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Performance and egg quality of laying hens fed flaxseed: highlights on n-3 fatty acids, cholesterol, lignans and isoflavones

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Abstract:	Flaxseed is a rich source of -linolenic acid and phytoestrogens, mainly lignans, whose metabolites (enterolactone - ENL and enterodiol - END) can affect estrogens functions. The present study evaluated the influence of dietary flaxseed supplementation on reproductive performance and egg characteristics (fatty acids, cholesterol, lignans and isoflavones) of 40 Hy-line hens (20/group) fed for 23 weeks control diet (C) or the same diet supplemented with 10 % of extruded flaxseed (F). Flaxseed diet, had approximately 3 times the content of lignans than control diet (2,608.54 ng/g), mainly SDG (1,534.24 vs. 494.72 ng/g). When compared with the control group, hens fed flaxseed showed a similar deposition rate (72.0 % vs. 73.9 %) and egg yield. Furthermore, there was no effect of flaxseed on chemical composition of egg and on its cholesterol content. Estradiol was higher in the plasma of control group (1419.00 vs. 1077.01 pg/ml) probably due to the effect of flaxseed on phytoestrogen metabolites. The plasma lignans were higher in F hens, while isoflavones were lower, mainly due to the lower equol value (50.52 vs. 71.01 ng/ml). Similar trend was showed in eggs: F group had higher level of END and ENL whereas the equol was lower (198.31 vs. 142.02 ng/g yolk). The secoisolariciresinol (SECO) was the main lignan in eggs of F group and its concentration was 3 times higher then control eggs. Flaxseed also improved the n-3 long chain PUFA of egg (3.25 vs. 0.92 mg/g egg), mainly DHA, however, its oxidative status (TBARS) was negatively affected. In conclusion, 10% dietary flaxseed did not affect the productive performance of hens, and the yolk cholesterol concentration, whereas the lignans and n-3 PUFA content of eggs improved. Further details on the competition between the different dietary phytoestrogens and their metabolites (estrogen, equol, END, ENL) should be investigated.
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Performance and egg quality of laying hens fed flaxseed: highlights on n-3 fatty acids, cholesterol, lignans and isoflavones

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Short title: **Dietary flaxseed in laying hens and egg quality**

Abstract

Flaxseed is a rich source of α-linolenic acid and phytoestrogens, mainly lignans, whose metabolites (enterolactone - ENL and enterodiol - END) can affect estrogens functions. The present study evaluated the influence of dietary flaxseed supplementation on reproductive performance and egg characteristics (fatty acids, cholesterol, lignans and isoflavones) of 40 Hy-line hens (20/group) fed for 23 weeks control diet (C) or the same diet supplemented with 10 % of extruded flaxseed (F). Flaxseed diet, had approximately 3 times the content of lignans than control diet (2,608.54 ng/g), mainly SDG (1,534.24 *vs.* 494.72 ng/g).

When compared with the control group, hens fed flaxseed showed a similar deposition rate (72.0 % *vs.* 73.9 %) and egg yield. Furthermore, there was no effect of flaxseed on chemical composition of egg and on its cholesterol content.

Estradiol was higher in the plasma of control group (1419.00 *vs.* 1077.01 pg/ml) probably due to the effect of flaxseed on phytoestrogen metabolites.

The plasma lignans were higher in F hens, while isoflavones were lower, mainly due to the lower equol value (50.52 *vs.* 71.01 ng/ml). Similar trend was showed in eggs: F group had higher level of END and ENL whereas the equol was lower (198.31 *vs.* 142.02 ng/g yolk). The secoisolariciresinol (SECO) was the main lignan in eggs of F group and its concentration was 3 times higher then control eggs. Flaxseed also improved the n-3 long chain PUFA of egg (3.25 *vs.* 0.92 mg/g egg), mainly DHA, however, its oxidative status (TBARS) was negatively affected.

In conclusion, 10% dietary flaxseed did not affect the productive performance of hens, and the yolk cholesterol concentration, whereas the lignans and n-3 PUFA content of eggs improved.

Further details on the competition between the different dietary phytoestrogens and their metabolites (estrogen, equol, END, ENL) should be investigated.

Keywords: flaxseed; isoflavones; lignans; equol; egg quality

Implications

Phytoestrogens of flaxseed affect the estrogen metabolism of hens reducing the 17β-estradiol without any effect on the egg deposition rate. Hens fed flaxseed produce eggs with higher n-3 fatty acids level and also enriched in lignans.

Accordingly, these eggs could be considered a functional food, not only for the n-3 enrichment but also for phytoestrogen amount.

Further research should analyse the interaction between flaxseed lignans and isoflavones and detail mechanism of these compounds on the physiology and health of hens' reproductive apparatus.

Introduction

There has been an increasing interest in the use of flaxseed (*Linum usitatissimum L.*) to improve the nutritional quality of human food (Oomah, 2001). Flaxseed is also used in animal feeding as main source of alpha linolenic acid (ALA) which is the precursor of important n-3 derivatives (eicosapentaenoic, EPA and docosahexaenoic acid, DHA).

