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Effects of diets containing linseed oil or lard and supplemented with pumpkin seeds on oxidative status, blood serum metabolites, growth performance and meat quality of naked neck chickens

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Complete List of Authors:	Meineri, Giorgia; University of Torino, Department of Veterinary Sciences Longato, Erica; University of Torino, Department of Veterinary Sciences Peiretti, Pier Giorgio; National Research Council, Institute of Sciences of Food Production
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RUNNING TITLE: *PUMPKIN SEEDS IN POULTRY NUTRITION***Effects of diets containing linseed oil or lard and supplemented with pumpkin seeds on oxidative status, blood serum metabolites, growth performance and meat quality of naked neck chickens**G. Meineri^{1,3}, E. Longato¹, and P. G. Peiretti²¹ Department of Veterinary Sciences, University of Torino, Grugliasco, Italy; and² Institute of Sciences of Food Production, National Research Council, Grugliasco, Italy.³ To whom correspondence should be addressed (giorgia.meineri@unito.it).

This study investigates the effect of two dietary fats, namely linseed oil and lard and supplementation with pumpkin seeds (PS) on the performance, oxidative status and blood serum metabolites of broilers, along with the composition, oxidative stability and fatty acid (FA) profile of broiler meat. Ninety-six 64 d-old naked neck chickens were randomly distributed into four groups of 24 chickens each (eight chickens per cage, three cages per treatment) and fed a linseed oil diet (LO), a LO diet supplemented with 50g PS kg⁻¹, a lard diet (LF) and a LF diet supplemented with 50g PS kg⁻¹. The antioxidant system is increased and reactive oxygen species decreased in chickens fed PS diets. Glycemia and cholesterol levels were significantly lower in the chickens fed PS diets than in those given diets without supplementation; the cholesterol levels was also significantly lower in the chickens fed LO diets than those fed LF diets. However, the LO diet affected the fresh meat quality by decreasing pH values of the meat at 24 h after slaughter. Adding PS to the LO diet improved the oxidative stability of the muscle at day 3 of refrigerated storage. The use of LO instead of LF favourably modulates the FA profile of meat, by increasing both linoleic and α -linolenic acid content and reducing the n-6/n-3 polyunsaturated FA ratio, atherogenic and thrombogenic index.

Key Words: *Cucurbita pepo*, broiler, fatty acid, muscle, meat quality

Effets des régimes alimentaires contenant de l'huile de lin ou de la graisse animale et complétés par des graines de citrouille sur l'état oxydant, les métabolites du sérum sanguin, les performances de croissance et la qualité de la viande des poulets de chair. Cette étude examine l'effet de deux graisses alimentaires, à savoir l'huile de lin (HL) et

le saindoux (SD) et la supplémentation en graines de citrouille (GC) sur performance, état oxydant et métabolites du sang des poulets de chair, ainsi que la composition, la stabilité à l'oxydation et le profil d'acides gras de la chair de poulet. Quatre-vingt-seize poulets de 64 jours ont été répartis au hasard en quatre groupes de 24 poulets de chair chacun (huit poulets de chair par cage et trois cages par traitement) et ont reçu un régime HL, un régime HL enrichi de 50 g GC kg⁻¹, un régime de SD et un régime de SD supplémenté avec 50 g de GC kg⁻¹. Le système antioxydant est augmenté et les espèces réactives d'oxygène ont diminué chez les poulets nourris avec GC. La glycémie et le taux de cholestérol étaient significativement plus faibles chez les poulets de chair nourris avec des régimes GC que chez ceux qui avaient reçu un régime sans supplémentation; le taux de cholestérol était également significativement plus faible chez les poulets de chair nourris avec HL que chez avec SD. Cependant, le régime HL a affecté la qualité de la viande fraîche en diminuant les valeurs de pH de la viande 24 h après l'abattage. L'ajout de GC au régime HL a amélioré la stabilité oxydante du muscle au jour 3 de stockage réfrigéré. L'utilisation de HL au lieu de SD module favorablement le profil d'acides gras de la viande, en augmentant la teneur en acide linoléique et en acide α -linoléique et en réduisant le rapport d'acides gras polyinsaturé n-6/n-3, l'indice athérogène et thrombogène.

Mots clés: *Cucurbita pepo*, poulet de chair, acide gras, muscle, qualité de la viande

Abbreviations: AI, atherogenic index; ALA, α -linolenic acid; BAP, Antioxidant potential test; FA, fatty acid; LA, linoleic acid; LF, lard fat; LO, linseed oil; LP-Cholox, Lipoperoxides test; MUFA, monounsaturated fatty acid; OA, oleic acid; OXI, Oxi-adsorbent test; PA, palmitic acid; PS, pumpkin seed; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; S/P, saturation index, SFA, saturated fatty acid; TBARS, thiobarbituric acid reactive substances; TI, thrombogenic index.

INTRODUCTION

An increased awareness of the relationship between human diet and disease prevention, has promoted interest in improving the nutritional quality of animal food products through nutritional strategies. Particular attention has been paid to the fat composition of these products.

The consumption of saturated fatty acids (SFAs) contributes to the increase of serum cholesterol and low-density lipoprotein levels, which are implicated in the pathogenesis of cardiovascular diseases (Szostak-Węgierek et al. 2013). On the other hand, polyunsaturated fatty acids (PUFAs), especially long-chain n-3, elicit several nutritional benefits in the human body (Sanders 2014). According to Simopoulos (2002), the low cardiovascular mortality in Mediterranean countries is due to a diet rich in PUFAs, with a n-6/n-3 ratio ranging from 2:1 to 1:1, compared to diet

rich in SFA and with a n-6/n-3 ratio ranging from 15:1 in Northern European diets to 16.7:1 in Northern American diets.

