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Effects of Linseed Expansion on its Dietary Molecular Structures, and on Broiler Chicks Digestive Enzymes Activity, Serum Metabolites, and Ileal Morphology

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Primary Audience: Nutritionist, Feed Manufactures

SUMMARY

Presence of various anti-nutritional factors such as mucilage, linatine, cyanogenic glycosides, and trypsin inhibitors in linseed has confined its usage as broiler feed ingredient. The objective of this study was to determine the effect of linseed, after a high-temperature, high-pressure expansion processing, on dietary anti-nutritional factors and nutrient molecular features. Further, an experiment was carried out in a completely randomized design with chicks fed either a control corn–soy diet or graded levels of expanded linseed (EL; 67, 135, and 203 g/kg). Each diet was fed to 8 groups of 15 chicks. Expansion of linseed increased live weight (LW) and reduced feed conversion ratio (FCR; g feed/g LW) considerably. EL incorporation in the diet of chicks increased digestive α-amylase, lipase, trypsin, and total alkaline protease activity. Incorporation of EL in the diet increased villi height and decreased crypt depth. Abdominal fat increased and pancreas weight decreased by inclusion of EL in diets. Gizzard and jejunum pH increased and duodenum pH decreased in chicks fed diets containing EL. The Fourier-transformed infrared spectroscopy showed only reduction in lipid to carbohydrates ratio in molecular structures. Positive correlation was found between FCR and α-helix to ß-sheet ratio and carbohydrate-to-protein ratio. Increased ß-sheet height reduced lipase activity, whereas an increased α-helix to ß-sheet ratio increased lipase activity. The outcome of this study was that expansion was effective for improving the nutritional value of linseed for broiler chicks.

Key words: expansion, linseed, enzyme activity, FTIR molecular structure

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DESCRIPTION OF PROBLEM

Because of beneficial health effects of linseed oil, especially omega-3 fatty acids [1–2], we tried to assess the effect of expansion on plasma lipid profile. Linseed, also known as flaxseed, (*Linum usitatissimum L.*) is high in omega-3 fatty acids and could be used to feed animals to improve their meat fatty acid profile. Linseed has received, however, little attention for poultry as a valuable source of energy, protein, and unsaturated fatty acids because it also contains various anti-nutritional factors such as mucilage, linatine, cyanogenic glycosides, trypsin inhibitors and phytic acid [3, 4]. Mucilage in linseed markedly increases the viscosity of ileal digesta in broilers and reduces nutrient

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digestibility and utilization resulting in reduced broiler chicken growth [3, 5]. Demucilaging linseed was found to increase its metabolizable energy and digestibility of fatty acid in broiler chicks [3, 6]. Treating linseed with carbohydrase enzymes was found to increase the fat content of breast, thigh, and adipose tissue [7]. Hence, using an appropriate, innovative and valuable processing technology will likely ameliorate the anti-nutritional factors and boost the nutritional value of linseed for broiler chicks.

The expander technology is a method with one of the best and most comprehensive conditioning capability, with low power consumption and high throughputs for compound feed and individual ingredients. The expander extrusion process includes a combination of moisture, high temperature, and pressure, over a short period of time that enhances digestibility of plant protein [8, 9] and improves soluble fiber content and digestibility [10, 11, 12]. One of the great features of the expander technology is that large quantity of liquids can be added, such as fat and molasses. This technology is therefore suitable for processing high oily seeds such as linseed. Björck et al. [11] reported that expanded extruding wheat flour increased soluble fiber digestion up to 75%. Barrows et al. [12] reported that extrusion increased soluble fiber content and improved fiber digestibility and digestible energy content of soybean meal. Extrusion and pelleting of high fibrous diet for growing pigs increased digestibility of starch and reduced ileal digesta viscosity [13]. However, over-processing of feed can have deleterious effect on nutrient molecular features and nutrient availability for the poultry. Fourier-transformed infrared spectroscopy (FTIR) is a method that can be used to determine the effect of different processing methods on molecular structures of proteins, lipids, and carbohydrates of feed [14]. Univariate and multivariate methods such as principal component analysis (PCA) and cluster analysis (CLA) are usually applied to describe the FTIR results [15]. Changed FTIR molecular structures have been related to nutrient availability of feed for the animal [16].

