Journal of Functional Foods 22 (2016) 454–462

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/jff

Alfalfa and flax sprouts supplementation enriches the content of bioactive compounds and lowers the cholesterol in hen egg

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ARTICLE INFO

Article history:
Received 28 November 2015
Received in revised form 29 January 2016
Accepted 4 February 2016
Available online 23 February 2016

Keywords:
Laying hens, eggs, sprouts
ω-3 fatty acid
Tocopherols
Phytochemicals

ABSTRACT

The effect of dietary supplementation with flax and alfalfa sprouts (40 g/d) on bioactive compounds and cholesterol contents of hen's egg was examined. Thirty White Leghorn hens, 26 weeks of age, were fed, for 66 days, three diets that included control (standard diet – C), standard diet + alfalfa sprouts (A), and standard diet + flax sprouts (F). Productive performance of hens was recorded daily. The cholesterol content of plasma and yolk, and the presence of bioactive compounds in the egg, were also analysed. Supplementation of flax and alfalfa sprouts reduced plasma and egg cholesterol probably due to the synergy between different compounds of the sprouts (polyunsaturated fatty acids - PUFAs, lignans, isoflavones and sterols). Eggs from A and F groups also had higher contents of n-3 PUFA, vitamins (α-tocopherol, α-, γ-tocotrienol, retinol), carotenes (β-carotene, lutein, zeaxanthin) and phytoestrogens (daidzein, equol, isolariciresinol) than eggs from the C group.

1. Introduction

No other single food of animal origin is eaten by so many people all over the world compared with the egg and none is served in such a variety of ways (Surai & Sparks, 2001). Therefore, the ability of the egg to be used as a functional food has been widely investigated (Stadelman, 1999) because the egg composition can be partly modified by changing the poultry feed (Lemahieu et al., 2015; Vaghefi, 2003). The majority of research has investigated the possibility of enriching eggs with fatty acids, vitamin E, selenium and lutein, but very few evaluated other bioactive phytochemicals (phytosterols, isoflavones, lignans, etc.) which are considered effective in human health (Finley, 2005; Webb & McCullough, 2005).

Phytochemicals are present in plant seeds and their contents are known to increase during germination in most plant species, from legumes, to oilseeds, to cereals (Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010; Baenas, Moreno, & Garcìa-Viguera, 2012; Benincasa et al., 2015; Marton, Mándoki, Csapo-Kiss, & Csapó, 2010). In fact, sprouts (i.e., the young seedlings obtained from seed germination) are becoming more and more popular in western countries as healthy foods, for their positive effects on the prevention of cardiovascular diseases

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Chemical compounds: Daidzein (PubChem CID: 5281708); Equol (PubChem CID: 91469); Phytosterols (PubChem CID: 12303662); Cholesterol (PubChem CID: 5997); Alpha-linolenic acid (PubChem CID: 5280934); Tocopherols (PubChem CID: 14986); Tocotrienols (PubChem CID: 9929901); Zeaxanthin (PubChem CID: 5280899); Lutein (PubChem CID: 5281243); Beta-carotene (PubChem CID: 5280489).

http://dx.doi.org/10.1016/j.jff.2016.02.007

and cancer (Ma et al., 2014). In particular, flax sprouts have been found to contain high levels of water-soluble proteins and free amino acids, free fatty acids and glycolipid fractions, lysophosphatidylcholine, and phosphatidic acid (Narina, Hamama, & Bhardwaj, 2012; Wanasundara, Shahidi, & Brosnan, 1999; Wanasundara, Wanasundara, & Shahidi, 1999). Alfalfa sprouts contain high amounts of vitamins A and C, coumestrol, liquiritigenin, isoliquiritigenin, loliolide, and saponins (Hong et al., 2011; Oleszek, 1998; Plaza, De Ancos, & Cano, 2003).

On the other hand, some public concern derives from the risk of bacterial contamination (e.g. *Escherichia coli*, *Salmonella enterica*, *Vibrio Cholerae*), because sprouts are normally home-made and used as components of salads, with no thermal or other sanitation treatment (Taormina, Beuchat, & Slutsker, 1999).

The use of sprouts in animal feeding could represent an alternative to transfer bioactive compounds from sprouts to livestock products and in turn to humans. This would be an attractive way to improve the quality and safety of food destined for human consumption together with animal health. However, very few studies have evaluated the possibility of transferring bioactive compounds from the sprouts to animal products (Dal Bosco et al., 2015), and none to hen's egg. Thus, the aim of the present study was to evaluate the effect of dietary supplementation of fresh alfalfa and flax sprouts on the bioactive compounds (phytosterols, phytoestrogens, tocopherols, carotenes, n-3 fatty acids) and the cholesterol contents of hen's egg.

2. Materials and methods

All used chemicals and reagents were at least of analytical grade and purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise specified.

