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Egg Production and Quality, Lipid Metabolites, Antioxidant Status and Immune Response of Laying Hens Fed Diets with Various Levels of Soaked Flax Seed Meal

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Abstract: Flax seed meal is a valuable source of fatty acids, particularly omega-3 such as alpha-linolenic acid (ALA), but its mucilage contents limit its use. This study assessed the effect of different levels of soaked flax seed meal (SFSM) on the production and health parameters in Rhode Island Red laying hens. A total of 120 hens were divided into three groups and fed diets containing 0, 6, and 12% SFSM for 10 weeks. The impact was determined based on the egg production and quality, lipid metabolites, antioxidant status, immune response, fertility, and hatchability. Diets containing up to 12% SFSM had no adverse effects on the laying rate, egg weight and mass, and feed conversion ratio (FCR). Hens supplemented with a diet containing 12% SFSM exhibited an improved egg-specific gravity, shell thickness, and yolk color. Moreover, egg albumen (%) and blood and meat spots were significantly increased. The Haugh unit score decreased substantially in chickens supplemented with a diet containing 12% SFSM. Moreover, the blood plasma and yolk lipid profiles were significantly reduced dose-dependently. The inclusion of SFSM improved the yolk lipoprotein by increasing the plasma and yolk high- density lipoprotein (HDL) and HDL/LDL (low-density lipoprotein). However, the antioxidant markers, namely, malondialdehyde (MDA), and the ratio between total antioxidants capacity (TAC)/MDA, were negatively affected in hens fed a 12% SFSM diet. Furthermore, the birds fed a 12% SFSM diet had a better immune response based on the phagocytic activity, phagocytic index, lymphocyte transformation test, lysozyme activity, and antibody titer for Newcastle disease virus. The fertility and hatchability increased significantly by 4.81 and 6.74%, respectively, when the hens were fed a diet of 12% SFSM compared with the control. In conclusion, up to 12% of SFSM in hens' diets had no adverse effects on the productive and reproductive performances; at the same time, they improved the yolk color, plasma lipid profiles, yolk lipid profiles, and immune parameters.

Keywords: soaked flax seed meal; laying hens; egg quality; plasma biochemistry; yolk lipid profiles; immunity; fertility; hatchability

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1. Introduction

The advancement of the economy is causing increased food waste, leading to environmental pollution and problems. As a result of the pandemic, war, and the changes in food chain supplies, agriculture coproducts and their valorization have received significant attention regarding animal nutrition. Moreover, some developing countries suffer from a shortage of animal feed resources due to water limitations and a lack of suitable land for agriculture production. Therefore, alternative feed resources, such as crop

coproducts, might be useful for animal nutrition. The 2030 Agenda for Sustainable Development includes the 17 Sustainable Development Goals. One of them is that countries should involve a plan to implement actions for sustainable management and the use of natural resources, reduction of food losses along supply chains, and the management of all waste throughout its life cycle, substantially reducing its production through reuse and recycle to minimize adverse effects on human health and the environment (https://www.fao.org/sustainable-development-goals/en, accessed on 23 May 2022).

Agro-industrial coproducts are produced during other products' processing, manufacture, use, or disposal. The coproducts still process residues that preserve and sometimes improve the nutritional composition of the products derived. The coproducts may represent a valuable source of bioactive compounds with substantial benefits for human and animal health and the environment [1]. However, many aspects of byproducts' nutritive value are being investigated for any effects of antinutritional factors and their effects on animal performance and product quality.

Flax (*Linum usitatissimum* L.) is a vital crop valued worldwide as a food and feed supplement because of its oil and fiber contents. Globally, the production of flax/linseed reached up to 8.7 million tons in 2016, which was valued at 70.2 $ (https://www.statista.com/statistics/916996/linseed-production-global/, accessed on 24 June 2022). Flax seed is an oilseed that is rich in omega-3 fatty acids (2-3). After oil extraction, cold-pressed flax seed meal is a valuable coproduct for animal nutrition. Cold pressing extraction is done at oil temperatures below 35 °C for oil production; the flax seed meal contains substantial amounts of residual oil—approximately 10% [2].

The cyanogenic glucosides and water-dispersible polysaccharide mucilage (80 g/kg) are the antinutritional factors in flax seed [3,4]. Mucilage is extremely sticky when wet and is indigestible by poultry. Nutritional limiting factors include trypsin inhibitors, pyridoxine antagonists, and phytic acid [5,6]. Soaking the flax seed in warm water, autoclaving it, or increasing the dietary level of Vitamin B6 could improve the use of linseed cake and could supply 50–75% of the protein required for a chick's diet, without affecting the bird's performance [3,5,7]. The mucilage removal of linseed with hot water improved the fat digestibility, major fatty acids, and apparent metabolizable energy corrected for nitrogen retention (AMEn), in addition to markedly reducing the digesta viscosity [4,8]. Improving the utilization of flax seed through soaking treatment may permit its inclusion at a higher level as a source of protein and n-3 fatty acids, despite the shortage in feedstuffs for poultry [9,10]. Flax seed or linseed oil is the primary source of α-linolenic acid (18:3, n-3) for human and animal nutrition [11–16]. It is also reported to enhance the n-3 fatty acid family of animal tissues for human health benefits [3,10–15]. Research on flax seed indicates that including flax in poultry diets can enhance the poultry performance and product quality [16–18]. Omega-3-enriched poultry products have improved the human health index [19]. Caston and Leeson [20] reported that including 10–20% flax in laying hen rations increased the alpha-linolenic acid (ALA) content in eggs by 10-fold or 20-fold, respectively. According to [21], the egg ALA content from hens fed flax seed increased linearly from 0.26 g/100 g of fatty acids in the control diet to 7.07 g/100 g for the eggs from hens fed 15% whole flax.