Moreover, flaxseed contains considerable amount of phytoestrogens, mainly lignans (Meagher and Beecher, 2000), recognized influencing the hormone metabolism, enzymes, protein synthesis, growth factors, malignant cell proliferation and angiogenesis (Lowcock *et al.*, 2013).

The lignans in question are pinoresinol (PINO), laricilresinol (LARI) and matairesinol (MATA); however, the more represented lignan is the secoisolariciresinol diglucoside (SDG) which is stored in the hull of the seed (Milder *et al.*, 2005).

Following ingestion, lignans are deglucosylated and then converted into enterolignans by intestinal microbiota (enterodiol, END and enterolactone, ENL) responsible for a large part of health benefit (Setchell *et al.*, 1981).

The structural similarity of END and ENL to estradiol, the most active estrogen in the body, allows these enterolignans to bind to estrogen receptors and exert weak estrogenic or anti-estrogenic effects (Carreau *et al.*, 2008) depending on dose and physiological state of animals. Similarly to humans, poultry possess both alpha- and beta-estrogen receptors and, thus, can efficiently bind END or ENL (Jenkins *et al.*, 1999) provided by dietary flaxseed metabolism.

Several studies in mammals (Prasad, 2016) have also shown that SDG reduces total and LDL-cholesterol of plasma and increases HDL-cholesterol.

Another class of phytoestrogens contained in flaxseed, the isoflavones, has a similar function and metabolic pathway of lignans. Daidzin, genistin, daidzein (the aglycone of daidzin) and genistein (the aglycone of genistin), although less represented than in soybean (Setchell *et al.*, 1981), are also found in flaxseed. An important metabolite of daidzein, produced by intestinal bacteria in some, but not in all, humans is the equol. It is retained that about 20-30 percent of North Americans and Europeans, who consume less soy than Asians, have the ability to produce equol (Setchell *et al.*, 2002). Equol possesses antioxidant properties (Kładna *et al.*, 2016) but it is mainly retained beneficial for its modulator properties on estrogen receptor, thus offering the beneficial effects of estrogen while not having the negative effects associated with it (Setchell *et al.,* 2002).

Considering that eggs are the food of animal origin more eaten in the world and that it is quite easy to modify their nutritional composition (Vaghefi, 2002), the aim of the study is to asses the long-term effect of dietary flaxseed on fatty acid profile, cholesterol, isoflavones and lignans of hen eggs. Productive performance and metabolic status of the hens were also investigated.

Material and methods

Reagents

All chemicals were analytical grade of highest purity or high performance liquid chromatography (HPLC) grade and purchased from the Sigma Chemical Company (St. Louis, MO, USA).

Diets

The diets were formulated according to the recommended nutrient requirements of hens (National Research Council, 1994). The control diet, was a standard corn-soybean diet; in the flaxseed diet the corn gluten meal and part of wheat bran were replaced by 10 % extruded flaxseed. Each diet was enriched of oyster shells in order to supply a suitable Ca contribution. The two diets were isonitrogenous and isocaloric; details of the ingredient and nutrient content are summarized in Table 1.

Study design

In this study, Hy-line hens, 10 months old and from the same hatch were randomly assigned to the 2 dietary groups (n=20 birds/group):

- Control;
- Flaxseed.

Feed and water were provided *ad libitum* for 23 consecutive weeks. For experimental reasons, the birds were housed in individual cages and maintained under the same photoperiod (16 light/ 8 dark), temperature and humidity. The study was carried out at the animal experimental section of University of Perugia (Italy). All procedures involving the care and handling of animals met the National Guidelines for Animal Care.

The hens were weighed at the beginning and at the end of the study. Feed intake and feed/weight conversion rate were calculated for each group. The baseline laying performance of the hens was measured for 2 weeks prior to the randomization to the different dietary treatments. All the eggs were collected, counted and weighed daily. The actual egg yield for each dietary group was determined by multiplying the egg production x egg weight. Feed efficiency was assessed by dividing total feed consumption by total egg mass. The percentage of egg-laying was calculated by dividing the number of laid eggs per number of animals.