2.1. Production of alfalfa and flax sprouts

Alfalfa (*Medicago sativa L.*) and flax (*Linum usitatissimum L.*) seeds were germinated on a substrate consisting of moistened tissue paper lying on a layer of silica sand sterilised at 105 °C in aluminium trays (22 cm × 30 cm for alfalfa and 30 cm × 36 cm for flax). In each tray, the sand (600 g for the alfalfa and 1 kg for the flax) was distributed to create a uniform layer on the bottom of the tray and moistened with demineralised water. The trays were placed in a temperature-controlled room at 20 °C in the dark and kept in these conditions for three days. Water was added periodically to compensate for sand water loss due to evaporation. In contrast to the usual sprouting procedure used for alfalfa in which sand water content is restored once a day, flaxseed requires several separate additions of water, as the seeds tend to produce a glue-like mucilaginous exudate in the presence of high water content that would hamper seedling development. For each species, the sprouts obtained on the third day from different trays were combined to prevent a possible tray effect and stored at 4 °C in plastic bags until use (i.e., within three days).

2.2. Animals and diets

The experimental protocol was devised according to the Italian directives (Gazzetta Ufficiale, 1992) on animal welfare and the research was carried out at the experimental farm of the Department of Agricultural, Food and Environmental Science of the University of Perugia (Italy) from November 2013 to February 2014.

Table 1 – Mean ingredients (g kg^{-1}) of diet and nutrient composition (g kg^{-1}) of diet and sprouts.

	Feed	Alfalfa	Flax
Ingredients			
Maize	450		
Extruded soybean flakes	200		
Maize gluten feed	160		
Sunflower meal	88		
Alfalfa meal	30		
Vitamin mineral premix*	10		
Calcium carbonate	50		
Dicalcium phosphate	5		
Sodium bicarbonate	5		
Salt	2		
Nutrient composition			
Water	11.0	87.6	87.5
Crude protein	178	68	61
Ether extract	53	5.2	6.3
Crude fibre	56	30	36
Ash	111	20	21

* Provided per kilogram of diet: vitamin A, 12,500 IU; cholecalciferol, 3,000 IU; DL-alpha-tocopheryl acetate, 60 mg; Vitamin B_1, 2 mg; Vitamin B_2, 6 mg; Vitamin B_6, 4 mg; pantothenic acid, 8 mg; PP 30 mg; folic 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, 0.5 mg.

Thirty White Leghorn hens, 30 wk. of age at the start of experiment, were randomly assigned to one of the following conditions:

- Standard diet (C);
- Standard diet + 40 g/d of alfalfa sprouts (A);
- Standard diet + 40 g/d of flax sprouts (F).

The hens of each group were kept in indoor pens under standard housing conditions with an artificial photoperiod of 16 h per day of light was applied. The building was under a controlled ventilation regime (10 m^3/hen/h): the temperature ranged from 23 to 27 °C, and the relative humidity ranged from 50 to 80%.

Standard feed and water were provided with manual bell feeders and automatic drinkers. Feed and water were provided *ad libitum* (Table 1) to all the groups and the daily residues were weighed for evaluation of voluntary feed intake. Fresh sprouts were placed daily near the feeders.

The overall experimental period lasted 66 days. Egg deposition was recorded daily; in particular the deposition rate was evaluated the wk. before the experiment started (baseline day 0) and at the end of sprout supplementation.

2.3. Eggs and blood sampling

For each dietary treatment, 10 pools of 10 egg yolks/per group were collected at day 0 and day 66 stored at 5 °C until the analyses (maximum 2 days after) that were performed in the laboratory of the department. The blood was sampled at 0 and 66 day from the brachial vein of 10 hens per group, in heparinised vacutainers and centrifuged at 1500 × g for 15 min at +4 °C, to measure the plasma cholesterol concentration.

2.4. Analytical determination

The chemical composition of the feed and sprouts was determined according to the method of the AOAC (1995).

2.4.1. Fatty acids

The fatty acid profiles of the feed, sprouts and egg yolk were determined by gas chromatography following lipid extraction according to the method described by Folch, Lees, and Sloane-Stanley (1957). In particular, 1 mL of lipid extract was evaporated under a stream of nitrogen and the residue was derived by adding 3 mL of sulphuric acid (3% in methanol). Following incubation at 80 °C for 1 h, the methyl esters were extracted with petroleum ether, and 1 μL was injected into a gas chromatograph (Mega 2 – model HRGC; Carlo Erba, Milan, Italy) equipped with a flame ionisation detector. The fatty acid methyl esters (FAMEs) were separated with an Agilent (J&W) capillary column (30 m × 0.25 mm I.D; CPS Analitica, Milan, Italy) coated with a DB-wax stationary phase (film thickness of 0.25 mm). The operating conditions used during the column injection of the 1 μL sample volume were as follows: the temperatures of the injector and detector were set at 270 °C and 280 °C, respectively, and the detector gas flows were H_2 at 50 mL min^{-1} and air at 100 mL min^{-1}. The oven temperature was programmed to provide a good peak separation as follows: the initial oven temperature was set at 130 °C; this temperature increased at a rate of 4.0 °C min^{-1} to 180 °C and was held for 5 min; the temperature was then increased at a rate of 5.0 °C min^{-1} to 230 °C; the final temperature was held for 5 min. Helium was used as a carrier gas at a constant flow rate of 1.1 mL min^{-1}. Individual fatty acid methyl esters were identified by referring to the retention time of FAME authentic standards. For the quantitative analysis, C19:0 methyl ester was used, added before extraction, as internal standard. The relative proportion of individual fatty acids was expressed as a percentage.