On the other hand, there have been conflicting reports about the recommended level of linseed cake in poultry diets, including 2–15% for growing chicks [2,4,7,22–24] and 6–15% for laying hens [25–28]. For broilers, up to 10% flax seed in pelleted diets did not affect growth, feed intake, and feed utilization [11,29]. Roth-Maier et al. [30] concluded that the tolerance of chicks to the effects of linseed seemed to be lower than that of the laying hens. Moreover, flax seed positively affects the plasma lipid profiles and reduces the apparent lipid digestibility and lipid synthesis while increasing lipid oxidation [31–33]. Therefore, this study aims to assess the response of the egg productivity, egg quality, lipid metabolites, antioxidant status, immune response, fertility, and hatchability of dual-purpose laying hens to diets containing different levels of soaked flax seed meal.

2. Material and Methods

2.1. Ethical Statement

This work was approved by the Deanship of Scientific Research, King Abdulaziz University, Saudi Arabia, under protocol no. G-246-155-1442 H. The protocol suggests general humane treatment of animals that does not cause distress, suffering, pain, or harm, as reported by Royal Decree number M59 in 14/9/1431H and institutional approval code ACUC-22-1-2.

2.2. Preparation of Soaked Flax Seed Meal (SFSM)

A flax seed meal (brown variety) was obtained from a commercial supplier after cold oil extraction using a screw-pressed method at ~40º. It was soaked in water at a *w/w* ratio for meal/water of 1:2 at 37 °C for 24 h in a water bath, according to Perlas and Gibson [34] and Attia et al. [4]. The soaked meal was then dried in a force ventilated oven at 65–75 °C until constant weight to contain ~10% moisture. Finally, it was ground into fine particles to pass through a 2 mm sieve.

2.3. Experimental Design

A total of 120 thirty-week-old Rhode Island Red laying hens with 1643 ± 39.6 g were used in this trial. The experiment lasted ten weeks, from 30 to 40 weeks. The first two weeks were the preliminary experimental period and were excluded from the investigation and used as an adaptation period. The experimental design included three groups, each containing eight replicates (five hens/each) housed in cages providing 0.36 m^2 per animal. Each cage was supplied with one 50 cm long tube feeder and one stainless steel nipple drinker. Chickens were fed food containing 0, 6, and 12% SFSM, respectively. Table 1 shows the ingredient profiles and nutrient composition of the experimental diets. The nutrient profile and fatty acid profile of the experimental diets were calculated based on the analytical values of feedstuffs National Research Council (NRC) [35] and the abovementioned chemical analyses of SFSM.

Table 1. Composition and chemical analysis of the experimental diets.

Ingredients	Levels of Soaked Flax Seed Meal (%)		
	0	**6**	**12**
Yellow corn	66.0	62.75	60.0
Soybean meal (44% Crude protein)	23.75	21.0	17.7
Soaked flax seed meal	0.0	6.0	12.0
Calcium di phosphate	1.3	1.3	1.3
Limestone	8.0	8.0	8.0
Sodium chloride	0.30	0.30	0.30
Vit. and Min. premix *	0.30	0.30	0.30
DL-methionine	0.10	0.10	0.15
L-Lysine	0.10	0.10	0.10
Sodium bicarbonate	0.10	0.10	0.10
Choline chloride 50%	0.05	0.05	0.05
Determined analysis			
Dry matter %	89.42	90.41	91.23
Crude protein %	15.78	15.83	15.64
Crude fat %	2.69	3.16	3.65
Crude fiber %	3.22	3.71	4.21
Ash	16.7	17.1	17.6

Table 1. *Cont.*

Ingredients	Levels of Soaked Flax Seed Meal (%)		
	0	**6**	**12**
Calculated analysis			
Crude protein %	16.1	16.1	16.0
Metabolizable energy (kcal /kg diet)	2771	2765	2766
Calcium %	3.62	3.62	3.61
Phosphorus available %	0.36	0.37	0.37
Methionine %	0.36	0.38	0.44
Methionine+Cystine %	0.64	0.60	0.60
Lysine (%)	0.88	0.87	0.85
n-6 Polyunsaturated fatty acids (PUFAs), g/1000 g fat	493	470	447
n-3 PUFAs, g/1000 g fat	20.2	49.2	77.9
n-6/n-3 ratio	24:4	9.55	5.73
Saturated fatty acids, g/1000 g fat	158	153	146
Unsaturated fatty acids, g/1000 g fat	739	745	751
Monounsaturated fatty acids, g/1000 g fat	237	236	236
Polyunsaturated fatty acids, g/1000 g fat	513	519	525
Polyunsaturated fatty acids/Saturated fatty acids	3.24	3.41	3.59

* Three kg of vitamin−mineral premix per ton of feed supplied each kg of diet with Vitamin A 12,000 IU; Vitamin D3 2000 IU; Vitamin E 10 mg; Vitamin k3 2 mg; Vit. B1 1 mg; Vitamin B24 mg; Vitamin B6 1.5 mg; Pantothenic acid 10 mg; Vitamin B12 0.01 mg; Folic acid 1 mg; Niacin 20 mg; Biotin 0.05 mg; Choline chloride (50% choline) 500 mg; Zn 55 mg; Fe 30 mg; I 1 mg; Se 0.1 mg; Mn 55 mg; ethoxyquin 3000 mg.