In a study that assessed European diets, Hulshof et al. (1999) showed that 21% of total fat intake came from meat and meat products. As the current n-3 PUFA consumption in most Western countries is less than the recommended values, the aim of researchers is to produce meat products with a more favourable fatty acid (FA) profile for the health of consumers (González-Esquerra and Leeson 2001).

In monogastric animals, dietary FAs have a direct effect on the lipid composition of products derived from them, and the amount of PUFAs in tissues can be increased by increasing dietary levels of PUFAs (Woods and Fearon 2009).

Poultry meat is considered a healthy product by consumers because of its nutritional characteristics; it contains a good amount of protein and a low fat content, in which the fraction of PUFAs predominates, compared to other meat products (Bonoli et al. 2007). According to Nieto and Ros (2012) cereal-based diets commonly offered to poultry, mainly provide n-6 PUFAs and only a small amount of n-3 PUFAs. Therefore, several studies were performed in an attempt to increase the nutritional quality of poultry meat using dietary supplementation with lipid sources rich in n-3 PUFAs (Zduńczyc and Jankowsky 2013).

However, the increase in the degree of FA unsaturation reduces the oxidative stability, resulting in a reduced shelf life and quality of the meat (González-Esquerra and Leeson 2001). Lipid oxidation is therefore a major cause of deterioration in the meat quality and is a limiting factor for the PUFAs to serve as nutritionally beneficial lipids in functional foods (Chaiyasit et al. 2007).

An interesting strategy to prevent or reduce the oxidative process in PUFA-enriched meat is the inclusion of antioxidants via the animal feeds. Antioxidants are chemicals that neutralize free radicals, thus preventing them from causing damage to cells. Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tertiary butyl hydroquinone have been widely used in the past as additives to meat products, but the potential toxicological effects have now restricted their use. The demand by consumers for natural antioxidants, particularly of plant origin, has therefore increased (Falowo et al. 2014; Shah et al. 2014). In recent years, several researchers have studied the effects on poultry products of diets supplemented with natural antioxidants (Nkukwana et al. 2014; Karre et al. 2013).

Pumpkin (*Cucurbita pepo*) seeds (PS) are a good source of natural antioxidants due to the presence of tocopherols, phytosterols and phenolic compounds (Patel 2013; Veronezi and Jorge 2012; Gohari-Ardabili et al. 2011; Stevenson et al. 2007), and are widely used in traditional medicine due to their beneficial anti-diabetic, anti-cancer, and anti-bacterial actions (Caili et al. 2006). These seeds are generally considered to be agro-industrial wastes, although in

some parts of the world they are consumed raw, roasted or cooked, but only at a domestic scale (Patel 2013). There is a growing interest for a broader use of PS thanks to their nutraceutical properties.

With the purpose of increasing the nutritional quality of the meat, the objective of this study was to investigate the effects of two dietary fats (linseed oil and lard) and PS supplementation (50 g kg⁻¹ of diet) on FA profile and oxidative stability of broiler breast meat, assuming that the linseed oil in the diet could result in an increase of oxidation in fresh meat, while PS could protect broiler breast meat from this effect.

MATERIALS AND METHODS

Animals and Diets

This study was carried out in accordance with the regulations of the Institutional Review Board for animal use and ethics of the Department of Veterinary Sciences, University of Turin.

A total of ninety-six 64 d-old female naked neck chickens were randomly distributed into four groups of 24 chickens each (eight chickens per cage, three cages per treatment) under controlled environmental conditions. Each cage measured 1.5 x 1.5 m and was equipped with a food trough and a watering provision. Experimental diets and clean drinking water were offered *ad libitum* for 49 d. Feed consumption per replicate was measured and each replicate was weighed weekly to evaluate growth performance. Mortality rates were recorded daily. Four different diets were formulated (Table 1), according to specific requirements for meat chickens (National Research Council 1994), namely a linseed oil diet (LO), a linseed oil diet supplemented with 50g PS kg⁻¹ (LO+PS), a lard diet (LF) and a lard diet supplemented with 50g PS kg⁻¹ (LF+PS). PS were purchased from Tavi S.p.A. (Genova, Italy). Each mixed feed was stored in silos sheltered from light to avoid self-oxidation of lipid components and was offered in flour form.

Slaughter procedures and sample collection

Immediately before slaughter, blood samples were collected into 5-ml heparin vacuum tubes and centrifuged at 3000 rpm for 10 min at room temperature. Serum was then separated and stored at -70 °C until analysis.

Then chickens were slaughtered according to current standards (EC Regulation 2009) in a poultry slaughterhouse. The *pectoralis major* muscle was removed from carcasses and divided into two parts: one part was used to measure pH and colour, and to assess thiobarbituric acid reactive substances (TBARS), the other part was frozen at -20 °C and freeze-dried to determine FA profile.

Analysis of oxidative status

Biological antioxidant activity and the reactive oxygen metabolites in serum were determined using Biological Antioxidant Potential (BAP) test, Oxi-Adsorbent (OXI) test, and Lipoperoxides (LP-Cholox) test run on an automated analyser (Free Carpe Diem, Diacron International, Grosseto, Italy) and using commercial kits (Diacron International) according to the manufacturer's instructions.

The BAP test exploits the ability of biological antioxidants to reduce ferric iron to ferrous iron which, on reacting with thiocyanate derivative solution, causes a decolouration, which intensity is measured photometrically at 505 nm (Pasquini et al. 2008). The results are expressed as $\mu\text{mol L}^{-1}$. In a study conducted in humans, Maruoka et al. (2013) classified BAP test results into two groups: normal ($>2200 \mu\text{mol L}^{-1}$) and stressed ($<2200 \mu\text{mol L}^{-1}$), indicative of a condition of oxidative stress by lowering of antioxidant defenses.