2.4.2. Sterols

Sterols were extracted and quantified in sprouts, plasma, and yolk samples. Cholesterol was extracted from plasma using the method of Folch et al. (1957). All samples were mixed with 8 mL of chloroform/methanol (2:1, v/v), and ultrasonicated at 30% intensity for 10 s (model IKA® U50, Staufen, Germany). The mixture was vortexed and filtered through Whatman paper (No. 1), then 1.5 mL of distilled water containing 1% of NaCl was added, vortexed and allowed to stand at room temperature for 1 h before centrifugation at 500 × g for 10 min. The upper layer was suspended in 8 mL chloroform/methanol/water containing NaCl (86:16:1, v/v/v) and extracts again. The chloroform extracts were pooled and dried under a flow of N_2. The residue was dissolved in 200 μL of mobile phase.

The sterols from sprouts and yolk were extracted with *n*-hexane. Briefly, 0.1 g of freeze-dried and finely ground sprouts or yolk was subjected to saponification in 2% KOH-ethanol solution for 1 h at 50 °C. At the end of the saponification two further extractions with *n*-hexane and sonication were carried out (30% for 10 sec; model IKA®, U50, Staufen, Germany). The mixture was vortexed and centrifuged at 900 × g for 5 min. The hexane extract was collected and dried under a stream of N2. The residue was suspended in 3 mL of mobile phase and filtered through a syringe with regenerated cellulose (RC Phenex 4 mm, 0.26 μM for size, Phenomenex srl, Bologna, Italy) before injecting into the HPLC system.

The quantification of sterols in the plasma, sprouts and yolks was performed through HPLC/UV-vis system (model pump PU-1850, equipped with an autosampler, model AS 950-10, Tokyo, Japan) with an analytical column of type C18 reverse phase (particle size ODS-2.5 M, 4.6 mm internal diameter; CPS analytical, Milan, Italy). The mobile phase is composed of a mixture of acetonitrile/isopropanol (70:30, v/v) and released with a flow of 1.5 mL min^{-1}. The injected volume was 10 μL. Sterols were identified using a UV detector (Model 2075 Plus Jasco, Tokyo, Japan) at λ 210 nm and quantified using a calibration curve outside with increasing amounts of pure standards solution (cholesterol, β-sitosterol, stigmasterol, campesterol, avenasterol, brassicasterol) in isopropanol.

2.4.3. Antioxidants

The different isoforms of vitamin E (α-, β + γ and δ tocopherol -T, and tocotrienols -T3) of the feed, sprouts and yolk were quantified by HPLC (Zaspel & Csallany, 1983).

Egg yolk was lyophilised (0.1 g) and was saponified in 1M KOH in ethanol in a thermostat bath at 50 °C for 1 hour. Then the content was sonicated and extracted 2 times with 10 mL of *n*-hexane. The upper phase was collected and dried with N_2 to be then reconstituted in 200 μL of acetonitrile and 50 μL was injected into the HPLC system (Perkin Elmer series 200, equipped with an autosampler system, model AS 950-10, Tokyo, Japan) on a Synergy Hydro-RP column (4 μm, 4.6 × 100 mm; Phenomenex, Bologna, Italy). The different isoforms were identified using an FD detector (model Jasco, FP-1525 – excitation and emission wavelengths of 295 and 328 nm, respectively) and quantified using external calibration curves prepared with increasing amounts of pure tocopherols.

The main carotenoids of the feed, sprouts and yolk were determined by the same HPLC system previously described. The solvent system consisted of solution A (methanol/water/acetonitrile, 10:20:70, v/v/v) and solution B (methanol/ethyl acetate, 70:30, v/v). The volume of injection was 20 μL and the flow rate was 1 mL min^{-1}, and the elution programme was a gradient starting from 90% A in a 20 min step to 100% B and then a second isocratic step of 10 min. The detector was UV–visible spectrophotometer (Jasco UV2075 Plus) set at a wavelength of 450 nm for lutein, zeaxanthin and β-carotene and at 325 nm for retinol. The different carotenoids were identified and quantified by comparing the sample with pure commercial standards (Sigma-Aldrich, Steinheim, Germany; Extrasynthese, Genay, France).