2.4. Assessment of Efficacy

2.4.1. Laying Performance

The laying house performance (e.g., laying rate (%), egg weight (g), egg mass (g/hen/d), feed intake, and FCR) was estimated on the replicate basis and was calculated for the whole experimental period.

2.4.2. Egg Quality

When the hens were 40 weeks old, four eggs were randomly collected from each replicate (32 eggs from each SFSM treatment) from two production days, which were then subjected to egg quality estimation. The egg quality traits, including the external and internal measurements, were estimated as outlined in [36–38]. The exterior measures included the egg shape index, egg-specific gravity, shell percentage, thickness, and weight per unit surface area. The internal egg quality trait included the yolk and albumen weight and yolk-to-albumen ratio. The albumen height was measured, and the Haugh unit was also calculated. The yolk index was estimated by dividing the height of the yolk by its diameter and multiplying it by 100. The egg yolk visual color score was determined by matching the yolk with one of the 15 bands using the Roche yolk color fan [39]. After breaking the eggs, the albumen pH value was determined immediately by measuring its pH using a pH Meter (microprocessor pH meter-HI 9321-Portugal).

2.4.3. Yolk Lipid Profile

Eight yolk samples were randomly collected per treatment—one egg from each treatment replicate. The eggs were used to examine the chemical composition of the yolk. The yolk lipids were extracted using the method of Folch et al. [40] to determine the total yolk lipids [41], triglycerides [42], cholesterol [43], high-density lipoprotein (HDL) [44], and low-density lipoprotein (LDL) [45]. The hypercholesteremia index was calculated as the ratio between the LDL and total cholesterol.

The proximate chemical composition of SFSM showed 91.3% dry matter (DM), 24.9% crude protein (CP), 10.2% ether extract (EE), 13.2% crude fiber (CF), and 6.3% crude ash. These percentages were determined according to AOAC using methods 934.01, 954.01,

920.39, 954.18, and 942.05, respectively [46]. The nitrogen-free extract (NFE) was calculated by differences as 36.7% using the following equation:

$$\text{NFE} = (100 - (\text{moisture} + \text{CP} + \text{EE} + \text{CF} + \text{crude ash})).$$

2.4.4. Blood Biochemical Constituents

Eight blood samples were randomly collected from each treatment to represent all treatment replicates for the hens at 40 weeks of age. The blood plasma was separated after centrifugation of the blood at $1500g \times 10$ min. Then, plasma lipid profiles were determined, including the total lipids [41], triglycerides [42], total plasma cholesterol (Allain, 1974), plasma LDL cholesterol [45], and plasma HDL cholesterol. Additionally, the plasma very-low-density lipoprotein (vLDL) was calculated by dividing the triglycerides by 5 [47].

The plasma total antioxidant capacity (TAC) and malondialdehyde (MAD) were determined according to [48] and [49], respectively. Then, the antioxidant balance was calculated as the ratio between TAC and MDA [50].

2.4.5. Immune System Parameters

The phagocytic activity (PA) and the phagocytic index (PI) were estimated using Kawahara et al.'s method [51]. PA is the percentage of phagocytic cells containing yeast cells and PI is the number of yeast cells phagocytized/number of phagocytic cells. The total antibody production specific to the Newcastle disease virus (NDV) vaccine was measured in the plasma collected on day 7 after vaccination using commercial ELISA kits [52]. The lymphocyte transformation test was determined following Balhaa et al.'s method [53]. Additionally, the bactericidal activity was assayed using Rainger and Rowley's method [54]. The turbidimetric method of Engstad et al. [55] was used to measure the serum lysozyme activity.

2.4.6. Hatching Characteristics

Fertility and Hatchability. At 36, 38, and 40 weeks of age, hens were artificially inseminated with 0.05 mL of pooled semen collected from 15 males. These males were kept in individual cages and fed the control diet. Hens were inseminated with fresh semen collected on 2 successive days. The eggs were collected from each replicate for 7 days and then kept in an egg room at 16.5 °C dry bulb (DB), and 71% relative humidity (RH). Next, the eggs were incubated (37.6 °C DB, 55% RH) and hatched (36.8 °C DB, 65% RH) in Jamesway incubators. The eggs were candled at 7 days; then, those that appeared infertile or had dead embryos were removed and opened to differentiate infertility from early dead embryos. Unhatched eggs were opened and tested to identify the infertile, embryonic mortality, and pipped eggs. Those containing milky white albumen, no embryo, or brownish albumen were considered infertile. Embryonic mortality includes embryos without visible formation of eyes, embryos with large black eyes but no feather formation, and embryos with feather formation that died in the eggs.

