed negative effects of the marigold extract on ash, P, and Mg utilization. In both supplemented groups, a significantly reduced P balance was measured. Reduced P ($p=0.105$) and Mg ($p=0.072$) ATTD compared to the olive leaf extract, and reduced ash ($p=0.055$) and Mg ($p=0.097$) balances compared to the control in these groups, was also noted. Additionally, marigold extract reduced the balance ($p=0.022$) and ATTD ($p=0.059$) of Zn. Our findings are not completely consistent with those of Chamorro *et al.* (2013), who showed that grape seed polyphenols reduced not only the absorption of Zn, but also that of Fe and Cu in broilers. In contrast, Afsana *et al.* (2004) reported a negative effect of tannic acid on the absorption of Fe but not of Zn, Cu, and Mn in rats. Similarly, Abdulkarimi and Daneshyar (2012) reported a negative effect on Fe absorption in chickens fed diets supplemented with thyme polyphenols. However, a lower absorption of Zn, but not of Fe or Cu were observed in rats fed various levels and types of tea (Greger and Lyle, 1988). The influence of different polyphenols on Zn absorption was assessed in an *in vitro* study by Kim *et al.* (2011) using human intestinal Caco-2 cell monolayers. While a grape seed extract inhibited Zn absorption, this was not altered by epigallocatechin-3-gallate and green tea extracts compared to the control group. The inhibition of Zn absorption was attributed to the procyanidins present in grape seed extracts, which bind Zn with high affinity. These results showed that different bioactive molecules may have different effects on mineral absorption, although further studies are needed to elucidate the influence of specific bioactive substances on mineral absorption and utilization.

Comparisons between studies are difficult, because the amount and composition of the studied supplements and the composition of the experimental diets differ. In our study, the effects of linseed and walnut oils were measured in relation to Cu utilization, for which linseed oil caused a lower ATTD than walnut oil. This might be due to the formation of insoluble Cu salts with free FAs (Robinet and Corbeil, 2003), which can deteriorate Cu digestibility. It is not known whether differences in the affinity of FAs to form Cu salts exists, but the difference in Cu ATTD might be linked to differences in the FA composition. In our study, different ratios observed between n-6 and n-3 PUFA in the oils used did not have any major effects on macro- and micro mineral utilization. This is not consistent with the findings of Kumar *et al.* (2015), who showed that inclusion of 0.5% n-3 FAs from linseed oil improved Ca, but not P retention. This could also be an effect of the inclusion level, since in our experiment, the content of n-3 fatty acids was 0.79% in walnut oil- and 3.55% in linseed oil-based diets.

Polyphenols can have positive effects on bone characteris-

tics, such as bone mineral density and strength. To date, around 500 known polyphenols with bioactive properties are known. These can protect bone health through different mechanisms, such as reducing bone loss. Given their anti-oxidative effects and anti-inflammatory action, they enhance osteoblastogenesis, suppress osteoclastogenesis, and have osteoimmunological action (Đukarič *et al.*, 2015). Previous studies have reported that under stressful situations, such as during an ovariectomy, inflammation (Puel *et al.*, 2006), and obesity (Shen *et al.*, 2012), polyphenols from olive oil and green tea improved bone strength and reduced bone loss in mice and rats. Polyphenol-containing plants, such as dried plum (Franklin *et al.*, 2006), blueberry (Devareddy *et al.*, 2008), green, black, red, and white tea (Shen *et al.*, 2012; Tomaszewska *et al.*, 2016), have demonstrated positive effects on bone health in different rat models (ovariectomy, inflammation, obesity, cadmium and lead exposure, etc.). A substantial amount of research on olive polyphenols has shown positive effects on bone health. Puel *et al.* (2006) showed that oleuropein alleviated bone loss following an ovariectomy and during inflammation in rats, probably by modulating inflammatory parameters. In addition, Hagiwara *et al.* (2011) showed that hydroxytyrosol and oleuropein decrease multinucleated osteoclasts in a dose-dependent manner, which helped prevent bone loss in the *femurs* of ovariectomized mice. Oleuropein in bone marrow-derived stem cells enhanced osteoblastogenesis and inhibited adipogenesis (Santiago-Mora *et al.*, 2011), which can positively affect bone mineralization and strength. Conversely, Anter *et al.* (2016) showed that hydroxytyrosol repressed the expression of osteoblastic markers in human bone marrow mesenchymal stromal/stem cells and increased the expression of adipogenic genes, which can lead to bone loss. However, our results showed that neither extract had an effect on bone mineralization, which is not consistent with previous findings. Frejnagel and Wroblewska (2010) reported similar results, showing that green tea, chokeberry, and honeysuckle polyphenols reduced Zn and Cu absorption in rats, but this was not accompanied by diminished concentrations of the *femur* bone. Different fatty acid compositions in the diets did not affect the *tibia* characteristics, which is consistent with the findings of other studies reporting no effect of the FA composition on bone characteristics in poultry. Tarlton *et al.* (2013) observed that PUFA reduced the bone strength in laying chickens, which differed from the results obtained by Baird *et al.* (2008), who did not observe an effect of n-3 PUFA.

Olive leaf and marigold extracts have been proposed as additives given their antioxidative, anti-inflammatory, and other possible effects reported in previous studies (Rahman *et al.*, 2007; Surai, 2014). However, further research is needed to elucidate their effects and appropriate dosage. In animal feed, a variety of plant extracts have been tested at different levels: from as low as 10 ppm (Liu *et al.*, 2013) to as high as 7.500 ppm (Namkung *et al.*, 2004). Thus, it is hard to develop practical guidelines for the use of olive leaf and marigold extracts in broiler feed.

In conclusion, supplementation with natural olive leaf and marigold extracts had no major effects on the digestion and utilization of the studied nutrients. The results highlight that the effect of both extracts on the digestion of nutrients is not negative, and that they can be used in low amounts. However, this needs to be further assessed before being implemented in practice. Furthermore, the utilization of P, Mg, and Zn, should be further addressed and elucidated. For practical use in commercial broiler diets, low concentrations of the extracts should be used to nullify some of the possible negative effects. We also show that the fatty acid profile of the diet (n-6/n-3 FA ratio) can have a minor effect on nutrient utilization, such as organic residue, ADF, and Cu and *tibia* characteristics.

The results of this balance experiment are also valuable for the practical considerations of broiler feeding. Supplementation of feed with natural olive leaf and marigold extracts to create meat with functional properties and a high content of n-3 fatty acids, by using feed mixtures with high concentration of n-3 fatty acid-rich oils like linseed oil, does not have an important influence on nutrient utilization and bone characteristics of broilers.

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