127 Approximately 5 ml of blood was obtained at the end of the study when the hens were
128 electrically stunned (110 V; 350 Hz) and slaughtered as normally happens to the hens at
129 the end of productive career. The blood was collected into EDTA-coated tubes and
130 samples were centrifuged at 2,500 rpm for 15 min and the plasma separated and stored
131 frozen at -80 ºC for later analysis.
132
133 *Analytical methods*
134 *Egg characteristics:* twenty eggs per dietary group were randomly collected every week for
135 the determination of egg composition. The percentages of albumen, yolk, and shell were
136 calculated relatively to the whole egg weight.
137 Albumen consistency was tested by calculating the Haugh unit (HU) value of each egg
138 defined as follows: $HU=100 \log [H-g^{0.5} (30W^{0.37}-100)/100 +1.9]$, where H is the maximum
139 height of thick albumen in millimeters, W is the weight of the egg in grams, and g is a
140 constant (32.2) related to the constant of gravitation.
141 Yolk colour intensity was established using the Roche colour fan scale (1-15) (Vuilleumier,
142 1969). The composition of egg-yolks (n=10 eggs/group per week) was determined using
143 the procedures of the Association of Official Analytical Chemists (AOAC, 1995).
144
145 *Fatty acids profile of diets and egg yolks:* the fatty acid profile was determined by gas
146 chromatography following lipid extraction according to the method described by Folch *et*
147 *al.* (1957). In particular, 1 ml of lipid extract was evaporated under a stream of nitrogen and
148 the residue was derived by adding 3 ml of sulfuric acid (3 % in methanol). Following
149 incubation at 80 °C for 1 h, the methyl esters were extracted with petroleum ether and 1 µl
150 was injected into a gas chromatograph (Mega 2 - model HRGC; Carlo Erba, Milan, Italy)
151 equipped with a flame ionization detector. The fatty acid methyl esters (FAMEs) were
152 separated with an Agilent (J&W) capillary column (30 m × 0.25 mm I.D; CPS Analitica,

153 Milan, Italy) coated with a DB-wax stationary phase (film thickness of 0.25 mm). The
154 operating conditions used during the column injection were as follows: the temperatures of
155 the injector and detector were set at 270 °C and 280 °C, respectively, and the detector gas
156 flows were H_2 at 50 ml/min and air at 100 ml/min. The oven temperature was programmed
157 to provide a good peak separation as follows: the initial oven temperature was set at 130
158 °C; this temperature increased at a rate of 4.0 °C/min to 180 °C and was held for 5 min;
159 the temperature was then increased at a rate of 5.0 °C/min to 230 °C; the final temperature
160 was held for 5 min. Helium was used as a carrier gas at a constant flow rate of 1.1 ml/min.
161 Individual fatty acid methyl esters were identified by referring to the retention time of FAME
162 authentic standards. For the quantitative analysis was used C19:0 methyl ester, added
163 before extraction, as internal standard. The relative proportion of individual fatty acids was
164 expressed as g/kg of feed and mg/g of yolk.
165
166 *Lignans and isoflavones of diets, plasma and egg yolks:* the composition of the diets was
167 determined by reverse-phase HPLC-ESI/MS as described by Setchell and Cole (2003).
168 The extraction of lignans, isoflavones and their metabolites (ENL, END and equol) was
169 performed on 1 ml of sample or 1 g of lyophilized yolk; proteins were removed by
170 precipitation and subsequent centrifugation. N-hexane was added to the supernatant for
171 liquid/liquid separation. The aqueous residue was added with β-glucuronidase, after
172 correction of the pH of the solution to 5. The sample is left overnight at 37 °C. Therefore
173 the extraction of the lignans and isoflavones fraction was performed by two extractions
174 with ethyl acetate. The two solutions extracted were unified and dried, then taken in 1 ml of
175 deionized water for the subsequent purification by silica gel dispersion chromatography.
176 The eluate obtained from the purification was dried again and derivatized for analysis by
177 gas chromatography mass spectrometry (GC/MS, Varian mod. MS Saturn 2000, ITD). The
178 identification of individual constituents of the fraction occurs by direct comparison of mass

spectra obtained from samples with those of pure standards and with the mass spectra contained in libraries NIST92 and Wiley5.

Cholesterol content of plasma and egg yolk: total plasma cholesterol concentration was determined by HPLC methods. Briefly, all samples were mixed with 8 ml of chloroform: methanol (2:1, v/v) (Folch *et al.* 1957) and ultrasonicated at 30 % of intensity for 10s (model IKA® U50). The mixture was vortexed and filtered through Whatman paper (No.1) to eliminate cell debris. Next, 1.5 ml of distilled water containing 0.6 % of NaCl was added and the mixture was vortexed and allowed to stand at room temperature for 1 h before centrifugation at 500 x g for 10 min. The upper layer was suspended in 8 ml of chloroform: methanol: water containing NaCl (86:16:1, v/v/v) and re-extracted. The chloroform extracts were pooled and dried under a flow of nitrogen. The residue was dissolved in 200 µl of mobile phase and injected on the HPLC system (pump model PU-1580, equipped with an auto-sampler model AS 950-10 Tokyo, Japan) on a Waters Spherisorb C18 reversed phase analytical column (ODS-2, 5um particle size, 250 x 4.6 mm internal diameter; CPS analitica, Milan, Italy). The mobile phase was composed of acetonitrile: isopropanol (70:30, v/v) and released at a flow rate of 1.5 ml/min. Cholesterol was indentified using UV detector (model Jasco 2075 Plus, Tokyo, Japan) set at 210 nm and was quantified by using external calibration curves prepared with increasing amounts of pure standard solution in isopropanol (range 0.25 to 2.5 mg). The recovery was > 89% and the volume of injection was 20 µl.