Fertility was calculated as the ratio between fertile eggs to the number of eggs set, as follows:

The hatchability percentage was estimated as the ratio of hatched chicks, excluding the pipped chicks, to the number of eggs set, as follows:

$$\text{Hatchability percentage} = \frac{Total\ number\ of\ hatched\ chicks}{Total\ number\ of\ fertile\ eggs\ set} \times 100.$$

2.5. Statistical Analysis

The statistical analysis was performed using the Statistical Analysis Software computer program [56]. Analysis of variance was done using one-way analysis of variance (ANOVA) of the effects of SFSM. The replicate was the experimental unit. Tukey's post hoc was also used for mean differences if a significant p-value was obtained. All per-

centages were transformed to $\log^{10}$ to normalize the data distribution before running the statistical analysis.

3. Results and Discussion

3.1. Effect of Different Levels of Soaked Flax Seed Meal on Laying Performance

Table 2 shows the effects of various levels of SFSM on the rate of laying, egg weight, and mass. Feeding hens 6% SFSM resulted in a greater laying rate than for the 12% SFSM diet, a higher egg mass than the other groups, and a better FCR than those fed the control diet. Increasing the SFSM to 12% had no harmful effects on egg weight and FCR, because these results did not differ from the outcomes of the control and the 6% SFSM groups. The findings also indicate that diets containing 12% SFSM supported laying performance and did not significantly differ from the SFSM-free diet. These results also agree with those observed in [25–27,57]. They indicated that laying hens could tolerate up to 15% flax seed meal without negatively affecting egg production, egg weight, or the feed efficiency of hens producing brown- and white-colored eggs [58]. However, other researchers observed a decline in egg production as a result of feeding 15% flax seed [28,59,60]. Roth-Maier et al. [30] similarly concluded findings that the tolerance of laying hens to linseed's effects seemed higher than that of chicks. In addition, Scheideler et al. [21] reported that laying hens fed flax diets (up to 15%) had outstanding egg production (%).

Table 2. Effect of different levels of soaked flax seed meal on the performance of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	Laying Rate (%)	Egg Weight (g)	Egg Mass (g/h/d)	Feed Intake (g/h/d)	FCR (g Feed: g Egg)	Body Weight Change (g)
0	58.7 ^{ab}	50.4	29.6 ^{b}	117.3 ^{a}	3.96 ^{a}	43.2 ^{a}
6	62.5 ^{a}	50.8	31.8 ^{a}	114.7 ^{b}	3.61 ^{b}	40.3 ^{ab}
12	57.1 ^{b}	51.9	29.6 ^{b}	110.1 ^{c}	3.72 ^{ab}	38.6 ^{b}
SEM	1.25	0.625	0.171	0.873	0.034	2.13
p value	0.004	0.196	0.001	0.005	0.001	0.001

$^{a–c}$ Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$; SEM = standard error of means.

The feed intake showed a stepwise decrease of 2.22% and 4.0%, respectively, with increasing the dietary SFSM of laying hens to 6% and 12%, which was reflected in a significant reduction (10.6%) in weight gain of the laying hens. This may reflect a palatability issue in the feed intake of SFSM diets due to the mucilage content. Similarly, Scheideler et al. [21] and Bean and Leeson [58] found that brown- and white-producing eggshell color hens fed 5% whole flax, 5% ground flax, and 15% ground flax had a lower feed intake and body weight. In this regard, Van Elswyk [32] suggested that laying hens fed an n-3 diet had decreased plasma triglycerides and circulating estrogen (E_2), which limited the availability of the lipids for essential body functions due to the high lipid oxidation and low lipid synthesis [33].

From the standpoints of the n6-to-n3 ratio, the experimental diets contained 24:4, 9.55, and 5.73:1 n6-to-n3 ratios when fed food containing 0%, 6%, and 12% SFSM concentrations, respectively (Table 1). In this regard, Burghardt et al. [61] reported that dietary ratios of n-6/n-3 PUFAs have been implicated in controlling the markers of metabolic syndrome, including insulin sensitivity, inflammation, lipid profiles, and adiposity. However, Carrillo et al. [62] found that the laying rate, egg weight, and egg mass were not significantly affected by the n-6/n-3 ratio, while the 14:1, 8:1, and 4:1 n-6/n-3 ratios had a decreased feed intake and improved FCR. However, Qota [63] observed that feeding different n-6/n-3 ratios did not affect the hens' feed intake and FCR. Furthermore, Diaz et al. [64] found no significant differences in the feed intake and FCR of laying hens fed two n-6/n-3 ratios

(2.34 and 15.99). The results of two n-6/n-3 ratios (1.24 and 15.99) were similar in Bovans laying hens [62].

3.2. Effect of Different Levels of Soaked Flax Seed Meal on Egg Quality

3.2.1. External Egg Quality

Table 3 presents data related to the external egg quality criteria as affected by different levels of SFSM. Hens fed different levels of SFSM had similar egg shape indices, shell weight percentages, and SWUSA. However, the egg-specific gravity and shell thickness significantly improved with the inclusion of SFSM in laying hen diets compared with the control group. The results revealed a different sensitivity of various egg quality measurements. In this regard, Panaite et al. [65] found that eggshell percentage and eggshell breaking strength were not affected by the addition of 6% linseed meal in laying hens' diets, but eggshell thickness increased significantly.