2.4.4. Phytoestrogens

Isoflavones (daidzein, genistein), cumestans and lignans (secoisolariciresinol diglucoside, isolariciresinol, hydroxymatairesinol, secoisolariciresinol and matairesinol) in the feed, sprouts and yolk were quantified by high performance liquid chromatography (HPLC-ECD). The extraction of lignans for alfalfa and flax sprouts was done according to Plaza et al. (2003) and Milder et al. (2004).

Lignans and isoflavones content in the egg yolk was determined as follows: 0.5 g of freeze-dried egg yolk was extracted by refluxing in aqueous ethanol (25 mL, 80%) for 30 min (Setchell,

2003; Setchell, Zhao, Jha, Heubi, & Brown, 2009). After filtering the sample through a Whatman No. 1 filter paper the ethanolic extract was made up to 50 mL with absolute ethanol and 5 mL was taken to dryness under a stream of nitrogen. The dried extract was reconstituted in 2 mL of 0.5 M acetate buffer (pH 4.5) and then subjected to hydrolysis at 37 °C overnight with a filtered solution (Grace & Teale, 2006) of 10,000 Fishman Units of a mixed β-glucuronidase/sulphatase (*H. pomatia*, Sigma Chemicals Inc.). After hydrolysis, lignans and isoflavones were isolated by solid-phase extraction on a Sampli Q C18 ODS SPE cartridge previously conditioned with methanol (2 mL) followed by distilled water (2 mL). Two mL of incubated sample was then passed through the cartridge, which was washed with 5 mL of distilled water. Finally, the eluate was collected with 5 mL of methanol, taken to dryness under a stream of nitrogen gas, suspended in 2 mL of methanol and injected.

Isoflavones, cumestans and lignans were determined by HPLC (Peñalvo et al., 2004) system equipment of two Jasco PU-1580 (L.i. Service, s.r.l., Rome, Italy), an HT310 L (HTA s.r.l., Brescia, Italy) autosampler with a 20 µL loop, and an Inertsil ODS-3 (C18 150 mm × 4.6 mm i.d.; particle size = 5 µm) (GL Sciences, Tokyo, Japan) protected with a guard column (an Inertsil ODS-3; C18 4.0 mm × 10 mm i.d.; particle size = 5 µm) (GL Sciences, Japan). For the binary gradient elution, mobile phase A was 50 mM acetate buffer (pH 5)/MeOH (80:20, v/v) and mobile phase B was 50 mM acetate buffer, pH 5/MeOH/ACN (40:40:20, v/v/v). The gradient cycle was as follows: 70% of phase A at the starting point followed by a decrease to 40% in 25 minutes, held for 10 minutes, decreased to 25% in 5 min, held for 10 min., brought back to the initial conditions in 5 min. and finally held to 70% of phase A for 5 min. The variation in gradient solvent was linear and the eight electrode potentials were set at 150, 230, 300, 420, 470, 550, 600 and 650 vs. palladium reference electrodes at room temperature (CoulArray; ESA, Inc., Chelmsford, USA). The injection volume was 20 µL and the total run time for each sample was 60 min. The flow rate was 0.9 mL min^{-1}. Molecules were identified comparing their retention times with pure standards.

2.5. *Statistical analysis*

Data were analysed with a linear model (STATA, 2015) having the diet as a fixed effect. Significance of difference among least squares means was performed using multiple t test ($P < 0.05$).

3. Results and discussion

The current study was designed to determine whether the dietary supplementation of laying hens with sprouts would enrich in some bioactive compounds the egg and affect its cholesterol level.

Fresh alfalfa and flax sprouts resulted to be very eatable by hens, and although sprouts had high water level (Table 1: 87.6 vs 11.0%, respectively) and the bioactive compounds were not very concentrated, they were able to enrich the eggs with several phytochemicals, contemporarily reducing the egg cholesterol.

The fatty acid profile (Table 2) of sprouts showed higher polyunsaturated fatty acid concentrations than the conventional feed; in particular, linoleic acid (LA: 33.6%) and α-linolenic acid (ALA: 62.5%) were highest in alfalfa and flax sprouts, respectively, whereas palmitic and stearic acid were higher in the conventional feed.

Table 2 – Main fatty acids (% total fatty acids) and antioxidant compounds (mg kg^{-1} d.m.) in feed and sprouts (mean ± s.e.m.).

