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

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Abstract:	<p>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).</p> <p>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.</p> <p>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.</p> <p>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.</p> <p>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.</p> <p>Further details on the competition between the different dietary phytoestrogens and their metabolites (estrogen, equol, END, ENL) should be investigated.</p>
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1 **Performance and egg quality of laying hens fed flaxseed: highlights on**
2 **n-3 fatty acids, cholesterol, lignans and isoflavones**

3

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14

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

16

17 **Abstract**

18 Flaxseed is a rich source of α -linolenic acid and phytoestrogens, mainly lignans, whose
19 metabolites (enterolactone - ENL and enterodiol - END) can affect estrogens functions.

20 The present study evaluated the influence of dietary flaxseed supplementation on
21 reproductive performance and egg characteristics (fatty acids, cholesterol, lignans and
22 isoflavones) of 40 Hy-line hens (20/group) fed for 23 weeks control diet (C) or the same
23 diet supplemented with 10 % of extruded flaxseed (F). Flaxseed diet, had approximately 3
24 times the content of lignans than control diet (2,608.54 ng/g), mainly SDG (1,534.24 vs.
25 494.72 ng/g).

26 When compared with the control group, hens fed flaxseed showed a similar deposition rate
27 (72.0 % vs. 73.9 %) and egg yield. Furthermore, there was no effect of flaxseed on
28 chemical composition of egg and on its cholesterol content.

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

31 The plasma lignans were higher in F hens, while isoflavones were lower, mainly due to the
32 lower equol value (50.52 vs. 71.01 ng/ml). Similar trend was showed in eggs: F group had
33 higher level of END and ENL whereas the equol was lower (198.31 vs. 142.02 ng/g yolk).

34 The secoisolariciresinol (SECO) was the main lignan in eggs of F group and its
35 concentration was 3 times higher then control eggs. Flaxseed also improved the n-3 long
36 chain PUFA of egg (3.25 vs. 0.92 mg/g egg), mainly DHA, however, its oxidative status
37 (TBARS) was negatively affected.

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

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

43

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

45

46 **Implications**

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

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

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

55

56 **Introduction**

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

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

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

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

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

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

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

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

94

95 **Material and methods**

96 *Reagents*

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

100

101 *Diets*

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

108

109 *Study design*

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

- 112 - Control;
- 113 - Flaxseed.

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

119 The hens were weighed at the beginning and at the end of the study. Feed intake and
120 feed/weight conversion rate were calculated for each group. The baseline laying
121 performance of the hens was measured for 2 weeks prior to the randomization to the
122 different dietary treatments. All the eggs were collected, counted and weighed daily. The
123 actual egg yield for each dietary group was determined by multiplying the egg production x
124 egg weight. Feed efficiency was assessed by dividing total feed consumption by total egg
125 mass. The percentage of egg-laying was calculated by dividing the number of laid eggs
126 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 °C 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 H₂ 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

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

181

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

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

205

206 *Plasma estradiol and oxidative status:* the 17 β -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 $\mu\text{mol H}_2\text{O}_2/\text{ml}$

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

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

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

233

234 *Egg-laying performance*

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

237

238 *Composition and chemical characteristics of eggs*

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

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

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

249

250 *Main biological compounds (lignans, isoflavones, cholesterol, estrogen) and oxidative 251 status (ROS and AOP) of plasma*

252 The isoflavones concentration in plasma of flaxseed hens was lower than in controls
253 (80.44 vs 103.84 ng/ml; Table 6). Such fact was mainly due to the lower amount of equol,
254 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 μ mol
263 H₂O₂/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

281 and n-3) was different. The amount of n-6 PUFA was higher in control and flaxseed group
282 and, as expected, the flaxseed enriched the egg yolk in ALA and n-3 PUFA. The long
283 chain derivatives of ALA (EPA and DHA) were almost 10 and 5-fold higher when flaxseed
284 was administered. As the consequence, the n-6/n-3 ratio was lower in flaxseed group
285 (10.62 vs. 2.11, respectively). The amount of DHA per kcal of egg was higher in flaxseed
286 group than control (3.42 vs 0.68 mg/kcal egg).

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

288

289 **Discussion**

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

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

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

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

304 In our trial the plasma estradiol concentration of hens fed flaxseed was lower than
305 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 17 β -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/17 β -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 17 β -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

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

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

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

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

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

358 (Hu *et al.*, 2007). Conversely, MATA is converted directly in ENL (Meagher and Beecher,
359 2000).

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

363 The concentrations of equol, as well as the daidzein and genistein of hens fed flaxseed,
364 were lower than in the control group, both in plasma and egg yolk. Equol is the main
365 metabolite of daidzein - the main isoflavone of soybean - which is a standard ingredient
366 (20-30 %) of poultry feed, whereas genistein is not involved in the equol metabolism
367 (Setchell *et al.*, 2002). In the control diet the phytoestrogen intake came from soybean
368 meal, and thus, equol was the main phytoestrogen detected.

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

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

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

384 (1999) reported that the consumption of flaxseed can reduce protein thiol groups, which
385 suggest an increased in oxidative activity. In our trial, the lignans of flaxseed although
386 represented a source of phenolic antioxidant (Antolovich *et al.*, 2000), as demonstrate by
387 an increase of AOP, however, were unable to counteract the serum ROS value probably
388 due to the increase of long chain PUFA (3.25 vs. 0.93 mg/g of yolk).

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

395 However, there are negative reports due to the enrichment of PUFA levels in food on the
396 development of abnormal flavours/aromas associated with lipid oxidation (Galobart *et al.*,
397 2001). As above mentioned, in this study the yolk TBARS was higher in the flaxseed
398 group, probably due to the higher concentration of PUFA, which can easily oxidize if not
399 adequately protected. Also Cherian and Hayat (2009) feeding flaxseed to laying hens
400 observed an increase in TBA reactive substances in plasma, liver and eggs.

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

403

404 **Conclusion**

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

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

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

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

414

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419

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

	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 B₁, 2 mg; Vitamina B₂, 6 mg; Vitamina B₆, 4 mg; pantothenic acid, 8 mg; PP 30 mg; folic
536 acid, 0.50 mg; vitamin B₁₂, 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.

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

541

	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

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

543 ISO: isolaricilresinol; SDG: secoisolaricilresinol diglucoside LARI: laricilresinol; PINO: pinoresinol;

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

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

	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

555 **Table 5.** The compositional analysis and quality assessment of eggs (n=20 per
 556 group/week) laid by hens fed control or flaxseed diet.
 557

	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

558 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, $\mu\text{mol H}_2\text{O}_2/\text{ml}$	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

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

568

	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

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

570 ISO: isolaricilresinol; SDG: secoisolaricilresinol diglucoside LARI: laricilresinol; PINO: pinoresinol;

571 END: enterodiol; ENL: enterolacton.

572

573

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