Table 3. Effect of different levels of soaked flax seed meal on egg shape index and eggshell quality of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	Shape Index (%)	Egg-Specific Gravity (g/cm^3)	Shell Weight (%)	Shell Thickness (μm)	SWUSA (mg/cm^2)
0	76.1	1.089 ^{b}	10.54	379 ^{b}	83.2
6	75.9	1.094 ^{a}	10.40	392 ^{a}	83.0
12	75.7	1.095 ^{a}	10.64	396 ^{a}	86.1
SEM	0.37	0.0006	0.121	31.5	2.68
p value	0.421	0.001	0.552	0.001	0.101

^{a,b} Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$; SWUSA = shell weight per unit of surface area; SEM = standard error of means.

The present results indicate that SFSM had no adverse effect on eggshell formation and provided adequate calcium for shell formation. Similarly, Caston et al. [66] found that eggshell deformation and absolute shell weight were not significantly affected by up to 20% dietary flax seed in the laying hens' diets. In the literature, shell weight and shell thickness were not ($p > 0.05$) different for hens fed up to a 10% flax seed diet [58]. In addition, Qota [63] and Bozkurt et al. [67] reported similar results when hens were fed soybean oil or fish oil as a source of n-3. However, Halle and Schone [28] indicated that the eggshell percentage was significantly decreased in hens fed linseed cake (5–15%) compared with rapeseed cake. Different dietary nutrient compositions of various linseed cakes could address this contradiction with the other results.

3.2.2. Egg Yolk Quality

Table 4 displays the effects of different concentrations of SFSM on internal yolk weight, yolk color, yolk index, and the yolk-to-albumen ratio. No significant differences were observed in yolk traits due to varying concentrations of dietary SFSM. However, the hens fed diets with increasing SFSM produced eggs with a significantly and similarly increased yolk color compared with the control diet, which could be attributed to the increased intake of SFSM pigmentation [65]. Additionally, these results agreed with those reported by Halle and Schone [28], who found that linseed cake increased the yolk color compared with rapeseed cake and hemp seed cake. The positive impact of increasing the yolk color on the consumer desire for eggs from hens fed SFSM is additional beneficial effect of increasing Omega-3 fatty acids [65,66].

Table 4. Effect of different levels of soaked flax seed meal on egg yolk quality of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	Yolk Weight (%)	Yolk Color	Yolk Index (%)	Yolk: Albumen Ratio
0	30.8	5.64 [b]	49.5	0.533
6	31.2	6.87 [a]	49.1	0.533
12	30.1	7.42 [a]	48.9	0.515
SEM	0.261	0.051	0.312	0.0071
p value	0.123	0.0001	0.523	0.174

[a,b] Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$; SEM = standard error of means.

Furthermore, according to the present results, Augustyn et al. [68] demonstrated that adding 2% dietary flax oil increased the egg yolk color by decreasing the n-6/n-3 ratio to 1.4 from 13 or 17 n-6/n-3 ratios. Additionally, Carrillo et al. [62] showed that hens fed a diet with a 1.24 n-6/n-3 ratio produced eggs with higher yolk color (9.6) than those fed a basal diet (9.22). However, Qota [63] found that providing different dietary ratios of n-6 to n-3 PUFAs gradually reduced the hen's yolk weight, without affecting the yolk color.

Similar to the current results, Caston and Leeson [20], Caston et al. [66], and Panaite et al. [65] reported that flax seed diets did not affect yolk weight and percentage. Nonetheless, Caston et al. [66] found that yolk weight was significantly decreased in the fifth experimental and attributed this to the low ME value of the 20% flax seed diet. Similar reports demonstrated that yolk weight (g) and percentage (%) were decreased ($p < 0.05$) in laying hens producing white and brown eggs that were fed 10% flax seed diets [28,58,69].

3.2.3. Egg Albumen Quality

Table 5 shows the impact of various concentrations of SFSM on albumen percentage, albumen pH, Haugh unit score, meat spots, and blood spots. Hens fed SFSM diets produced eggs with significantly higher albumen percentages and increased blood and meat spot incidences than the control diet, but with a lower Haugh unit score than the control diet. These results agree with those of Halle and Schone [28], who noted that linseed cake increased the albumen percentage (%) compared with hemp seed cake.

Table 5. Effect of different levels of soaked flax seed meal on the egg albumen quality traits of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	Albumen (%)	Albumen pH	Haugh Unit	Meat & Blood Spots (%)
0	58.1 [b]	8.09	93.1 [a]	0.078 [c]
6	58.7 [ab]	8.02	91.7 [b]	0.112 [b]
12	59.5 [a]	7.75	91.3 [b]	0.178 [a]
SEM	0.251	0.138	0.231	0.0338
p value	0.003	0.295	0.005	0.006

[a–c] Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$; SEM = Standard error of means.

The increase in blood and meat spots of hens fed high levels of SFSM could be attributed to the increasing incidence of hemorrhages. In addition, Bean and Leeson [58] reported that the livers of brown- and white-eggshell-producing laying hens fed flax seeds had a higher ($p < 0.05$) incidence of liver hemorrhages. The increase in blood and meat spots of hens fed 12% SFSM could be attributed to increasing PUFA contents, which are subjected to oxidative rancidity [18,70,71]. However, Schuman et al. [72] and Caston et al. [66] reported that hens did not have more hemorrhages when consuming flax seed.