Fatty acid	Feed	Sprouts	
		Alfalfa	Flax
C16:0	16.0 ± 1.0	14.2 ± 0.6	6.99 ± 0.8
C18:0	20.3 ± 2.2	3.57 ± 0.2	2.4 ± 0.3
C18:1n-9	17.1 ± 1.3	12.5 ± 1.0	6.81 ± 0.9
C18:2n-6	21.4 ± 1.9	33.6 ± 3.2	12.6 ± 1.5
C18:3n-3	15.3 ± 0.8	26.5 ± 2.7	62.5 ± 4.4
α-tocopherol	113.7 ± 10.2	361.6 ± 23.1	338.3 ± 26.2
α-tocopherol acetate	52.3 ± 2.1	n.d.	n.d.
γ-tocopherol	0.3 ± 0.1	41.6 ± 1.9	30.4 ± 2.9
δ-tocopherol	3.0 ± 0.6	1.6 ± 0.1	0.8 ± 0.1
α-tocotrienol	4.7 ± 1.7	2.8 ± 0.2	1.6 ± 0.1
γ-tocotrienol	6.7 ± 1.9	2.6 ± 0.2	1.7 ± 0.1
Retinol	63.0 ± 3.9	81.2 ± 1.7	98.2 ± 8.8
Lutein	5.7 ± 1.6	121.2 ± 10.17	18.3 ± 1.2
Zeaxanthin	16.2 ± 1.5	788.7 ± 40.1	100.6 ± 12.0
β-carotene	10.5 ± 0.8	152.1 ± 10.5	18.3 ± 0.9
Total carotenoids	26.7 ± 2.6	1063.0 ± 70.9	137.2 ± 10.8

n.d.: not detectable. Each value represents the mean of three replications.

The amount of α-tocopherol (Table 2) was lower in the feed than in alfalfa and flax sprouts (113.7 vs 361.6 and 338.3 mg kg^{-1} d.m., respectively). Total carotenoids and mainly zeaxanthin were higher in alfalfa than in flax sprouts and control feed (788.7 vs 100.6 and 16.2 mg kg^{-1}, respectively).

Isoflavones, cumestans, lignans and phytosterols compounds were also evaluated in the feed and sprouts (Table 3).

Table 3 – Main isoflavones, cumestans, lignans and phytosterols in control diet and sprouts (mg 100 g^{-1} d.m; mean ± s.e.m.).

		Sprouts	
	Control diet	Alfalfa	Flax
Secoisolariciresinol diglucoside	40.7 ± 5.4	28.2 ± 1.0	4514.1 ± 158.2
Isolariciresinol	0.10 ± 0.01	6.25 ± 1.8	100.9 ± 7.2
Hydroxymatairesinol	n.d.	3.3 ± 0.7	68.3 ± 4.5
Secoisolariciresinol	n.d.	5.9 ± 1.0	122.7 ± 6.1
Matairesinol	n.d.	1.25 ± 0.3.	70.9 ± 3.2
Coumestrol	n.d.	6.8 ± 0.8	142.1 ± 12.0
Daidzein	6.83 ± 0.6	22.2 ± 1.2	21.4 ± 1.7
Genistein	4.02 ± 0.4	n.d.	n.d.
Glycitein	2.51 ± 0.3	n.d.	n.d.
β-sitosterol	7.28 ± 0.39	33.07 ± 1.23	61.98 ± 4.40
Stigmasterol	n.d.	84.56 ± 5.46	44.14 ± 2.36
Campesterol	4.25 ± 0.65	n.d.	19.26 ± 1.44
Avenasterol	3.55 ± 0.49	38.55 ± 3.84	n.d
Brassicasterol	n.d.	n.d.	30.87 ± 0.41
Total sterols	31.76	237.57	286.67

n.d.: not detectable. Each value represents the mean of three replications.

Table 4 – Effects of sprouts on productive performance.

		Group			
		Control	Alfalfa	Flax	Pooled SE
Baseline deposition rate*	%	78.5	77.1	78.5	
Final deposition rate*	%	77.1[a]	81.4[b]	80.0[a]	3.2
Egg weight*	g	57.6	57.4	57.6	2.3
Yolk weight**	g	17.30	17.20	17.00	1.2
Feed consumption*	g d^{-1}	120.3	115.4	114.0	8.2
Feed and sprouts consumption*	g d.m. d^{-1}	106.8	107.5	106.1	7.9
Baseline body weight*	kg	1.83	1.88	1.82	0.20
Final body weight*	kg	1.85	1.89	1.90	0.18

* N = 10 hens per group/day.
** 10 pools of 10 yolks per group, a-b: $P < 0.05$.

The main isoflavones of control diet were genistein (4.02 mg 100 g^{-1}) and glycitein (2.51 mg 100 g^{-1}), mainly derived from extruded soybean and maize; on the contrary the daidzein content was higher in alfalfa and flax sprouts than control diet (22.2 and 21.4 mg 100 g^{-1} d.m., respectively). Sprouts also had a higher content of lignans: in particular, the flax sprouts contained very high amounts of secoisolariciresinol diglucoside (SDG); even the other lignans (isolariciresinol, hydroxymatairesinol, secoisolariciresinol, matairesinol) and coumestrol were higher than in control diet and alfalfa sprouts.