The objective of the current study was to determine the effect of linseed expansion on FTIR molecular structures. Secondly, to determine the effect of inclusion of expanded linseed (EL) in the diet of broiler chicks on gastrointestinal enzymes activities, gut morphology, plasma metabolites, and growth parameters. Our hypothesis was that the expansion of linseed would change its nutrient profile and availability, and improve chicks' performance and physiological responses.

MATERIALS AND METHODS

The animal experiment was carried out in accordance with the procedure and guidelines approved by the Animal Care Committee of Iranian Council of Animal Care, 1995.

Expansion Processing of Linseed

A batch of the linseed was wet expanded at a high temperature (115 ± 5°C) with high pressure for a period of 40 s using a single screw (speed of 450 rpm, diameter of 10 cm), single conditioner expander machine [17]. Standard temperature was 105 to 127°C and standard duration was 25 to 40 s for linseed expansion to maintain an optimum balance between reducing anti-nutritional factors and increases fiber solubility and starch gelatinization, while maintaining fatty acids integrity and bioavailability of essential amino acids. Expanded linseed was analyzed for its crude protein and amino acids profiles and also for apparent metabolizable energy content using near infrared spectroscopy [18]. Crude fiber was determined by sequential extraction with diluted acid and alkali [19] and ether extract by Soxhlet fat analysis after 3 *N* HCl acid hydrolysis [19]**.** Composition analysis of EL is shown in Table 1.

Bird Management and Experimental Design

In total, 480 one-day-old male Ross-308 broilers with an initial body weight (BW) of 40 ± 1.1 g were housed in an environmentally controlled building. Chicks were randomly allotted to 3 experimental groups (67, 135, and 203 g/kg EL) or the control group, each with 8 replicated pens with 15 chicks each. Formulated control and experimental diets (Table 2) were fed to chickens in the mash forms. Weekly feed consumption for chicks in each pen was calculated by total amount of feed placed in the feeder minus the residual feed in the feeder.

Table 1. Chemical Composition, Apparent Metabolizable Energy (AME), Amino Acids, and Anti-Nutritional Factors in Raw and Expanded Linseed (EL).

	Raw linseed	Expanded linseed
	(g/kg DM)	
AME (MJ/kg DM)	4,100	4,140
Crude protein	220.3	220.8
Crude fat	354	356
Crude fiber	67	63
Methionine	4.05	4.10
Cystine	3.61	3.64
Methionine + Cystine	7.66	7.74
Lysine	7.82	7.83
Threonine	7.54	7.61
Tryptophan	3.31	3.42
Arginine	17.87	17.87
Isoleucine	8.43	8.45
Leucine	11.47	11.65
Trypsin-inhibitor (units/g)	11,360	783
Trypsin-inhibitor activity (mg/g)	4.7	1.3
Urease activity (Δ pH units)	0.14	0.00

Automatic bell drinkers supplied ad libitum water. Chicks were brooded following standard temperature regimens, gradually decreasing from 32 to 24°C, under a 23:1 light:dark cycle. The BW was measured weekly and cumulative feed intake was measured at 14, 28, and 42 d of age for each pen, which allows for calculating the feed conversion ratio (FCR).

Molecular Spectroscopic Analysis

The IR absorbance band of samples was determined using FTIR spectroscopy [20] coupled with a universal attenuated total reflectance accessory. The samples (3 per each treatment) were finely ground and pressed uniformly against the diamond surface using a spring-loaded anvil. The mid-IR spectra were recorded from a resolution of 4,000 to 800 cm^{-1} at 2 cm^{-1} (Figure 1), and also from a resolution of 2,770 to 3,000 cm^{-1} for total lipid area (Figure 2), 1,485 to 1,725 cm^{-1} for total protein area (amide I and

Table 2. Ingredient and Chemical Composition of the Control Diet and Diets Containing EL.