In the current study, the concentration of SFSM had no significant effect on the albumen pH. Similarly, Bean and Leeson [58] and Panaite et al. [65] found that the albumen height was not significantly ($p > 0.05$) influenced by feeding laying hens 0% and 10% flax seed. However, Grobas et al. [73] stated that the group fed the 0.6:1 n-6/n-3 PUFAs ratio had higher Haugh unit scores than those provided 7.8:1 n-6/n-3 PUFAs, while no significant differences were observed in the yolk weight, albumen weight, and egg yolk color. Shang et al. [74] found that increasing the ratio of n-6/n-3 PUFAs (6:1, 8:1, and 14:1) increased the albumen percentage of brown dwarf hens. However, Panaite et al. [65] reported that a 6% linseed meal diet did not affect the egg albumen percent and Haugh unit score.

The decrease in the Haugh score (1.93%) coincided with increases in the albumen percentage (2.41%) and albumen blood and meat spots (128%). The rise in albumen percentage (%) was correlated with a noticeable decrease (2.27%) in the yolk percentage (%). The decline in yolk percentage could be due to a reduction in the yolk weight (%) of hens consuming a flax seed diet because of the decrease in dietary plasma triglycerides. In addition, the decline could limit the availability of lipids for yolk formation because of the low lipid digestibility, high lipid oxidation, and low lipid synthesis [32,33,58]. Additional causes may include the low ME value of a flax seed diet, as reported in [66].

3.3. Effect of Different Levels of Soaked Flax Seed Meal on Plasma Lipid Profile

Table 6 presents the plasma lipid profiles. The results showed that the plasma total lipids, triglyceride, and LDL cholesterol were markedly decreased with increasing the dietary SFSM ($p < 0.05$). Similarly, extruded linseed dietary concentrations of up to 12% were significantly correlated with a reduction in the total plasma cholesterol, triglycerides, and vLDL lipoprotein levels [75]. On the other hand, the total plasma cholesterol decreased significantly, much like the results of feeding SFSM at 6% and 12% compared with the control diet.

Table 6. The effect of different levels of soaked flax seed meal on the content of various lipid fractions of blood plasma of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	Total Lipids (g/dL)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	HDL/LDL
0	4.12 ^{a}	503 ^{a}	121 ^{a}	73.9 ^{a}	34.9 ^{b}	0.472 ^{b}
6	3.01 ^{b}	441 ^{b}	111 ^{b}	68.8 ^{b}	39.1 ^{b}	0.568 ^{b}
12	2.78 ^{c}	331 ^{c}	113 ^{b}	56.7 ^{c}	46.2 ^{a}	0.814 ^{a}
SEM	0.135	12.81	2.13	1.43	1.86	0.033
p value	0.001	0.001	0.001	0.001	0.001	0.001

$^{a–c}$ Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$. LDL = low-density lipoprotein; HDL = high-density lipoprotein; HDL/LDL = high-density lipoprotein/low-density lipoprotein ratio; SEM = standard error of means.

The decrease in plasma lipid profiles could be attributed to the reduction of ME in the experimental diets. Hence, procures for lipid synthesis were reduced [66], which could be due to the negative effect of flax seeds on lipid digestibility and metabolism. Additionally, flax seed mucilage alone or in combination with calcium negatively affects the apparent digestibility of fat in dogs, rats, humans, and broilers chickens [31,76–78]. Moreover, Van Elswyk [32] revealed that a decrease in circulating triglycerides in birds due to n-3 consumption could limit the availability of lipids for yolk formation. The author also suggested that n-3 could affect circulating estradiol. Moreover, Kristensen [79] demonstrated that dietary flax seed fibers decreased cholesterol and increased fecal fat excretion. In the application of omega-3 eggs in human nutrition, Shakoor et al. [70] stated that offering omega-3 eggs to human subjects reduced the total serum cholesterol by 16.6 mg/dL ($p < 0.001$) and the triglyceride by 18 mg/dL, while increasing the HDL cholesterol concentration by 0.48 mg/dL ($p < 0.001$), as compared with those fed diets with no eggs.

The plasma HDL and HDL/LDL were significantly increased in those fed 12% SFSM compared with those fed other diets (0 and 6% SFSM). The increase in the serum HDL of hens fed 12% SFSM shows the beneficial effects of SFSM on lipid metabolism and the increase in beneficial lipoprotein due to increasing the omega-3 intake [18,70,71].

Feeding hens a 4:1 n-6/n-3 ratio diet decreased the total plasma lipid, triglyceride, cholesterol, and LDL and increased the plasma HDL and HDL/LDL ratios. In addition, Celebi and Utlu [80] reported that ISA brown laying hens fed the control diet (13 n-6/n-3 ratios) had significantly higher triglycerides, total cholesterol, and LDL-C levels than those fed flax oil with (1.4) n-6/n-3 ratios or soybean oil (17) with n-6/n-3 ratios. However, in the same hens, the plasma HDL increased significantly with the addition of 4% flax oil compared with the control group. Furthermore, Qota [63] found that adding 2.5% and 5% flax oil to the laying hen diets for 11 weeks gradually reduced the total lipids and cholesterol levels in the blood plasma.