Sterols were abundant in flax sprouts (286.67 vs 237.57 and 31.76 mg 100 g^{-1} d.m, in flax, alfalfa and feed, respectively) and the main phytosterol was the β-sitosterol, followed by stigmasterol, brassicasterol and campesterol. In the alfalfa sprouts the campesterol was not detectable and the most represented sterol was the stigmasterol.

About the productive performance of hens, the addition of both the sprouts increased the deposition rate, whereas the weight of eggs and yolk was not affected (Table 4). The consumption of both sprouts slightly decreased the voluntary intake of the solid diet, but when the sprouts were expressed in term of dry matter the intake of all the groups was almost the same.

The hens supplemented with sprouts decreased the cholesterol of plasma and eggs with respect to baseline and C group (Table 5). On the contrary, the amount of phytosterols in the yolk was low in all the groups and was not affected by the sprouts.

Dietary supplementation with sprouts increased the phytoestrogens content in the yolk compared to the control group (Table 6). This change was mainly due to isolariciresinol content, which was higher in A and F group (20.9 and 15.8 vs 6.5 μg 100 g^{-1}, respectively). A similar trend was observed for daidzein and equol, while coumestrol was detectable only in the F group. Secoisolariciresinol contents did not show significant differences.

Regarding antioxidant compounds (Table 7), retinol was the most abundant in egg yolk, especially when hens feed alfalfa sprouts (48.6 vs 34.4 and 42.2 mg kg^{-1} yolk, in the C and F groups, respectively). Such result was confirmed by the higher amount of β-carotene in the two groups fed sprouts since the retinol is the metabolic product of β-carotene. Even the tocopherols and tocotrienols were higher in alfalfa and flax eggs, particularly α-T, α-T3 and γ-T3. Lutein and zeaxanthin were also more concentrated in the eggs of sprout groups; in particular, lutein was greater in the A and zeaxanthin in the F group.

The fatty acid composition of the yolk was affected by the administration of sprouts, whereas the lipid level did not show any difference (Table 8). At the end of experimental period the eggs from hens of the F group showed a higher concentration of ALA, eicosapentaenoic (EPA) and docosahexaenoic (DHA)

Table 5 – Level of cholesterol in plasma (mg/dl) and egg yolk (mg g^{-1} yolk) and phytosterols (mg 100 g^{-1} yolk).

Day	Group	Plasma cholesterol*	Egg yolk cholesterol**	Campesterol**	β-Sitosterol**
0	Baseline	76.3[b]	11.8[b]	12.3	4.8
66	Control	79.2[b]	11.5[b]	13.0	5.0
	Alfalfa	65.2[a]	10.4[a]	11.8	3.8
	Flax	62.3[a]	10.5[a]	14.3	1.8
Pooled SE		2.8	0.7	2.5	1.9

* N = 10 hens per group/day.
** 10 pool of 10 yolks per group, a–b: $P < 0.05$.

Table 6 – Isoflavones, and lignan in the egg yolk (μg 100 g^{-1} yolk).

Day	Group	Isolariciresinol	Secoisolariciresinol	Daidzein	Equol	Coumestrol
0	Baseline	5.82[a]	44.6	5.28[a]	52.7[a]	n.d.
66	Control	6.54[a]	38.5	6.84[a]	52.8[a]	n.d.
	Alfalfa	20.9[b]	46.3	18.7[b]	67.5[c]	n.d.
	Flax	15.8[b]	34.9	29.2[c]	60.6[b]	0.11
Pooled SE		2.9	7.5	3.2	3.6	–

N = 10 pools of 10 yolks per group, a–c: $P < 0.05$.
n.d.: no detectable.

Table 7 – Main antioxidants in the egg yolk (mg kg^{-1} yolk).

Day	Group	α-T	γ-T	δ-T	α-T3	γ-T3	β-carotene	Retinol	Lutein	Zeaxanthin
0	Baseline	16.1^{b}	0.27	0.02	0.01^{a}	0.30^{a}	0.31^{a}	36.9^{a}	2.91^{a}	8.35^{a}
42	Control	14.5^{a}	0.29	0.02	0.01^{a}	0.37^{a}	0.30^{a}	34.4^{a}	3.02^{a}	8.46^{a}
	Alfalfa	20.3^{d}	0.28	0.04	0.13^{b}	0.57^{b}	0.39^{b}	48.6^{c}	6.21^{c}	11.94^{c}
	Flax	19.4^{c}	0.23	0.03	0.14^{b}	0.57^{b}	0.33^{a}	42.2^{b}	3.69^{a}	9.7^{b}
Pooled SE		1.3	0.09	0.008	0.06	0.1	0.05	2.0	0.9	1.2

N = 10 pools of 10 yolks per group, a–d: $P < 0.05$.
α-T: α-tocopherol; γ-T: γ-tocopherol; δ-T: δ-tocopherol; α-T3: α-tocotrienol; γ-T3: γ-tocotrienol.

acid with respect to control diet, whereas the A group showed intermediate values.