Total cholesterol of eggs yolk was determined by HPLC with the same condition of plasma. About 0.1 g of lyophilized yolk was incubated at 50 °C for 60min in KOH (2% in ethanol) solution. After was extracted with n-hexane and the upper layer was dried under a stream of nitrogen and the residue was reconstituted in 1 ml of mobile phase and 50 µl were injected in HPLC.

205

206 *Plasma estradiol and oxidative status:* the 17β-Estradiol concentration (E2) in plasma was
207 assayed using a commercial 125I RIA kit (ICN Pharmaceuticals Inc., Diagnostic Division,
208 Costa Mesa, CA, USA). The limit of detection was 0.8 pg/ml and the intra- and inter-assay
209 coefficients of variation were <6 and <10 %, respectively.
210 The reactive oxygen substances (ROS) of the plasma were evaluated with a commercial
211 kit (Diacron, Grosseto, Italy) and are expressed as μmol H_2O_2/ml
212 The antioxidant power of plasma (AOP) was measured with a commercial kit (Diacron,
213 Grosseto, Italy) that evaluates the ability of plasma to oppose the massive oxidative action
214 of HClO solution. The AOP of the samples are expressed as nmol of neutralized HClO/ml.
215

216 *Statistical Analysis*
217 The effect of the diets on egg production performance, lignans, isoflavones, cholesterol
218 fatty acid profile and on egg quality were analyzed by one way ANOVA and significant
219 differences were determined using the post-hoc Bonferroni test at the level of $P<0.05$
220 (STATA, 2015).
221

222 **Results**
223 *Fatty acids profile, lignans and isoflavones content of diets*
224 The fatty acids profile of the diets is reported in Table 2. The control diet was richer in
225 SFA, PUFA and n-6 PUFA respect to the flaxseed one; on the same time the main fatty
226 acid of flaxseed was the ALA, which was almost 8 fold higher than in control diet.
227 In the control diet the content of total lignans was of 1,201.55 ng/g diet (Table 3) mainly
228 represented by LARI and SDG. Flaxseed diet had approximately double the content of
229 lignans than control diet (2,608.54 ng/g). The relative proportion of ISO, SDG and LARI

was quite the same in both the diets whereas SDG was higher in flaxseed diet (1,534.24 *vs.* 494.72 ng/g).

The isoflavone concentration did not show differences between the two diets.

Egg-laying performance

The baseline laying performance was about 72 %. During the trial, the dietary groups showed the same egg-laying performance and final body weights of hens (Table 4).

Composition and chemical characteristics of eggs

The composition and the nutritional content of the eggs from each group are summarized in Table 4. Major differences were found in the egg fractions (albumen and yolk) of the two experimental groups. Eggs produced by flaxseed group had respectively, the highest percentage of albumen and the lowest of yolk, compared to controls, whereas, the shell percentage, was similar.

The chemical composition, the quality of albumen and the yolk colour were similar in the two dietary groups.

The cholesterol concentration (mg/g yolk) was not affected by dietary treatment, and the mg cholesterol per egg of flaxseed group was slightly lower ($P>0.05$) than controls (189.14 *vs.* 195.22 mg/egg, respectively) mainly due to the lower proportion of yolk.

Main biological compounds (lignans, isoflavones, cholesterol, estrogen) and oxidative status (ROS and AOP) of plasma

The isoflavones concentration in plasma of flaxseed hens was lower than in controls (80.44 vs 103.84 ng/ml; Table 6). Such fact was mainly due to the lower amount of equol, since the daidzein and genistein values were similar.

255 Dietary supplementation of flaxseed resulted in a higher plasma lignans respect to control
256 (144.52 *vs.* 195.24 ng/ml), which is about 200 times that of normal circulating levels of
257 endogenous estrogen (~1 ng/ml); in particular, the most abundant lignans were SECO and
258 ISO followed by LARI. Enterolignans (END and ENL) were 2 and 9 times higher in
259 flaxseed than in control group.
260 The plasma cholesterol concentration of flaxseed hens was the same than controls
261 whereas the estradiol was lower (1077.01 *vs.* 1419.00 pg/ml).
262 ROS and total antioxidant capacity were higher in hens fed flaxseed (14.05 *vs.* 9.21 μmol
263 H_2O_2/ml; and 269.81 nmol *vs.* 251.44 nmol HClO/ml, respectively), compared to the
264 controls.
265
266 *Lignans, daidzein and equol in egg yolk*
267 The major isoflavone of the eggs was the daidzein and its concentration was the similar in
268 the two groups (Table 7). However, equol and the total isoflavones were lower in the eggs
269 from flaxseed group than controls.
270 The concentration of total lignans was higher in hens of flaxseed group. In particular ISO,
271 SECO and PINO were three-fold higher whereas MATA is totally absent in both groups.
272 The LARI was not detectable in controls while was 76.11 ng/g yolk in the flaxseed group.
273 The enterolignans (END and ENL) showed similar trend than lignans with almost 10 fold
274 higher value for END and 4-fold for ENL in flaxseed group. As a consequence, lignans
275 were higher in eggs of hens fed flaxseed than in controls.
276
277 *Fatty acid profile and TBARS of egg yolk*
278 In Table 8 is reported the fatty acid profile of egg yolk.
279 The MUFA proportion was higher in control group due to the higher amount of oleic acid.
280 The total PUFA did not showed difference, however, the repartition of the two series (n-6