	Basal diets (g/kg DM)			
Ingredients	Control	67EL	135EL	203EL
Maize	556	527	497	467
Soybean meal	380	347	314	281
Expanded linseed (EL)	0	67	135	203
Maize oil	26	21	16	10
Dicalcium phosphate	18	18	18	18
Limestone	10	10	10	10
Sodium chloride	3.5	3.5	3.5	3.5
Vitamin and mineral premix[1]	3	3	3	3
DL-Met	2.2	2.1	1.9	1.9
L-Lys HCL	1.1	1.6	2.1	2.7
L-THR	–	–	0.1	0.3
Composition				
ME (MJ/kg)	12.45	12.34	12.24	12.13
Crude protein	214	215	216	216
Crude Fiber	6.93	7.10	8.80	10.5
NSP[2]	14.40	15.10	15.90	16.60
Lys	12.51	12.92	12.78	12.64
Met	5.3	5.2	5.17	5.25
Met + Cys	8.83	8.80	8.51	8.43
Thr	8.17	8.39	8.28	8.16
Calcium	10	10	10	10
Phosphorus (available)	5	5	5	5

[1]Vitamin and mineral mix supplied the following per kg of diet: transretinol: 11 mg; cholecalciferol: 0.5 mg; a tocopherol acetate: 80 mg; menadione: 3 mg; thiamine: 3 mg, riboflavin: 8 mg; pyridoxine: 5 mg; cyanocobalamin: 0.024 mg; nicotinic acid: 60 mg; folic acid: 2 mg; Ca pantothenate: 15 mg; choline chloride: 250 mg; Mn: 120 mg; Zn: 100 mg; Cu: 15 mg; Se: 0.3 mg; I: 1 mg; and Fe: 30 mg.

[2]Non-starch polysaccharides.

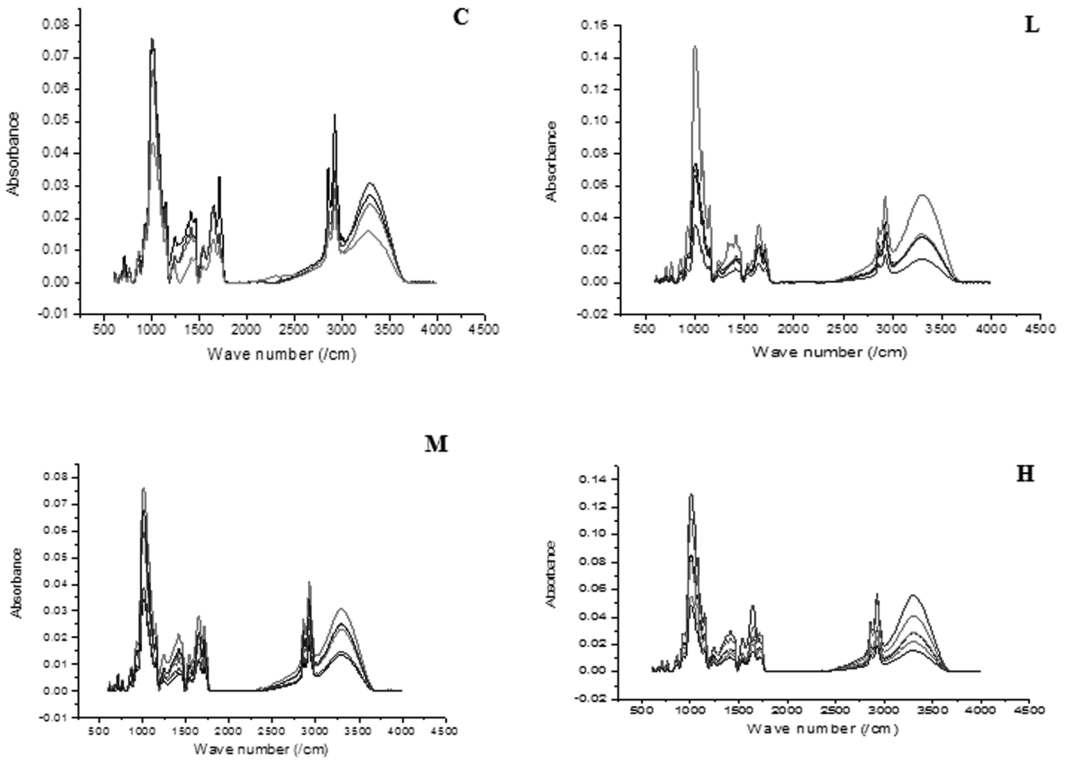

Figure 1. The mid-IR spectra recorded from a resolution of 4,000 to 800 cm^{-1} at 2 cm^{-1}, (c) Control, i.e., raw linseed; or low (L; 67 g/kg), medium (M; 135 g/kg), and high (H; 203 g/kg) inclusion level of expanded linseed (EL).

amide II; Figure 3), and 800 to 1,184 cm^{-1} for total carbohydrates [21] area (Figure 4). Each sample was scanned twice (3 samples $\times$ 2 scans $=$ 6 scans for each treatment). The collected spectra were corrected against air as background. The second derivative spectrum in the protein amide I region was used for Lorentzian and Gause modeling to determine the protein second structures, including α-helix and ß-sheets [14, 15] (Figure 3).