The OXY-Adsorbent test allows the colorimetric assessment of the capacity of serum sample to oppose to a massive oxidant insult induce from a HClO solution. According to the manufacturer's recommendations, 10 μl of serum diluted 1:100 with distilled water were mixed with 1 ml of oxidant solution (HClO) and 10 μl of a chromogenic mixture (N,N diethylparaphenyldiamine). The absorbances were measured immediately at 505 nm or 546 nm (Trotti et al. 2001). Results are expressed as $\mu\text{mol HClO L}^{-1}$.

The LP-Cholox test evaluates lipid peroxidation, an important marker of oxidative stress. The test is based on the ability of peroxides to promote the oxidation of ferrous iron to ferric iron. The ferric iron bound to thiocyanate develops a coloured complex that can be measured spectrophotometrically. The increase in absorbance is directly proportional to the concentration of lipoperoxides in the sample, and the values are related to specific standard solution (Macri et al. 2015).

Biochemical parameters

Biochemical analysis was carried out on serum samples and levels of glycemia, cholesterol, triglycerides, and albumin were determined by an automated clinical biochemistry analyzer (ILab Aries analyzer, Instrumentation Laboratory, Milan, Italy) using commercial dedicated kits (Instrumentation Laboratory).

Proximate analysis

The composition of the PS and diets was analysed according to AOAC procedures (AOAC 1990) to determine dry matter, total N content, ash by ignition to 550°C, and gross energy by means of an adiabatic bomb calorimeter (IKA C7000, Staufen, Germany). Neutral detergent fibre without sodium sulphite or α -amylase, and acid detergent fibre, as described by Van Soest et al. (1991), expressed as being exclusive of residual ash, were determined on the experimental diets. Lipid extraction was performed on PS, diets and meat samples according to Hara and Radin (1978). Raw muscle samples were weighed, dried at 125°C for 5 h and reweighed to determine the water content. Freeze-dried muscle

samples were analysed to determine the total nitrogen content using a nitrogen analyser (Rapid N III, Elementar Analysensysteme GmbH, Germany), according to the Dumas method, and the crude protein was calculated as total N $\times$ 6.25.

pH and colour measurement

The pH of the breast muscle was measured in duplicate at 45 min and 24 h after slaughter, using a Crison portable pH-meter equipped with a spear-type electrode and an automatic temperature compensator probe (Crison Instruments, S.A., Alella, Spain). At 24 h after slaughter, meat colour was measured on a freshly cut surface of the muscle at room temperature (20°C) using a Minolta Colorimeter CR-331C (Konica Minolta, Tokyo, Japan) (25 mm measuring area, 45° circumferential illumination/ 0° viewing angle geometry) with the D65 illuminant and a 2° standard observed angle. Colour measurements are reported as lightness (L*), redness (a*), and yellowness (b*) in the CIELAB colour space model (CIE 1976). The colour values were obtained from the average of three readings per meat sample.

TBARS assay

Breast muscle was stored under refrigeration (at 0 to 4°C) in order to evaluate lipid oxidation of meat at three storage times: 1, 3 and 9 days after slaughter. Lipid oxidation of the meat was determined through the TBARS assay according to Peiretti et al. (2015), and was performed for each meat sample in triplicate.

Fatty acid determination

FAs were analysed as their methyl esters. Analysis was carried out by gas chromatography, as reported by Peiretti et al. (2007). The saturation (S/P), atherogenic (AI) and thrombogenic (TI) indexes were calculated according to Ulbricht and Southgate (1991) as follows:

$$S/P = (C14:0 + C16:0 + C18:0) (\Sigma MUFA + \Sigma PUFA)^{-1}$$

$$AI = (C12:0 + 4 \times C14:0 + C16:0) (\Sigma MUFA + \Sigma (n-6) + \Sigma (n-3))^{-1}$$

$$TI = (C14:0 + C16:0 + C18:0) (0.5 \times \Sigma MUFA + 0.5 \times \Sigma (n-6) + 3 \times \Sigma (n-3) + \Sigma (n-3) / \Sigma (n-6))^{-1}$$

where MUFAs and PUFAs are monounsaturated fatty acids and polyunsaturated fatty acids, respectively.

Statistical analyses

The experimental unit of growth performance parameters was considered as each cage, while the individual animal was the experimental unit for meat quality traits, composition of FAs and TBARS. Data were analysed as a factorial arrangement of two dietary fat sources (linseed oil vs lard) and absence or presence of PS in the mixed feed (0 vs 50 g kg⁻¹). Data were evaluated by means of the Linear Mixed Model procedure of the SPSS software package (version

11.5.1 for Windows, SPSS Inc., USA) and were presented as the means of each group and the standard error of the means (s.e.m.), together with the significance levels of the main effects.

RESULTS AND DISCUSSION

Chemical composition and FA profiles of PS and diets

The chemical composition and FA profile of PS is reported in Table 2. PS are characterized by a high proportion of linoleic acid (LA, C18:2n-6) and oleic acid (OA, C18:1n-9), which represent about 80% of total FA content.

The chemical composition of diets is similar in crude protein and gross energy content, even though the experimental diets differ in the lipid source and the PS content (Table 3). As expected, the diets containing LF have higher palmitic acid (PA, C16:0), stearic acid (C18:0) and OA contents than those of the diets containing LO, while α -linolenic acid (ALA, C18:3n-3) is the most abundant FA in the diet with LO, but the ALA levels decreased with the inclusion of PS (Table 4). OA and LA content increased in both experimental diets supplemented with PS.