and n-3) was different. The amount of n-6 PUFA was higher in control and flaxseed group and, as expected, the flaxseed enriched the egg yolk in ALA and n-3 PUFA. The long chain derivatives of ALA (EPA and DHA) were almost 10 and 5-fold higher when flaxseed was administered. As the consequence, the n-6/n-3 ratio was lower in flaxseed group (10.62 *vs.* 2.11, respectively). The amount of DHA per kcal of egg was higher in flaxseed group than control (3.42 vs 0.68 mg/kcal egg).

The TBARS value of egg yolk was higher in flaxseed than in control group.

Discussion

Our study showed that dietary supplementation of 10 % flaxseed to adult hens had no effect on egg production, egg weight and main components of the yolk.

Scheideler and Froning (1996) stated that up to 15 % dietary flaxseed can be safely added to layer diets without any detrimental effects on egg production.

Many of the discrepancies obtained in laying performance depend on the dose, flaxseed cultivar and age of hens. Indeed, flaxseed use should be limited in the diet of young birds due to the potential anti-nutritional effects of mucilage, linatine and linamarin. Several studies have demonstrated that feeding flaxseed can potentially reduce feed intake, body weight and egg production when administered to young hens in the early stages of production (Van Elswyk, 1997). Bean and Leeson (2003) confirmed that feeding 10 % flax did not impact egg production if the flaxseed diet gradually start at 28 weeks of age.

Flaxseed is a rich source of phytoestrogens, mainly lignans and isoflavones, plant substances which possess to a greater or lesser extent functional similarity to the mammalian estrogen 17β-estradiol (Ososki and Kennelly, 2003).

In our trial the plasma estradiol concentration of hens fed flaxseed was lower than controls. Different mechanisms have been proposed to explain the effect of lignans on

306 estradiol. One potential explanation is the competitive inhibition exerted by ENL on the
307 binding of estradiol at the type II nuclear estrogen (Mousavi and Adlercreutz, 1992),
308 An alternative mechanism is the inhibition of the aromatase, the enzyme responsible for
309 converting androgens to estrogens (Evans *et al.*, 1995), acted by enterolignans (Brooks
310 and Thompson, 2005). Another possible way to explain how lignans reduce estrogen
311 involves steroid hormone binding globulin. Lignans are known to stimulate the production
312 of this globulin in the liver, resulting in a reduction of free circulating estradiol (Shultz *et al.*,
313 1991). It is also reported that flaxseed affects the catabolism of 17β-estradiol (2 and 16-
314 hydroxyestrone) as well as the expression of the estrogen metabolising enzymes
315 (Cyp3A4, Cyp1B1) and reduces estrogen receptors in the ovaries (Dikshit *et al.*, 2015).
316 Morton et al. (1994), measuring plasma lignans in response to dietary isoflavones or
317 lignans (flaxseed), found that enterolignans were higher in flax diets and the plasma
318 concentration of ENL was 5 to 20 times that of the estradiol. In our study, the
319 ENL/17β–estradiol ratio changed from 2 to 25 suggesting a strong antagonism between
320 these molecules (Morton et al., 1994).
321 Despite this “down-regulation” of flaxseed on the release of body estrogens and probably
322 on their metabolism, the flaxseed group showed a good laying activity. To our knowledge
323 no studies have specifically analyzed this relationship in flaxseed-fed hens. Thus, indirect
324 evidences on the hypothesis that the lowering of 17β-estradiol produced by 10 % flaxseed
325 can help promote hormone balance and reproduction functions could be deduced from
326 studies on other species and other aims. In aged hens, which naturally develop ovarian
327 cancer, dietary flaxseed improves the health of the reproductive apparatus by modulating
328 inflammation mediators, estrogen metabolism (Speckman *et al.*, 2012) and reduces the
329 progression of carcinogenesis.
330 Studies in women, with normal ovulatory cycles, confirm that flaxseed addition (10 g/day)
331 lengthens the luteal-phase, increases the ratio of progesterone/estradiol and reduces the

number of anovulatory cycles (Phipps *et al.*, 1993). Petit and Twagiramungo (2006), in milking cows, found that flaxseed reduce the embryo mortality and improve the CL size and progesterone synthesis.