Univariate Molecular Spectral Analysis

The FTIR spectra were baseline correction and data normalization were performed using Origin software [22]. Protein molecular structure determined were amide I and II peak area and height, α-helix and ß-sheet peak height intensities, and their ratios. Chemical function groups were identified according to published reports [23]. In this study, the analyzed spectral baseline was ca. 1,720 to 1,485 cm^{-1} for proteins, ca. 1,720 to 1,575 for amide I, ca. 1,575 to 1,490 cm^{-1} for amide II and ca. 1,452 to 1,486 cm^{-1} for cellulosic compounds. The α-helix and ß-sheet peaks fell in the range of ca. 1,650 to 1,660 and 1,620 to 1,640 cm^{-1}, respectively. The different ratios were calculated according to respective absorbance intensity value [14, 15].

Multivariate Analysis

Protein, lipid, and carbohydrate IR fingerprint data of the diets were analyzed using agglomerative hierarchical CLA and PCA using Origin software [14, 15]. These results were used to classify and distinguish 4 combinations, inherent lipid, protein and carbohydrate structural differences (Figures 2, 3, and 4), and the main sources of variation within the protein amide I and II fingerprint spectra (ca. 1,720 to 1,575 and 1,575 to 1,490 cm^{-1}) and cellulosic compound (ca. 1,452 to 1.486 cm^{-1}) (Figures 3 and 4).