Growth performances

Results on growth performances are given in Table 5. Parameters were significantly influenced by supplementation of PS and not by dietary fat.

Regarding PS supplementation, this result is in disagreement with a study by Martínez et al. (2010a), who demonstrated that including 100 g kg⁻¹ of PS in the diet of broilers did not influence the final live weight, total feed intake or feed conversion, while Hajati and Hassanabadi (2011) reported that PS oil supplementation, of up to 50 g kg⁻¹ diet, did not have any significant effect on growth performances of broilers, but when it was added at a quantity of 100 g kg⁻¹ diet, it negatively affected growth performances. In a study conducted by Suleiman and Uko (2010), broilers that were fed diets supplemented with 225 g kg⁻¹ diet of raw or autoclaved PS significantly decreased ($P < 0.05$) consumption of feed compared to broilers fed a diet without PS, while weight gain and feed utilization was not influenced by this supplementation. The use of dietary PS in combination with other herbal substances can improve growth performance of broilers, as shown by Tabari et al. (2015). These authors demonstrated that supplementation of PS oil (0.5 g kg⁻¹ of diet) in combination with nettle root improved the final live weight and feed conversion ratio of broilers.

Regarding dietary fat sources, in our experiment, the type of lipid content in the diet did not influence growth performances. This is in agreement with Crespo and Esteve-Garcia (2001), but it is in contradiction with Tufarelli et al. (2015), who found that the incorporation of different lipid sources influenced growth performances in broilers.

Oxidative status

The BAP test estimates the Biological Antioxidant Potential, determined by concentration of major component about serum antioxidant barrier, such as vitamin C and E, uric acid and bilirubin. Instead, the OXY-Adsorbent test evaluates the structural component concentration of serum antioxidant barrier characterized by antioxidants with high molecular weight and whose recovery time is slower, in comparison to substances included in the BAP determination. Therefore, the use of both tests allows to evaluate the cumulative antioxidant capacity rather than the only that relating to the time of collection (Abuelo et al. 2013).

Both dietary fat and PS inclusion significantly influenced results on oxidative status of chickens fed experimental diets (Table 6). The BAP test and the OXI-Adsorbent test showed that blood antioxidants concentration significantly decreased ($P < 0.005$) in chickens fed diets with LO and significantly increased ($P < 0.005$) in chickens fed diets with PS supplementation.

The low antioxidants concentration of blood in the LO group is probably due to their consumption; indeed, the high concentration of PUFAs in this oil results in an increased susceptibility against free radicals attack, that involve in a production of a wide variety of reactive oxygen species (ROS). Despite its remarkable efficiency, the body endogenous antioxidant system does not suffice to contrast lipid peroxidation when the pro-oxidizing processes increase (Carocho and Ferreira 2013). In agreement with this result, Husvth et al. (2000) reported that feeding diet with oil rich in PUFAs, decreased blood total antioxidant status and serum glutathione content of experimental broilers.

Dietary PS supplementation can enhance the body's antioxidant defences. The observed range of BAP data reported in our study are slightly lower than those reported in broilers fed diet supplemented with organic selenium, an essential element with important role in antioxidant enzymes for protection against oxidative stress (Celi et al. 2013). In a study conducted in broilers fed diets supplemented with grape seed extract, Azad et al. (2013) evaluated the improvement of BAP levels compared to control diets, probably due to the high concentration in polyphenols of this extract.

The OXY Adsorbent test values obtained in our study are not in agreement with previous range reported by Trotti et al. (2001) in humans. The OXY Adsorbent test was also evaluated in chickens (Castellini et al. 2002; Castellini et al. 2006) and thoroughbreds (Piccione et al. 2007) and the difference may be explained by diversities in the antioxidant plasma components between different species (Ninfali and Aluigi 1998). The values obtained in these studies were higher compared to those of our research.

The LP-Cholox test showed that lipoperoxides were significantly decreased ($P < 0.005$) in serum of chickens fed LF diets in comparison with LO diets and in serum of chickens fed PS diets compared with un-supplemented diets (Table 6). The lipoperoxides are terminal oxygenated compounds derived from lipid peroxidation, especially from

cholesterol and PUFA, and their serum level are used as an indicator of systemic oxidative stress in humans and experimental animals (Repetto et al. 2012).

In our study, we evaluated an high oxidative stress levels in the serum of chickens fed diet containing LO. On phytochemical screening, PS revealed the presence of phenolics as major compounds and this characteristics might contribute to its antioxidant activity (Peiretti et al. 2016). In agreement with these findings, some research have shown that the administration of PS reduces the degree of lipid peroxidation in the tissues of experimental animals. Makni et al. (2011) reported that diet supplemented with flax and pumpkin seed mixture in alloxan diabetic rats enhanced antioxidant enzymes activity and glutathione plasma levels and decreased plasma and liver malonaldehyde concentration. Eraslan et al. (2011) showed that the administration of PS oil alleviate the aflatoxin-induced lipid peroxidation in all of the tissues examined of mice received aflatoxin at a dose of 625 $\mu\text{g kg}^{-1}$ of body weight per day and PS oil at a dose of 1.5 ml kg^{-1} of body weight per day for a period of 21 days.

Therefore, the concomitant increase of BAP and OXY concentration and the decrease of lipoperoxides level in serum found in our study, suggest that the antioxidant system of chickens fed PS could have coped with an overproduction of pro-oxidant compounds from PUFA.

Blood serum metabolites

Results of serum biochemical analysis are shown in Table 6. Glycemia and cholesterol levels were significantly lower ($P < 0.005$) in the serum of chickens fed on the PS-supplemented diets than in those fed diet without supplementation, regardless of the type of fat used.