Concerning the increase of the egg albumen and the decrease of the yolk percentage observed in flaxseed group. Novak and Scheideler (2001), showed similar results in Hy-Line hens fed flaxseed-based diets (10 %). Probably, the increase of albumen percentage in flaxseed fed hens was due to the induction of ovalbumin synthesis mediated by phytestrogens. Palmiter *et al.* (1971) found that 17β-estradiol administration to chicks increases the incorporation of ^{3}H-uridine and ^{S}H-lysine into oviduct magnum RNA and protein, respectively, and then enhance the albumin synthesis; whereas, when the estrogen receptors were blocked this effect was lacking.

In agreement with Sultan *et al.* (2015) and Scheideler and Froning (1996) flaxseed did not affect egg cholesterol. In other mammalian species, several Authors reported a reduction in serum cholesterol when animals fed flaxseed enriched diet (Prasad *et al.*, 2016). The poor effectiveness of dietary strategies to reduce egg yolk cholesterol probably depends on the homeostatic mechanism to maintain an adequate level of cholesterol in the yolk for the survival of the developing of embryo (Milinsk *et al.*, 2003). Sometimes, the modulation of egg cholesterol, associated with a dietary plan, is a side effect due to a change in the deposition rate and thus to dilution/concentration of egg constituents (Mattioli *et al.*, 2016).

Flaxseed increased the plasma lignans in particular SECO and ISO followed by LARI, PINO and MATA; also enterolignans (END and ENL) were 2 and 9 times higher in flaxseed than in the control group. It is reported that human consumption of flaxseed linearly increases the excretion of END, ENL, and total lignans, but not MATA (Dikshit *et al.*, 2015).

Human studies have suggested that END is synthesized from SECO, LARI, ISO and PINO by facultative anaerobes in the colon and can be further oxidized by the microflora to ENL

(Hu *et al.*, 2007). Conversely, MATA is converted directly in ENL (Meagher and Beecher, 2000).

We showed that hens are able to convert lignan into enterolignans. Few studies are available on such topics: Kennedy (1997) showed that the poultry cecal microflora is able in converting lignan to END and ENL with a low efficiency than human.

The concentrations of equol, as well as the daidzein and genistein of hens fed flaxseed, were lower than in the control group, both in plasma and egg yolk. Equol is the main metabolite of daidzein - the main isoflavone of soybean - which is a standard ingredient (20-30 %) of poultry feed, whereas genistein is not involved in the equol metabolism (Setchell *et al.*, 2002). In the control diet the phytoestrogen intake came from soybean meal, and thus, equol was the main phytoestrogen detected.

Murray *et al.* (2007) suggest a competition for the tissue uptake of isoflavones when lignans are concomitantly administered in the diet: co-administration of SDG with isoflavones (60 mg/kg) modulates the metabolism of these substances increasing END and decreasing daidzein concentration. Such competition could be the reason for less equol in the flaxseed group. Indeed, the preferential conjugation of some blood enzymes of the isoflavones compared to END and ENL could result in a minor proportion of equol (Holder *et al.*, 1999).

A part the reduction in equol, the lignans (ISO, LARI, PINO) and the main metabolic derivatives (SECO, ENL and END) are higher in the egg yolk of hens fed flaxseed. This study shows for the first time that the lignans of flaxseed increase ENL and END in the egg. Such a result could be desirable in order to enrich the egg with compounds retained beneficial for human health (Setchell *et al.*, 2001).

It is reported that flaxseed has antinflammatory and antioxidant properties (Oomah, 2001; Kładna *et al.*, 2016). Part of such effect is probably due to the isoflavones and part to n-3 fatty acids which affect the energy metabolism and disease prevention. Jenkins et al

(1999) reported that the consumption of flaxseed can reduce protein thiol groups, which suggest an increased in oxidative activity. In our trial, the lignans of flaxseed although represented a source of phenolic antioxidant (Antolovich *et al.*, 2000), as demonstrate by an increase of AOP, however, were unable to counteract the serum ROS value probably due to the increase of long chain PUFA (3.25 *vs.* 0.93 mg/g of yolk).

In this study the eggs of hens fed the control diet provide 0.68 mg/kcal egg of DHA, whereas an egg of hens fed 10 % flaxseed, almost 4 times more. These results are a consequence of the higher α-linolenic intake due to flaxseed administration. This essential fatty acid has been efficiently converted into long chain derivatives by the hens' metabolism and transferred into the egg, where they furnish an important source of nutriment for the chick, and also for human consumption.

However, there are negative reports due to the enrichment of PUFA levels in food on the development of abnormal flavours/aromas associated with lipid oxidation (Galobart *et al.*, 2001). As above mentioned, in this study the yolk TBARS was higher in the flaxseed group, probably due to the higher concentration of PUFA, which can easily oxidize if not adequately protected. Also Cherian and Hayat (2009) feeding flaxseed to laying hens observed an increase in TBA reactive substances in plasma, liver and eggs.

In such view, the level of vitamin E used in this trial (60 mg/kg feed) should be further enhanced to protect hens' body and eggs from oxidation.

Conclusion

Dietary supplementation with 10 % flaxseed does not affect the yolk concentration of cholesterol nor the chemical characteristics of egg and the productive performance.