3.4. Effect of Different Levels of Soaked Flax Seed Meal on Egg Yolk Lipids Profile

Table 7 shows the results for the egg yolk lipids profiles of the hens fed different levels of SFSM. All of the lipid profiles were significantly affected by the SFSM levels. Compared with the control diet, the results indicate that the total lipids, triglycerides, and vLDL decreased due to the 12% SFSM diet. In addition, the total cholesterol, LDL cholesterol, and risk of hypercholesteremia had a stepwise decrease with an increase in SFSM ($p < 0.05$).

Table 7. Effect of different levels of soaked flax seed meal on lipid fractions in the egg yolk of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	TL (mg/g)	TRIG (mg/g)	TC (mg/g)	LDL (mg/g)	HDL (mg/g)	HDL/LDL Ratio	vLDL (mg/g)	RH
0	287 ^{a}	222 ^{a}	13.9 ^{a}	9.32 ^{a}	3.33 ^{b}	0.357 ^{c}	44.4 ^{a}	0.671 ^{a}
6	269 ^{b}	211 ^{ab}	12.7 ^{b}	7.92 ^{b}	4.93 ^{a}	0.622 ^{b}	42.2 ^{ab}	0.624 ^{b}
12	258 ^{b}	197 ^{b}	11.4 ^{c}	6.57 ^{c}	5.36 ^{a}	0.816 ^{a}	39.4 ^{b}	0.576 ^{c}
SEM	5.61	3.41	0.293	0.431	0.516	0.038	0.673	0.132
p value	0.01	0.029	0.001	0.001	0.001	0.001	0.028	0.001

$^{a–c}$ Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$; SEM = standard error of means; TL = total lipids; TRIG = triglycerides; TC = total cholestero; LDL = low-density lipoprotein; HDL = high-density lipoprotein; HDL/LDL = high-density lipoprotein/low-density lipoprotein ratio; vLDL = very-low-density lipoprotein; RH = risk of hypercholesteremia = LDL/TC.

The high-density lipoprotein cholesterol (HDL cholesterol) and the HDL/LDL ratio were increased significantly with an increase in SFSM. These results indicated that increasing omega-3 and PUFA, which were correlated with an elevated SFSM in the hens' diets, showed similar effects on the plasma (Table 6) and yolk lipids profiles (Table 7). The precursors for yolk lipids are synthesized in the liver and transported by the portal vein to the ovary for yolk formation [81].

The decrease in yolk lipid profiles could be caused by the reduction in the circulating triglycerides of birds due to a 12% SFSM diet, increasing the n-3 consumption and potentially limiting the availability of lipids for yolk formation. Increasing dietary n-3 sources has been shown to affect the circulating estradiol [32,33], reduce the apparent digestibility of fat [31,76–78], and lower the ME of the flax seed diet [66]. Additionally, Caston et al. [66] found that the liver weight, dry liver matter, and lipid levels decreased significantly due to feeding 10% and 20% flax seed to the laying hens. In the literature, the yolk cholesterol levels were not affected by the dietary levels of 24.5:1, 2.7:1, 1.8:1, 1.2:1, and 1:1 n-6-to-n-3 ratios [20,82,83]. Caston et al. [66] found that liver fat (%) was markedly lower in birds fed all levels of flax seed ($p < 0.05$), associated with a significant increase in n-3 fatty acids (especially linolenic acid) in the livers of hens fed different concentrations of flax. Fat accumulation in animal tissues such as the yolk, liver, oviduct, and abdominal adipose is affected by the levels and types of lipids [84]. The contradiction among the

results reported herein may be due to the variations in the experimental diet composition and the feeding duration.

3.5. Effect of Different Levels of Soaked Flax Seed Meal on Antioxidant Markers

Table 8 shows the effects of SFSM on the antioxidant status of breeding hens. No significant differences in TAC due to different SFSM concentrations were observed; however, MAD and TAC/MDA were found to be negatively correlated. The antioxidant balance showed a stepwise decline with increasing SFSM concentrations, reflecting the increase in MAD production due to the higher PUFA and free fatty acids in SFSM; these free fatty acids are subjected to peroxidation [81].

Table 8. Effect of different levels of soaked flax seed meal on antioxidant markers in the blood plasma of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	TAC (Umol/L)	MAD (Umol/L)	TAC/MDA (mg/dL)
0	421	1.03 ^{b}	408 ^{b}
6	436	0.971 ^{b}	450 ^{a}
12	428	1.23 ^{a}	348 ^{c}
SEM	5.17	0.097	7.42
p value	0.419	0.003	0.0001

$^{a–c}$ Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$. TAC = total antioxidant capacity; MDA = malondialdehyde; SEM = standard error of means.

According to the existing literature, flax seeds have a low-fat digestibility and high free fatty acids that may promote free radical production and decrease the antioxidant balance. However, in the liver, superoxide dismutase in erythrocytes, glutathione peroxidase in the plasma, and 8-oxo-2′-deoxyguanosine concentrations were unchanged [85]. However, it has been reported that 30% flax seed cake enhanced the plasma total antioxidant status and 30% genetically modified flax seed cake lowered liver thiobarbituric acid reactive substances (TBARS). The latter findings are contrary to the present findings regarding the harmful effects of SFSM on the antioxidant's conditions. In agreement with the current results, Caston et al. [66] found that MDA levels in the liver of birds fed 20% dietary flax seed were moderately elevated. Nonetheless, this did not indicate serious lipid peroxidation. However, Panaite et al. [65] observed that compared with the hens on the control diet, laying hens fed 6% linseed meal had significantly reduced TBARS. These differences in antioxidant status due to feeding SFSM could be attributed to different dietary factors such as the level fed, method of oil extractions, and duration of feeding period [4].