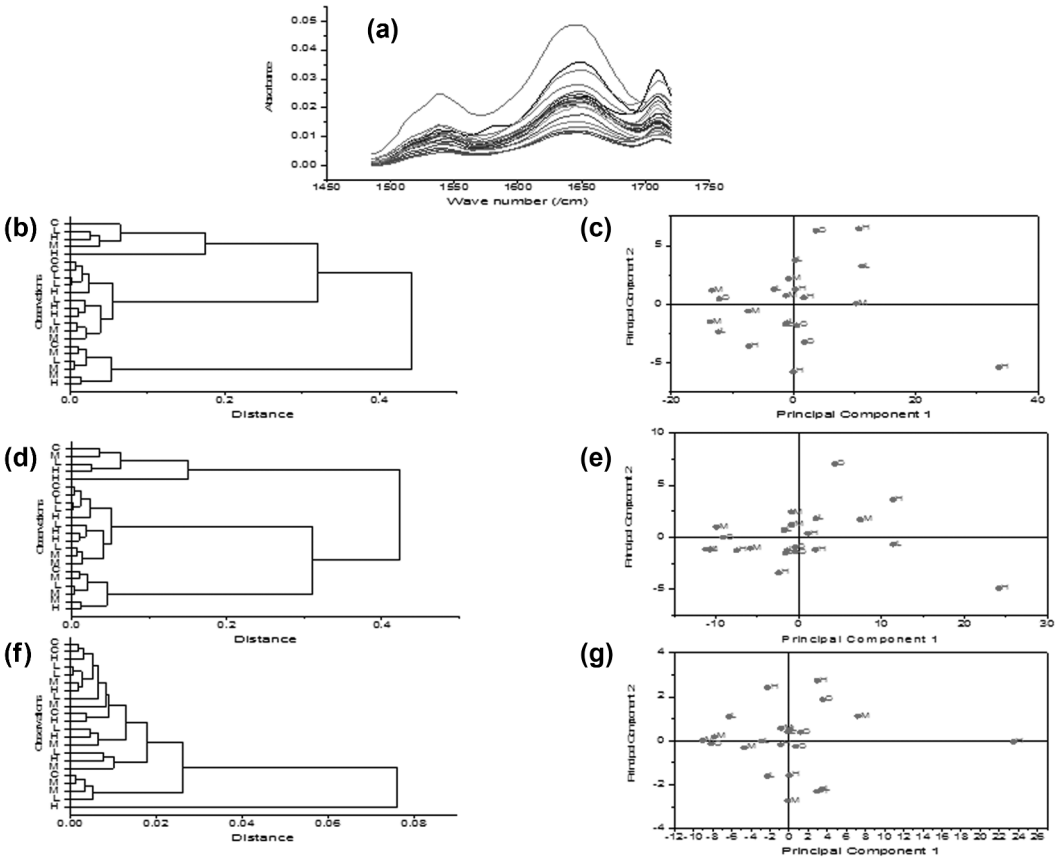

Figure 2. (a) The mid-IR spectra recorded from a resolution of 1485 to 1725 cm^{-1} for total protein area; (b) Cluster analysis (CLA) and (c) Principal component analysis (PCA) from protein area (baseline ca. 1,485 to 1,725 cm^{-1}); and (d and e) CLA and PCA from amide II area (ca. 1,720–1,575 cm^{-1}); and (f and g) CLA and PCA from amide I (baseline ca. 1,575 to 1,720 cm^{-1}) of Control, i.e., raw linseed; or low (L; 67 g/kg), medium (M; 135 g/kg), and high (H; 203 g/kg) inclusion level of expanded linseed (EL).

Digestive Enzymes Activity Analysis

Digestive enzymes activities were determined at 21 and 42 d of age. Two chicks from each pen were chosen randomly, weighed, and euthanized in accordance with the procedure and guidelines approved by the animal care committee of the university. Feed was removed from the feeders at 6 h before slaughter to facilitate intestinal emptying of birds. The duodenum and a 10-cm segment of the jejunum adjacent to the distal pancreas, free of residual food, were removed and frozen in liquid nitrogen. Samples were stored in liquid nitrogen until preparation for assay. The frozen intestine was partially thawed in a refrigerator at 4°C for 2 h. Each sample was homogenized (dilution 1:5, w/v) in cold buffer (50 mM Tris–HCl, pH 8.0 containing 10 mM CaCl2) on ice at 11,000 rpm for 2 min. Thereafter, the homogenate was centrifuged at 14,000 *g* for 45 min at 4°C. The supernatant was collected and aliquots were stored at –80°C until digestive enzyme analysis. Assay dilution tests were previously performed for each enzyme activity to ensure optimum ratio between enzyme and substrate. All enzyme activities were measured at 37°C using a spectrophotometer, with specific assay conditions for each enzyme as described hereafter.

Trypsin (EC 3.4.21.4) activity was determined using Nα-benzoyl-Larginine 4-nitroanilide hydrochloride as substrate according to the method of Erlanger et al. [24]. One unit of activity was defined as the enzyme releasing 1 μmol p-nitroaniline per min at