Our observation of a glycemia-lowering effect of PS in chickens is consistent with findings from previous research in other animals and humans (Perez-Gutierrez 2016). Pumpkin seed exhibited hypoglycaemic and hypocholesterolemic activity in alloxan-induced diabetic rats (Sedigheh et al, 2011) and rabbits (Caili et al., 2006) and in humans affected by *diabete mellitus* (Kwon et al. 2007). The detailed mechanism of antidiabetic action of PS remains to be clarified. Different components were isolated to investigate their hypoglycemic capacity: in a human clinical study, the administration of water-extracted pumpkin polysaccharides for four weeks reduced both urinary and plasma glucose (Shi et al., 2003). Additionally, Teng and Chen (2016) showed that phenolic phytochemicals of PS have an anti-diabetic effect and act as α -glucosidase inhibitor.

Cholesterol levels were also significantly lower ($P < 0.005$) in the serum of chickens fed LO diets than in those fed LF diets and the lowest value was obtained in chickens fed LO+PS diet. Neither triglyceride nor albumin levels were significantly influenced by dietary fat or by PS supplementation. Pumpkin seeds oil has been reported to possess also cholesterol lowering effect and these results are in agreement with the observations of previous researchers (Fruhworth and Hermetter, 2007). Reduction on serum total cholesterol was observed in alloxan-induce diabetic rats

applied with pumpkin powder (Makni et al. 2011; Sedigheh et al. 2011), while Shobana and Jayachitra (2015) investigated the hypolipidemic activity of pumpkin ethanolic extract in rats.

Our study shows that diets high in PUFA content reduces serum concentration of total cholesterol. Other research also reported lower plasma cholesterol level in broilers fed diets with lipid sources rich in PUFA than other rich in SFA. Febel et al. (2008) reported that plasma total cholesterol level in broilers fed diets with added sunflower oil, soybean oil or linseed oil were significantly lower than those of broilers fed diet supplemented with lard. In a study of Crespo and Esteve-Garcia (2003), lower plasma cholesterol level was lower in broilers fed diets with sunflower oil or linseed oil with respect to those fed tallow or olive oil. The decrease in cholesterol concentration may be explained by suppression of hepatic cholesterol synthesis and to enhance hepatic cholesterol breakdown. Dietary n-3 PUFAs has been reported to increase the hepatic bile acid synthesis by up-regulation and to decrease blood levels of cholesterol by down-regulation of specific enzymes in rats (Oh et al. 2015). Probably the lowering effect of higher n-3 PUFA content on serum cholesterol found in chickens fed LO diets could reflect this inhibition.

Meat quality traits

The meat quality traits of chickens fed experimental diets are shown in Table 7. Dry matter, lipid and crude protein contents and color parameters of breast muscle did not differ significantly. The pH of the meat immediately after slaughter was identical for all four dietary groups, while the pH of the meat at 24 h after slaughter was significantly increased ($P<0.05$) in chickens fed a diet with LF in comparison with LO, while PS supplementation did not affect pH after 24 h. The pH value plays a key role in maintaining the meat quality during storage, and depends on the muscle energy metabolism (Gaafar et al. 2014). The reduced pH of meat in chickens fed with LO was in agreement with Betti et al. (2009), who reported that administering flaxseed in the diet strongly decreased the ultimate pH of breast meat. These authors reasoned that the decrease of the pH in the meat was due to the accumulation of lactic acid derived from the activation of adenosine monophosphate protein kinase, an important enzyme involved in regulating glycolysis, which can be activated by ROS (Carling 2004). Another study, by Zhang et al. (2011), showed that oxidative stress in live broilers accelerated post-mortem glycolysis, causing a reduction in the pH value of the meat.

Lipid oxidation of the meat

Susceptibility of meat to lipid oxidation increased during storage for all dietary groups (Table 8). Both dietary fat and PS inclusion influenced TBARS content of meat at day 3 of storage: in the LF groups, the TBARS concentrations were significantly lower ($P<0.05$) than those of the LO groups, and groups supplemented with PS showed a significant decrease ($P<0.05$) of TBARS values compared with groups without PS. At day 9 of refrigerated storage, TBARS values were not significantly different between dietary groups, probably because the oxidation products were so high that the

antioxidants present in the PS were unable to prevent their formation. Oxidative damage to lipids occurs due to an imbalance between the production of ROS and the animal's defence mechanisms (Morrissey et al. 1998).

The high oxidative deterioration of meat in the LO group is due to its high concentration of PUFAs. In fact, LO is a rich source of ALA and, therefore, its use here in the broiler diet increased the degree of unsaturation of fatty acids and, as a result, the oxidative stress of the meat. This result is in agreement with Betti et al. (2009), who reported that lipid susceptibility to oxidation increased in breast broiler meat upon increasing the LO inclusion in animal diets. In our study, breast muscle of chickens fed LO+PS diet showed the lowest increment of TBARS values during storage (+ 0.27 mg Malondialdehyde kg⁻¹ meat) compared to the other groups (+ 0.35, + 0.39 and + 0.41 for LO, LF and LF+PS, respectively).

PS were revealed to have a high antioxidant activity because the oil was rich in tocopherols (Murkovic et al. 1996; Stevenson et al. 2007), carotenoid pigments (Murkovic et al. 2002) and small quantities of phenolic compounds (Zduńcyc et al. 1999). Tocopherols are the major lipid soluble antioxidants, they break the chain of lipid peroxidation in cell membranes and prevent the formation of lipid hydroperoxides (Di Mascio et al. 1991). Several studies have shown that tocopherol supplementation in broiler diets reduced the lipid peroxidation in fresh meat samples (Guo et al. 2001; Grau et al. 2001; Voljc et al. 2011).