It is well known that the egg composition can be modified by changing the poultry feed and that enriched eggs can increase the consumption of beneficial compounds without modifying the dietary regimen of humans (Bruneel et al., 2013; Sim & Sunwoo, 2002).

Nutrients in foods are present as complex carbohydrates, proteins, and lipids, and many studies suggest that the interactive effect of such compounds may be superior to isolated ones (Thiel, 2000). Moreover, dehydration or treatment of extraction reduce the bioactive compounds of plants (e.g. phenolics, volatiles, carotenoids) diminishing their physiological actions (Abascal, Ganora, & Yarnell, 2005).

Probably, the lowering effect on cholesterol herein attained (about 9%) with the sprout administration is the result of a synergy among several plant substances (phytoestrogens, sterols, fatty acids, chlorophyll, polyphenols, catechins), which likely affect the cholesterol biosynthesis.

Anyway, part of the cholesterol reduction in the sprouts groups probably derives from the increase of deposition rate and thus from a "dilution" effect of the cholesterol synthesised by the hens. Indeed, when the same deposition rate is assumed, the calculated reduction of egg cholesterol was lower (4.3 and 5.1% in alfalfa and flax sprouts, respectively). Isoflavones, lignans and cumestans might have an effect on oestrogen release and thus on deposition rate. Previous observations showed that daidzein increased the egg production in ducks and silky hens (Ni et al., 2007).

In plants, isoflavones occur as glycosides, which are deconjugated by intestinal glucosidases to aglycones and metabolised into hormone-like compounds by the intestinal microflora (Setchell, 2003). The oestrogenic activity of these compounds depends on their affinity with oestrogen receptors (Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996). An important metabolite of isoflavones that has a strong affinity for oestrogen receptors and a proper activity on the deposition rate is the equol (Setchell & Clerici, 2010).

Apart from the probable effect of phytoestrogens on the deposition rate, our results showed that hens supplemented with fresh sprouts produced eggs with a significant increase of isoflavones and lignans.

Saitoh, Sato, Harada, and Takita (2001), Saitoh, Sato, Harada, and Matsuda (2004), and Lin, Wu, Abdelnabi, Ottinger, and Giusti (2004) showed that isoflavones can be transferred from the feed into the egg yolk of chickens and quails, respectively.

Laying hens are generally fed with high level of dietary isoflavones due to the soymeal, which is a relevant source of isoflavones (100–250 mg 100 g^{-1}; Vincent & Fitzpatrick, 2000). However, the phytoestrogen content in egg yolk is lower than in milk products and standard egg can be considered a modest source of isoflavones (Kuhnle et al., 2008). Since the intake of phytoestrogens necessary to achieve a biological effect in humans appears to be 30–50 mg d^{-1} (Setchell, 2003), one egg per day provides about 25% of isoflavones requirements. On the contrary, in contrast to milk-based products, the equol amount is relevant, particularly in sprout-fed hens.

There is no clear knowledge on the effect of diet on phytoestrogen content in egg; e.g. the grass intake, trough pasture, in *free-range* chickens does not seem to increase the phytoestrogens in egg (Kuhnle et al., 2008).

Other substances from sprouts that could modulate the egg cholesterol are the sterols. The question on whether plant sterols can effectively lower the egg cholesterol is still debated. Peterson (1951) was the first who showed that dietary soybean sterols reduced plasma and liver cholesterol in cholesterol-fed chickens. Presently, this lowering effect is considered more ambiguous and limited to the plasma. Elkin and Lorenz (2009), utilising sterols from dehydrated plant, observed minor reductions in the egg yolk cholesterol, suggesting that plant sterols, even when present in bioavailable form, have a low intestinal absorption. The same authors suggested that the co-transporters of sterols are able to discriminate between cholesterol and other sterols and do not pump cholesterol back into the intestinal lumen and into the bile (Igel, Giesa, Lutjohann, & Von Bergmann, 2003; Wang et al., 2006).

Our results confirmed that the plant phytosterols given by sprouts (3.3 control vs 4.4 and 4.7 mg d^{-1} hen in alfalfa and flax-seed sprouts, respectively) do not increase the phytosterols in the egg yolk. In agreement with Elkin and Lorenz (2009), only a small amount of campesterol and β-sitosterol was detected into the eggs corresponding to the efficiency of the intestinal absorption (cholesterol > cholestanol > campesterol > sitosterol; Yu, 2008).

Table 8 – Level of lipids (%) and omega-3 fatty acids (ALA, EPA and DHA; g kg^{-1}) in the egg yolk.