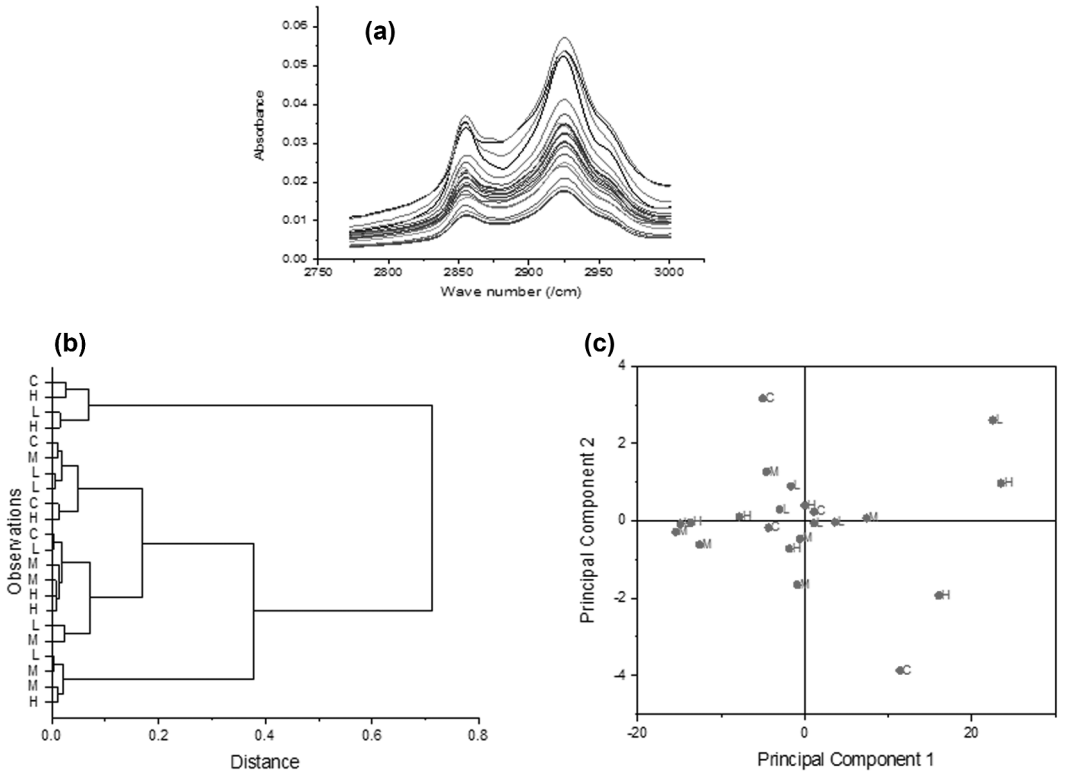

Figure 3. (a) The mid-IR spectra recorded from a resolution of 2,770 to 3,000 cm^{-1} for total lipid area. (b) Cluster analysis (CLA) and (c) Principal component analysis (PCA) from lipid component for Control, i.e., raw linseed; or low (L; 67 g/kg), medium (M; 135 g/kg) and high (H; 203 g/kg) inclusion level of expanded linseed (EL). (baseline ca. 2770 to 3000 cm^{-1}).

410 nm. α-amylase (EC 3.2.1.1) activity was estimated using the Bernfeld [25] procedure, with starch as substrate. One unit of activity was defined as 1 μmole of maltose released per min, with absorbance was measured at 540 nm. Lipase (EC 3.1.1.3) activity was measured by hydrolysis of ρ-nitrophenyl myristate as substrate according to the method of Iijima et al. [26]. One unit of enzyme activity was defined as 1 μmol of ρ-nitrophenol released per min at 405 nm. Total alkaline protease activity (APA) was determined based on the casein hydrolysis assay of Kunitz [27] as modified by Walter [28]. One unit of activity was defined as the amount of enzyme needed to produce 1 μmol tyrosine per min at 280 nm. The concentration of soluble protein in extracts was determined by the method of Lowry et al. [29], using bovine serum albumin (0 to 1 mg mL^{-1}) as a standard, with absorbance measured at 750 nm.

Blood Metabolites

Four chicks from each pen were randomly selected at days 21 and 42 of age and 2-mL blood samples collected from the brachial vein into heparinized and non-heparinized tubes. Two samples (non-heparinized tubes) were centrifuged at 1,734 $\times$ g at 0°C for 20 min to harvest serum, which was used for analysis of triglyceride, cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) using an automatic biochemical analyzer [30] by the colorimetric method [31]. The blood samples in the heparinized tubes were centrifuged at 1,734 $\times$ g at 0°C for 20 min and plasma harvested, which was used for analysis of uric acid, albumin and total protein concentration. Serum (0.5 mL) from 2 birds was collected and pooled by replicate pen. The pooled serum was then analyzed for plasma uric acid [32] and serum urea