Flaxseed in laying hens is mainly employed for improving the fatty acid profile of eggs (n-3 PUFA) and, a part n-3 enrichment, this paper highlighted also relevant changes in the phytoestrogen content of eggs. The eggs of flaxseed fed hens had higher content lignans

and enterolignans whereas, the lower amount of equol suggests a probable competition for tissue uptake when these compounds are simultaneously present in the diet.

The effect of flaxseed on whole phytoestrogen metabolism and on the reproductive health of laying hens should be further investigated.

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532 **Table 1.** Ingredients and nutrient composition of the control and flaxseed diets.
533

	Control	Flaxseed
Ingredients (g/kg of diet)		
Corn flour	516	516
Extruded soybean flour, 44 %	230	230
Corn Gluten meal	80	-
Wheat bran	85	65
Extruded flaxseed	-	100
Soybean oil	4	4
Dicalcium Phosphate	5	5
Calcium carbonate	55	55
Sodium Bicarbonate	5	5
NaCl	5	5
Vitamin and mineral premix[1]	15	15
Chemical composition (g/kg t.q)		
Dry matter	882	882
Crude protein	178	180
Ether extract	36	37
Crude fiber	53	50
Ash	126	125
Estimated Metabolisable energy (MJ/kg)	11.3	11.0

534 [1] Provided per kilogram of diet: vitamin A, 12,500 IU; cholecalciferol, 3,000 IU; DL-alpha-tocopheryl acetate,
535 60 mg; Vitamina B_1, 2 mg; Vitamina B_2, 6 mg; Vitamina B_6, 4 mg; pantothenic acid, 8 mg; PP 30 mg; folic
536 acid, 0.50 mg; vitamin B_{12}, 0.02 mg; vitamin K, 2 mg; choline, 750 mg; Fe, 35 mg; Zn, 42 mg; I, 0.5 mg; Co,
537 0.5 mg.

538 **Table 2.** Main fatty acid profile of hens diets (g/kg)

	Control	Flaxseed	SED
C14:0	0.12	0.10	0.07
C16:0	1.31[b]	0.55[a]	0.27
C18:0	0.43[b]	0.22[a]	0.09
Σ SFA	1.86[b]	0.87[a]	0.34
C16:1	0.08	0.09	0.07
C18:1	2.43[b]	1.73[a]	0.45
Σ MUFA	2.51[b]	1.82[a]	0.59
C18:2 n-6	4.95[b]	1.86[a]	0.98
C18:3 n-3	0.68[a]	5.43[b]	0.01
Σ PUFA	5.63[a]	7.29[b]	0.90
n-6/n-3	7.27[b]	0.34[a]	0.52

539 Each value represents the mean of five samples per diet.

Table 3. Main isoflavones, lignans and fatty acids of diets.

	Control	Flaxseed	Pooled SE
Lignans, ng/g			
ISO	130.31	180.66	32.54
SDG	494.72[a]	1,534.24[b]	186.22
LARI	402.61	532.68	83.53
MATA	0[a]	25.02[b]	3.84
PINO	173.91[a]	335.94[b]	12.32
TOTAL	1,201.55[a]	2,608.54[b]	101.61
Isoflavones, µg/g			
Daidzein	75.28	68.59	7.35
Genistein	41.44	38.91	4.81
TOTAL	116.72	107.50	15.91

a...b, $P<0.05$: values with different letters on the same raw are significant different.
ISO: isolaricilresinol; SDG: secoisolaricilresinol diglucoside LARI: laricilresinol; PINO: pinoresinol;
Each value represents the mean of five samples per diet.

545
546 **Table 4**. Effects of control or flaxseed diet on productive performance of laying hens
547 (n=20 per group/week)
548

	Control	Flaxseed	Pooled SE
Hens' egg productivity			
Average egg weight, g	62.61	62.05	5.31
Total egg yield [x], g/week	315.02	316.10	20.22
Egg-laying rate[y], %	72.00	73.89	6.55
Feed efficiency [z]	2.20	2.20	0.11
Baseline body weight, kg	1.85	1.80	0.12
Final body weight, kg	1.90	1.87	0.14

549 a...b, P<0.05: values with different letters on the same raw are significant different.
550 [x] (total number of laid eggs) x (egg weight in g);
551 [y] (total number of laid eggs) x 100/(length of experiment in days) x (number of hens);
552 [z] (total feed consumption in g/total egg yield in g).
553
554

Table 5. The compositional analysis and quality assessment of eggs (n=20 per group/week) laid by hens fed control or flaxseed diet.