Day	Group	Lipids	ALA	EPA	DHA
0	Baseline	30.2	1.25^{a}	0.09^{a}	0.88^{a}
66	Control	32.1	1.30^{a}	0.11^{a}	0.92^{a}
	Alfalfa	30.5	1.58^{a,b}	0.18^{b}	1.12^{a,b}
	Flax	30.1	2.01^{b}	0.23^{b}	1.75^{b}
Pooled SE		3.3	0.31	0.08	0.22

N = 10 pools of 10 yolks per group, a and b: $P < 0.05$.

Also polyunsaturated fatty acids can contribute to reduce the plasma and egg cholesterol (Horrobin & Manku, 1983). Our results show that sprouts significantly increased ALA and DHA in the eggs. Komprda (2012), in a review, summarised that the cholesterol reduction can be modulated by ALA, and mainly DHA dietary supplementation in mice, trough different metabolic pathways, i.e. the 3-hydroxy-3-methylglutaryl-CoA reductase synthesis (HMG-CoA), the transcription factors peroxisome proliferator-activated receptor (PPAR), the sterol-response element binding protein (SREBP) and nuclear factor kB (NF-kB) or with the modification of the lipid raft configuration.

The dietary strategies for improving the fatty acid profile of egg mainly consist of adding ingredients rich in ALA (e.g., linseed, rapeseed; Jiang, Ahn, & Sim, 1991; Lemahieu et al., 2015) or directly in LCPUFA (e.g., fish oils, algae; Hargis, Van Elswyk, & Hargis, 1991; Huopalahti, Lopez-Fandino, & Anton Mand Schade, 2007).

The supplementation of ALA produces less dramatic polyunsaturated fatty acids (LCPUFA) increases being affected by the n-6/n-3 ratio of the diet and by the hen metabolism. However, the former strategy is more physiologically and environmentally sustainable because the fish sources, very rich in LCPUFA, are quickly decreased due to decades of overfishing (Tocher, 2010).

The present experiment confirms the efficiency of the hens (Cherian & Sim, 2001; Lemahieu et al., 2015) to elongate and desaturate ALA into n-3 LCPUFA and to transfer in the eggs where they play a crucial role during chick development (Donaldson, 1996). Many authors suggested that the intake of grass improves the n-3 content of egg (Horsted & Hermansen, 2007; Mugnai, Dal Bosco, & Castellini, 2009) and lowers its n-6/n-3 ratio (Simopoulos & Salem, 1989). The higher LCPUFA content of eggs from hens fed sprouts might be due to their greater antioxidant content too, thanks to the presence of isoflavones (Dixit et al., 2012), vitamin E and carotene.

It should be underlined that high levels of LCPUFA, if not adequately protected, can transform LCPUFA benefits into risks due to the formation of unstable and extremely injurious by-products. In our study, the amount of tocotrienols and carotenoids was two-fold higher after sprouts enrichment. Many researches clearly indicate that members of the vitamin E family are not redundant with respect to their biological functions (Parker, Pearce, Clark, Gordon, & Wright, 1993; Qureshi et al., 1991). The different isomers emerged as vitamin E molecules with functions clearly distinct from that, widely recognised, of α-tocopherol.

Even carotenoids are tissue-specific in their biological activity since α- β- and γ-carotenes have vitamin A activity. These compounds, which are highly pigmented, being yellow, orange and red, are present in fruits and vegetables, and when consumed by birds are incorporated into the yolk. When the sprouts are administrated on the laying hens, lutein concentration doubles. The carotenes, including γ-carotene, lycopene and lutein, protect against uterine, prostate, breast, colon-rectal and lung cancer (Hayes, Pronczuk, & Liang, 1993). It is also known that carotenes may have a high power scavenging of free radicals and are considered among the main elements of defence from coronary heart disease (Osganian et al., 2003).

Greene, Waters, Clark, Contois, and Fernandez (2006) underline the importance of carotenoids on the eye health and the protective effect against the degeneration of retina that can lead to blindness. In particular, lutein and zeaxanthin have a filter effect and protect the macula from degenerative, oxidative and photochemical damages (Burke, Curran-Celentano, & Wenzel, 2005). Although the egg yolk contains smaller amounts of lutein compared to some vegetables (i.e. spinach), it has higher bioavailability.

4. Conclusions

This study demonstrated relevant transfer of bioactive compounds from fresh sprouts to egg yolk, which simultaneously reduced the egg cholesterol. The results herein obtained sustain the possibility to enrich egg not only in LCPUFAn-3 but also in isoflavones, antioxidant and vitamins. The fatty acids and phytoestrogens present in both alfalfa and flax sprouts could be the main responsible for the effect recorded by us, whereas the phytosterols seem to have a lower influence. These positive results encourage further studies to evaluate the effects of different supplementation levels and strategies (e.g., inclusion of lyophilised sprouts in the hen's diet).

Acknowledgements

We gratefully acknowledge Mr. Silvano Locchi for his assistance in sprout production in the Seed Laboratory, and Giovanni Migni and Osvaldo Mandoloni for animal handling.

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