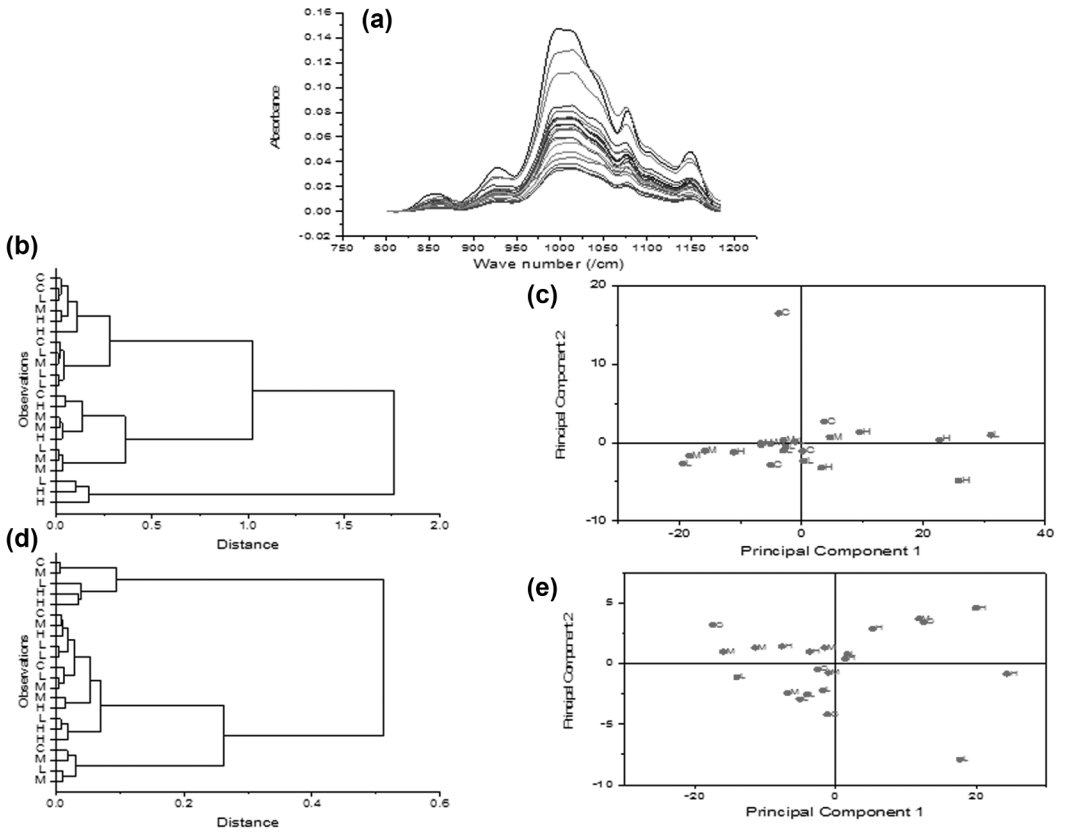

Figure 4. (a) The mid-IR spectra recorded from a resolution of 800 to 1,184 cm^{-1} for total carbohydrates. (b) Cluster analysis (CLA) and (c) Principal component analysis (PCA) from carbohydrates area (baseline ca. 800 to 1,184 cm^{-1}); and (d and e) CLA and PCA from cellulosic compound (baseline ca. 1,452 to 1,486 cm^{-1}) of Control, i.e., raw linseed; or low (L; 67 g/kg), medium (M; 135 g/kg), and high (H; 203 g/kg) inclusion level of expanded linseed (EL).

nitrogen [33] analyzed using commercial reagent kits.

Ileal Morphology

Two chicks from each pen were euthanized at 21 and 42 d of age for evaluation of ileal morphology. The digestive tract along with contents was removed properly with great care from each chick and the ileum was separated from the Meckel's diverticulum up to 1 cm proximal to the ileocecal junction, which were then dried with desiccant paper. A 2-cm section of ileum was taken from the middle of the ileum, which was gently flushed with phosphate buffered saline (pH 7.2). Tissue sections were immediately fixed in 10% neutral buffered formalin, which was changed 2 times for completion of the fixation. A single 0.5-cm sample was cut from each ileal section, dehydrated with increasing concentrations of ethanol, cleared with xylene, and placed into polyfin embedding wax. Tissue sections (2 μm) were cut by microtome [34] floated onto slides, and stained with hematoxylin and eosin [35]. To measure villus height and crypt depth, images from samples and micrometer were taken using a digital camera on a light microscope [36]. Forty images from 20 tissue sections of each ileal section were taken and villus heights and crypt depths were measured using imaging software. Measurements for villus lengths were taken from the tip of the villus to the valley between individual villi, and measurements for crypt depth were taken from the valley between individual villi to the basolateral membrane.

Carcass Analysis and Gastrointestinal pH

Two chicks from each pen were euthanized at days 21 and 42 of age and the weight of carcass, breast, thigh, abdominal fat, liver, pancreas, gizzard, and bursa of Fabricius recorded, and the length of the duodenum, jejunum, and ileum were measured. The weight of the empty organs was expressed relative to live weight. The gizzard, duodenum, jejunum, and ileum were cut longitudinally and the pH of their contents measured in triplicate using a digital pH meter [37].

Statistical Analysis

Data were analyzed in a completely randomized design using the GLM procedure of SAS [38] with 4 treatments (control and 3 extruded linseed inclusion level) and 8 replicated pens per treatment. Normal distribution of the residuals was tested by the UNIVARIATE procedure in SAS. Trypsin activity data were not normally distributed and these data were therefore log transformed before analysis. When the effect of treatment was significant, then differences among individual treatment means were analyzed using the Duncan multiple comparison test.