FA profile of the meat and relation to nutritional quality

The FA composition of breast muscle of chickens fed experimental diets is shown in Table 9. The FA composition of the meat from the chickens of the four different dietary groups can be attributed to the FA composition of their respective diets.

Dietary fat significantly influenced the proportion of all the FAs analysed, with the exception of margaric acid (C17:0) and n-6 PUFA content. This is in agreement with previously studies that showed a correspondence between dietary FA composition and FA composition of broiler meat (Scaife et al. 1994; Nyquist et al. 2013). In particular, the LO groups showed a higher content of both LA and ALA than those of the LF groups, which instead had the highest content of OA. Few studies have been carried out into the effects of linseed or LO-supplemented diets on FA composition of poultry meat (Kouba and Mourot 2011). In agreement with our results, Lin et al. (1989) reported that meat from broilers fed linseed oil contained a higher amount of n-3 PUFAs and n-6 PUFAs.

Supplementation with PS significantly affected the FA composition of breast muscle of chickens (Table 9). The muscles from the LO+PS and LF+PS groups contained a significantly lower amount of PA, palmitoleic acid (C16:1n-7), octadecatrienoic acid (C18:3n-4), ALA, conjugated linoleic acid and eicosapentaenoic acid (C20:5n-3), than those of the LO and LF groups. Significantly higher concentrations of LA and arachidonic acid (C20:4n-6) were found in breast muscles of chickens fed diets supplemented with PS. There are no reports in the literature on the effects

of dietary PS inclusion on the FA profiles of broiler meat. In a study by Celik et al. (2011), it was observed that ALA and OA contents in egg yolks of layer hens increased with the increase of dietary PS oil after an 8 week-long supplementation, thus increasing egg quality.

The nutritional quality of meat can be assessed in terms of the SFA, MUFA, PUFA, PUFA n-3, PUFA n-6 contents and their ratios, along with the saturation (S/P), atherogenic (AI) and thrombogenic (TI) indexes (Table 9). Breast meat of animals fed LO diets showed higher n-3 PUFA levels than those found in meat of LF groups, while the breast meat of chickens fed diets supplemented with PS presented significantly lower n-3 PUFA levels and significantly higher n-6 PUFA levels than those of birds fed diets without PS. The effect of PS on the FA composition of breast meat is due to its high amount of unsaturated FAs and in particular the n-6 PUFA content, as reported by Gohari-Ardabili et al. (2011). The n-6/n-3 PUFA ratio was influenced by both dietary fat and PS supplementation in the diet, and it increased from 0.98 to 1.71 in meat of chickens fed diets with LO and LO+PS, respectively, and from 3.9 to 14.2 in meat of chickens fed LF and LF+PS diets, respectively. These ratios obtained from LO and LO+PS groups are in line with dietary recommendations for human nutrition (Simopoulos 2002), while those obtained from LF and LF+PS group were very high. The atherogenic and thrombogenic indexes significantly decreased in breasts of chickens fed LO diets compared to chickens fed LF diets. In addition, LO+PS and LF+PS groups showed that AI was significantly lower, and TI was significantly higher than LO and LF groups, respectively (Table 9). The AI decreased with PS inclusion in the diet, as previously reported by Aguilar et al. (2011) in serum broilers fed up to 100 g kg⁻¹ of PS meal. However, the AI values reported are much higher than those obtained in our study; in the serum of broilers fed a diet supplemented with 33 g of PS meal kg⁻¹ of diet, the AI value was 1.92 against 0.29 and 0.40 obtained in our study from chickens fed LO+50g PS kg⁻¹ of diet and LF+50g PS kg⁻¹ of diet, respectively. Similar results were obtained by Martínez et al. (2010b) in serum from laying hens in which the AI significantly decreased with increased content of PS meal in the feed.

In conclusion the use of linseed oil instead of lard as a lipid source in broiler feed reduces serum cholesterol level, favourably modulates the FA profile of breast muscle, by increasing ALA and LA content and reducing the n-6/n-3 PUFA ratio, AI and TI indexes, without causing adverse effects on growth performances. However, dietary LO reduces serum antioxidant system, increases ROS production and affected the fresh meat quality by decreasing pH values of the meat 24 h after slaughter. The addition of PS to the LO diet reduce serum glycemia and cholesterol level, reduces ROS production and increased the serum antioxidant system and muscle oxidative stability at day 3 of refrigerated storage, which improved the meat quality. Additional research is required to determine if PS dietary supplementation higher than 50 g kg⁻¹ could further increase the shelf-life of the meat.

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Table 1. Ingredients (%) of experimental diets.

	Diet/group ^a			
	LO	LO+PS	LF	LF+PS
Wheat	60.0	59.0	60.0	59.0
Soybean meal (46% CP)	27.0	21.7	27.0	21.7
Lucerne	2.0	5.8	2.0	5.8
Pumpkin seeds	0.0	5.0	0.0	5.0
Linseed oil	8.5	6.0	0.0	0.0
Lard	0.0	0.0	8.5	6.0
Vitamin–mineral premix ^b	2.5	2.5	2.5	2.5

Note: ^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

^b Per kg of diet: vitamin A 25000 IU, vitamin D3 10000 IU, a-tocopheryl acetate 70 mg, niacin 56 mg, vitamin B1 4 mg, vitamin B2 8 mg, vitamin B6 4 mg, vitamin B12 0.04 mg, vitamin K3 7 mg, folic acid 1 mg, biotin 0.3 mg, dicalcium phosphate 1000 mg, ferric oxide 600 mg, FeSO₄·H₂O 331.97 mg, CuSO₄·5H₂O 151.3 mg, MnO₂ 128.48 mg, KJ 1.79 mg, Na₂SeO₃ 1.53 mg, ZnO 130.2 mg.