3.6. Effect of Different Levels of Soaked Flax Seed Meal on Immune Responses

Table 9 shows the effects of SFSM on the immune indices breeding hens. Although the longevity rate and bactericide activity (BA) were not significantly affected by the dietary SFSM concentration, PA, PI, lymphocyte transformation test (LTT), lysozyme activity (LA), and hemagglutination-inhibition for Newcastle disease virus (HINDV) increased markedly. Likewise, the improvement was noticeable when the 12% SFSM was offered. Furthermore, the 6% SFSM induced a similar trend in the PA, LTT, and LA.

The results indicate that SFSM could enhance the immune function of laying hens due to the n-3 fatty acid content. Sijben et al. [86] noticed that the dietary n-6-to-n-3 ratios of 29.3:1, 56.7:1, 1.35:1, and 2.41:1 PUFAs in the laying hen diets resulted in total antibody titers of 7.36, 7.74, 6.87, and 8.07, respectively. Similarly, increasing the n-3 PUFA levels improved the immune response in newly hatched chicks [63,87].

Table 9. Effect of different levels of soaked flax seed meal on the immune characteristics of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	PA (%)	PI (%)	LTT (%)	BA (%)	LA (IU%)	HINDV (Log^2)	Longevity
0	18.1 ^{b}	1.28 ^{b}	18.0 ^{b}	40.9	0.033 ^{b}	3.92 ^{b}	98
6	22.6 ^{a}	1.53 ^{ab}	20.9 ^{a}	42.3	0.037 ^{a}	4.16 ^{ab}	95
12	20.4 ^{a}	1.72 ^{a}	20.3 ^{a}	41.4	0.039 ^{a}	4.36 ^{a}	98
SEM	0.213	0.061	0.274	0.354	0.00012	0.115	1.96
p value	0.001	0.015	0.001	0.452	0.003	0.006	0.763

^{a,b} Means within a column within each factor not sharing similar superscripts are significantly different $p > 0.05$. PA = phagocytic activity; PI = phagocytic index; LTT = lymphocyte transformation test; BA = bactericidal activity; LA = lysozyme activity; IU = international unit; HINDV (Log_2) = antibody titration against Newcastle disease virus; HI = hemagglutination inhibition; SEM = standard error of mean.

It has been reported that n-6-derived eicosanoids propagated inflammatory signals, while many n-3-derived eicosanoids were less inflammatory or anti-inflammatory [88]. Although there were apparent effects of feeding SFSM to laying hens, there were also clear trends in the longevity of feeding. A small number of the experimental population and/or the good hygienic conditions in the current experiment could explain the lack of significant differences in longevity. These results agree with those reported by Hudson and Wilson [89]. They noted that there was no significant effect on the mortality of male broiler breeders when they were fed two n-6/n-3 ratios—16:1 and 4:1.

3.7. Effect of Different Levels of Soaked Flax Seed Meal on Hatching Performance

Table 10 shows the impact of different levels of SFSM on embryonic mortality (%), piped chicks (%), fertility (%), and hatchability of the total eggs (%). The fertility (%) and hatchability of total eggs (%) were significantly improved with increasing the SFSM, reaching their greatest point from the hens fed 12% SFSM. On the other hand, the embryonic mortality and pipped chicks were not affected by different n-6/n-3 ratios. The improved fertility and hatchability of the hens fed SFSM could be attributed to increasing the n-3 fatty acids in SFSM diets and decreasing the obesity of laying hens. The negative effects of SFSM on lipid digestibility, dietary lipid deposition, and ME were associated with reduced broiler breeders' obesity, thus improving fertility and hatchability [36,37]. In the literature, supplementation with PUFAs improved the reproductive performance of young roosters by increasing hormone secretion and function [90]. Therefore, balancing the n-3/n-6 PUFA ratio with dietary omega-3 supplementation in broiler breeders can lower late embryonic mortality [91].

Table 10. Effect of different levels of soaked flax seed meal on the hatching characteristics of dual-purpose breeding hens.

Soaked Flax Seed Meal (%)	Embryonic Mortality (%)	Pipped Eggs (%)	Fertility (%)	Hatchability (%)
0	2.31	4.11	89.4 ^{b}	83.1 ^{b}
6	1.71	5.11	91.3 ^{ab}	86.5 ^{ab}
12	2.35	6.86	93.7 ^{a}	88.7 ^{a}
SEM	0.451	0.358	0.428	0.516
p value	0.186	0.487	0.001	0.001

^{a,b} Means within a column within each factor not sharing similar superscripts are significantly different, $p > 0.05$; SEM = standard error of means.

4. Conclusions

From findings, it was assessed that feeding laying hens up to 12% SFSM did not adversely influence performance traits, fertility and hatchability, and eggshell quality.

Moreover, it improved yolk color, plasma, yolk lipid profiles, and immune indices while decreasing the risk of yolk hypercholesteremia.

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