	Control	Flaxseed	Pooled SE
Egg constituents and quality markers			
Albumen, (% of weight)	59.81[a]	61.08[b]	4.24
Yolk, (% of weight)	27.79[b]	26.72[a]	1.55
Yolk g	17.41	17.22	10.21
Shell, (% of weight)	12.40	12.20	1.61
Haugh unit	115.61	115.24	10.21
Roche colour scale	13.00	13.00	1.00
Yolk constituents			
Dry matter, (mg/g of wet yolk)	504.21	506.33	42.12
Ether extract, (mg/g of wet yolk)	323.22	321.34	28.22
Crude protein, (mg/g of wet yolk)	160.00	158.34	15.27
Ash, (mg/g of wet yolk)	16.71	17.74	1.35
Cholesterol, mg/g yolk	11.22	11.09	1.05
Cholesterol, mg/egg	195.22[b]	189.14[a]	1.62

a...b, P<0.05: values with different letters on the same raw are significant different

559 **Table 6.** Main biological compounds and oxidative status of plasma in hens fed
560 control or flaxseed diet (n=20 per group).
561

	Control	Flaxseed	Pooled SE
Plasma			
Isoflavones (ng/ml)			
Daidzein	20.51	19.64	1.51
Genistein	12.32	10.55	0.90
Equol	71.01^{b}	50.25^{a}	4.02
TOTAL isoflavones	103.84^{b}	80.44^{a}	8.71
Lignans (ng/ml)			
ISO	43.84	49.37	4.25
SECO	53.00	64.01	5.55
MATA	1.22^{a}	5.82^{b}	0.84
LARI	20.21	25.48	2.01
PINO	19.61	16.94	1.63
END	3.82^{a}	8.21^{b}	0.33
ENL	2.82^{a}	25.41^{b}	1.04
TOTAL lignans	144.52^{a}	195.24^{b}	15.41
Biological compounds			
Estradiol, pg/ml	1419.00^{a}	1077.01^{b}	310.02
Cholesterol, mg/dl	67.91	70.00	30.01
Oxidative status			
ROS, µmol H_2O_2/ml	9.21^{a}	14.05^{b}	4.23
AOP, nmol HClO /ml	251.44^{a}	269.81^{b}	13.72

562 a...b, P<0.05: values with different letters on the same raw are significant different.
563 ISO: isolaricilresinol; SDG: secoisolaricilresinol di glucoside; LARI: laricilresinol; PINO: pinoresinol;
564 END: enterodiol; ENL: enterolacton.
565

Table 7. Isoflavones and lignans concentration of eggs laid by hens fed control or flaxseed diet (n=20 eggs/group per week).

	Control	Flaxseed	Pooled SE
Yolk			
Isoflavones (ng/g yolk)			
Daidzein	298.21	285.95	25.22
Genistein	193.54	179.22	18.05
Equol	198.31[a]	142.02[b]	12.11
TOTAL	690.06[b]	607.19[a]	46.31
Lignans (ng/g yolk)			
ISO	40.51[a]	152.92[b]	13.35
SECO	54.22[a]	192.77[b]	14.54
LARI	0[a]	76.11[b]	2.52
PINO	39.21[a]	97.93[b]	3.55
END	0[a]	9.82[b]	0.33
ENL	17.91[a]	82.65[b]	1.51
TOTAL	151.85[a]	612.20[b]	18.22

a...b, $P<0.05$: values with different letters on the same raw are significant different.

ISO: isolaricilresinol; SDG: secoisolaricilresinol diglucoside LARI: laricilresinol; PINO: pinoresinol; END: enterodiol; ENL: enterolacton.

574 **Table 8.** Fatty acid profile (mg/g yolk), nutritional indexes and TBARS of egg
575 yolk (n=20 eggs/group per week).
576

		Control	Flaxseed	Pooled SE
C14:0		1.03	0.95	0.25
C16:0		54.87	46.98	1.90
C18:0		28.54	18.24	1.11
Others		1.98	1.76	0.52
SFA		85.68^{b}	68.07^{a}	2.04
C16:1n-7		9.22	7.25	0.59
C18:1n-9		92.58^{a}	114.36^{b}	1.18
Others		0.78	0.61	0.12
MUFA		105.71^{a}	125.34^{b}	3.42
C18:2n-6		27.48^{b}	18.50^{a}	1.45
C20:4n-6		4.14^{b}	1.15^{a}	1.14
Others		1.22^{a}	2.10^{b}	0.50
C18:3n-3		1.25^{a}	2.68^{b}	0.38
C20:5n-3		0.11^{a}	1.11^{c}	0.15
C22:5n-3		0.08^{a}	0.91^{b}	0.06
C22:6n-3		0.95^{a}	4.55^{c}	0.5
Others		0.70	1.03	0.84
PUFA		38.07	33.30	1.987
Σ n-3 >20C	mg/g egg	0.93^{a}	3.25^{b}	0.34
Σ n-6	“	57.71^{b}	39.39^{a}	2.14
n-6/n-3		10.62^{b}	2.11^{a}	1.8
DHA	mg/kcal egg	0.76^{a}	3.42^{b}	0.45
TBARs	mg MDA/kg yolk	0.20^{a}	0.44^{b}	0.11

577 a...b, P<0.05: values with different letters on the same raw are significant different