For Review Only

Table 2. Chemical composition (based on dry matter) and fatty acid profile of pumpkin seeds (Mean $\pm$ s.d.; n=2).

Dry matter (%)	93.9 $\pm$ 0.02
Organic matter (%)	95.6 $\pm$ 0.36
Crude ash (%)	4.40 $\pm$ 0.36
Ether extract (%)	54.3 $\pm$ 0.30
Crude protein (%)	37.2 $\pm$ 0.26
Gross energy (MJ/kg DM)	29.0 $\pm$ 0.39
C16:0 (% total FAME)	12.0 $\pm$ 0.18
C17:0 (% total FAME)	0.84 $\pm$ 0.27
C18:0 (% total FAME)	7.16 $\pm$ 0.23
C18:1n-9 (% total FAME)	34.1 $\pm$ 0.44
C18:2n-6 (% total FAME)	45.1 $\pm$ 0.10

For Review Only

Table 3. Chemical composition (based on dry matter) of the experimental diets (Mean±s.d.; n=4).

	Diet/group ^a			
	LO	LO+PS	LF	LF+PS
Dry matter (%)	89.7±0.07	90.4±0.64	89.9±0.18	90.2±0.17
Organic matter (%)	94.4±0.28	94.5±0.24	94.7±0.12	94.7±0.14
Crude ash (%)	5.55±0.28	5.48±0.24	5.31±0.12	5.27±0.14
Neutral detergent fibre (%)	12.9±0.26	14.1±0.60	13.9±1.36	14.1±0.22
Acid detergent fibre (%)	6.06±0.13	6.31±0.45	5.06±0.44	6.57±0.20
Lipid (%)	6.23±0.78	4.60±0.29	6.06±1.17	5.42±0.50
Crude protein (%)	22.7±0.23	23.4±1.68	22.5±1.13	21.6±1.28
Gross energy (MJ kg DM ⁻¹)	19.2±0.14	19.5±0.15	19.6±0.18	19.6±0.16

Note:^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

For Review Only

Table 4. Fatty acid profile (% total FAME) of the experimental diets (Mean $\pm$ s.d.; n=4).

	Diet/group ^a			
	LO	LO+PS	LF	LF+PS
C14:0	0.00 $\pm$ 0.00	0.01 $\pm$ 0.02	0.81 $\pm$ 0.17	0.84 $\pm$ 0.02
C16:0	7.53 $\pm$ 0.66	8.03 $\pm$ 0.18	19.7 $\pm$ 1.86	20.2 $\pm$ 0.15
C16:1	0.24 $\pm$ 0.21	0.08 $\pm$ 0.00	1.46 $\pm$ 0.22	1.39 $\pm$ 0.02
C17:0	1.30 $\pm$ 0.06	0.47 $\pm$ 0.03	0.61 $\pm$ 0.26	0.46 $\pm$ 0.08
C18:0	3.89 $\pm$ 0.19	4.16 $\pm$ 0.07	10.2 $\pm$ 0.95	10.4 $\pm$ 0.31
C18:1n-9	18.6 $\pm$ 0.91	20.6 $\pm$ 0.16	33.6 $\pm$ 2.02	35.1 $\pm$ 0.30
C18:1n-7	1.14 $\pm$ 0.17	0.83 $\pm$ 0.03	2.52 $\pm$ 0.22	2.49 $\pm$ 0.01
C18:2n-6	23.5 $\pm$ 0.50	29.9 $\pm$ 0.64	20.4 $\pm$ 1.57	26.8 $\pm$ 0.58
C18:3n-4	1.94 $\pm$ 0.10	0.15 $\pm$ 0.03	0.49 $\pm$ 0.18	0.11 $\pm$ 0.02
C18:3n-3	39.8 $\pm$ 2.40	34.2 $\pm$ 0.97	9.85 $\pm$ 3.46	2.29 $\pm$ 0.27
C18:4n-3	1.99 $\pm$ 0.14	1.66 $\pm$ 0.04	0.44 $\pm$ 0.19	0.05 $\pm$ 0.00

Note:^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

For Review Only

Table 5. Performance of chickens fed the experimental diets (n=24).

	Diet/group ^a				<i>P</i> value		
	LO	LO+PS	LF	LF+PS	SEM ^b	Fat	PS
Number of cages	3	3	3	3			
Number of chickens/cage	8	8	8	8			
Mortality	1/24	0/24	1/24	1/24			
Initial weight (g)	1677	1623	1659	1649	9.7	0.840	0.113
Final weight (g)	2984	2238	2304	2335	96.6	0.081	0.039
Total feed consumption (g)	6800	4998	5218	5324	222	0.100	0.035
Total weight gain (g)	1307	615	645	686	92.1	0.067	0.047
Daily feed (g)	138.8	102.0	106.5	108.6	4.5	0.100	0.035
Daily weight gain (g)	26.7	12.6	13.2	14.0	1.9	0.067	0.047
Feed/ gain ratio (g g ⁻¹)	5.3	8.3	8.1	7.9	0.5	0.171	0.107

Note:^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

^b Standard error of mean.

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Table 6. Biological antioxidant potential (BAP), oxi-adsorbent test (OXI), lipoperoxides and blood serum metabolites in blood samples taken at slaughter (n=24).

	Diet/group ^a				<i>P</i> value		
	LO	LO+PS	LF	LF+PS	SEM ^b	Fat	PS
BAP ($\mu\text{mol L}^{-1}$)	2328	2474	2414	2489	12.3	0.002	<0.001
OXI ($\mu\text{mol HClO L}^{-1}$)	181	230	210	237	4.2	0.002	<0.001
Lipoperoxides ($\mu\text{mol L}^{-1}$)	667	501	455	351	19.4	<0.001	<0.001
Glycemia (mg dL ⁻¹)	221	219	222	219	0.27	0.501	<0.001
Cholesterol (mg dL ⁻¹)	102	89.7	150	130	3.9	<0.001	<0.001
Triglycerides (mg dL ⁻¹)	62.4	62.1	60.7	63.1	1.10	0.868	0.632
Albumin (g dL ⁻¹)	1.53	1.50	1.49	1.53	0.03	0.924	0.924

Note: ^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

^b Standard error of mean.

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Table 7. Meat characteristics of chickens fed the experimental diets (n=24).

	Diet/group ^a				<i>P</i> value		
	LO	LO+PS	LF	LF+PS	SEM ^b	Fat	PS
Dry matter (%)	28.2	27.6	28.0	27.6	0.15	0.743	0.097
Lipid (%)	3.28	3.26	3.56	3.92	0.14	0.087	0.520
Crude protein (%)	89.5	92.4	90.3	90.0	0.80	0.645	0.450
pH after 0 h	5.62	5.69	5.68	5.67	0.02	0.512	0.505
pH after 24 h	5.70	5.70	5.77	5.74	0.01	0.050	0.627
L* ^c	53.2	53.0	53.6	56.2	0.62	0.161	0.312
a* ^d	-2.03	-2.00	-2.28	-2.14	0.15	0.532	0.7799
b* ^e	6.28	5.44	5.84	6.32	0.17	0.972	0.702

Note: ^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.
^b Standard error of mean.
^c Lightness.
^d Redness.
^e Yellowness.

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Table 8. Content of thiobarbituric acid reactive substances (TBARS; mg Malondialdehyde kg⁻¹ meat) in the meat of chickens (n=24) fed the experimental diets after 1, 3 and 9 days of refrigerated storage.

	Diet/group ^a				<i>P</i> value		
	LO	LO+PS	LF	LF+PS	SEM ^b	Fat	PS
TBARS _{Day 1}	0.18	0.17	0.17	0.15	0.01	0.258	0.266
TBARS _{Day 3}	0.38	0.28	0.27	0.26	0.02	0.009	0.025
TBARS _{Day 9}	0.53	0.44	0.56	0.56	0.04	0.393	0.561

Note: ^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

^b Standard error of mean.

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Table 9. Fatty acid composition (% total FAME) and indexes related to human health in the meat of chickens (n=24) fed the experimental diets.

	Diet/group ^a				P value		
	LO	LO+PS	LF	LF+PS	SEM ^b	Fat	PS
C14:0	0.50	0.53	0.94	0.74	0.04	<0.001	0.112
C16:0	18.9	18.0	24.0	22.9	0.48	<0.001	0.012
C16:1n-7	1.50	1.38	2.31	1.78	0.08	<0.001	0.012
C17:0	1.55	1.28	1.13	1.78	0.20	0.951	0.634
C18:0	8.76	9.02	10.1	10.5	0.20	<0.001	0.360
C18:1n-9	26.8	26.6	35.3	33.1	0.77	<0.001	0.154
C18:1n-7	1.38	1.54	2.68	2.60	0.11	<0.001	0.661
C18:2n-6	17.1	22.3	15.4	18.7	0.54	0.001	<0.001
C18:3n-4	0.95	0.73	0.20	0.06	0.07	<0.001	<0.001
C18:3n-3	18.9	14.5	4.44	2.20	1.28	<0.001	<0.001
CLA ^c	0.87	0.65	0.15	0.06	0.06	<0.001	0.003
C20:1n-9	0.20	0.25	0.40	0.43	0.02	<0.001	0.076
C20:4n-6	1.66	2.84	2.70	5.21	0.39	0.016	0.008
C20:5n-3	0.81	0.51	0.15	0.06	0.06	<0.001	0.003
SFA ^d	29.8	28.8	36.3	35.8	0.68	<0.001	0.355
MUFA ^e	29.9	29.8	40.7	37.9	0.95	<0.001	0.135
PUFA ^f	40.4	41.5	23.0	26.3	1.52	<0.001	0.077
PUFA n-3 ^g	20.6	15.6	4.71	2.30	1.39	<0.001	0.001
PUFA n-6 ^h	18.8	25.1	18.1	23.9	0.66	0.276	<0.001
n-6/n-3 ⁱ	0.98	1.71	3.90	14.2	1.01	<0.001	<0.001
S/P ^l	0.40	0.39	0.55	0.53	0.01	<0.001	0.180
Atherogenic Index	0.30	0.29	0.44	0.40	0.01	<0.001	0.015
Thrombogenic Index	0.33	0.38	0.80	0.92	0.05	<0.001	0.014

Note: ^a LO=linseed oil diet; LO+PS=LO+pumpkin seed diet; LF=lard fat diet; LF+PS=LF+pumpkin seed diet.

^b Standard error of mean.

^c Conjugated Linoleic Acid.

^d Saturated Fatty Acids.

^e Monounsaturated Fatty Acids.

^f Polyunsaturated Fatty Acids.

^g Polyunsaturated Fatty Acids series n-3.

^h Polyunsaturated Fatty Acids series n-6.

ⁱ PUFA n-6/ PUFA n-3 ratio.

^l Saturated Fatty Acid/Unsaturated Fatty Acid ratio.