The relationship between the FTIR lipid, carbohydrate and protein molecular structures amide I and II area, the amide I and II ratio and α-helix and ß-sheet and their ratios and measured animal characteristics were analyzed using PROC CCORR of SAS software [38]. Having appropriate correlation, data are considered at Table 5. For all statistical analyses, significance was declared at $P < 0.05$. Treatment means were compared using the Tukey multiple range test.

RESULTS

Molecular Spectroscopic Features

All FTIR molecular spectral features were similar among the 4 treatments, except FTIR lipid to carbohydrate ratio, which decreased linearly ($P \leq 0.05$) with increasing EL inclusion in the diet (Table 4). Using multivariate analysis, PCA and CLA, differences among treatments for protein, amid I, amid II, carbohydrate, cellulosic compounds, and lipid could not be distinguished among the 4 diets (Figures 2, 3, and 4).

Feed conversion ratio had a strong positive correlation with α-helix to ß-sheet ratio (r = 0.94), carbohydrate-to-protein ratio (r = 0.97) and ratio of amide I to amide II (r = 0.96) (Table 5). Plasma uric acid concentration had a strong positive correlation with carbohydrate-to-protein ratio (r = 0.97) at 21 d of age. Plasma cholesterol had a strong negative correlation (r = –0.95) with ß-sheet height and a strong positive correlation (r = 0.97) with ß-sheet to α-helix ratio at 21 d of age. There was a negative correlation between triglyceride and lipid-to-protein ratio (r = –0.98) and lipid-to-carbohydrate ratio (r = –0.9) at 21 d of age.

At 42 d of age, there were strong negative correlations for feed intake (r = –0.96) and trypsin activity (r = –0.98) with lipid-to-protein ratio. Also, FCR had strong negative correlation with protein amid I and II molecular structures (r = –0.99) and tended to have a negative correlation with carbohydrate-to-protein ratio (r = –0.93; $P = 0.07$). Lipase activity had a negative correlation with ß-sheet height (r = –0.95) and a positive correlation with α-helix to ß-sheet ratio (r = –0.95; $P \leq 0.04$) at 42 d of age.

Molecular Spectral Multivariate Analysis

The CLA and PCA analysis for classifying the structural differences in treatments for lipid, protein, and carbohydrate were shown in Figures 2, 3, and 4. The spectral cluster of treatments were without EL (C) and diets containing different levels of EL (L = 67 g/kg, M = 135 g/kg, and H = 203 g/kg) were not different from each other in regards to the lipid, protein, and carbohydrate structure. Also the CLA of cellulosic compound and protein amid I and II for control and diets containing EL revealed no differences between treatments. PCA also displayed that the lipid, protein, and carbohydrate structural makeup for control and diets containing EL (L, M, and H) were not distinguished.

Performance

Including 67 and 135 g/kg of EL in the diet reduced FCR and increased BW,

Table 3. Performance of Broilers Fed Control or Graded Levels of EL.[1]

	14 d			28 d			42 d		
Treatments	FCR,[3] g of feed/g of BW	FI,[2] (g)	BW (g)	FCR,[3] g of feed/g of BW	FI,[2] (g)	BW (g)	FCR,[3] g of feed/g of BW	FI,[2] (g)	BW (g)
Control	1.13[b]	503.88[b]	445.69	1.43[b]	1,848.44	1,373.75[a]	1.78[a]	4,124	2,478.8[b]
67EL	1.08[b]	533.59[a]	450.51	1.44[b]	1,956.02	1,425.74[a]	1.69[a,b]	4,379.1	2,748.8[a]
135EL	1.12[b]	535.98[a]	444.5	1.49[b]	1,983.98	1,400.92[a]	1.63[b]	4,192.7	2,532.5[a,b]
203EL	1.28[a]	533.23[a]	416.6	1.61[a]	1,895.26	1,239.2[b]	1.85[a]	4,288.6	2,486.3[b]
SEM	0.03	8.13	10.71	0.02	43.7	38.4	0.04	107.85	89.34
P-value	0.02	0.04	0.1	0.0004	0.17	0.02	0.05	0.67	0.08

[a–d]Means within a column without a common superscript significantly differ ($P < 0.05$).

[1]Pens means were used to calculate means per diet.

[2]FI = feed intake. [3]FCR = feed conversion ratio.

especially at 42 d of age compared to the control diet and 203 g/kg of EL in the diet (Table 3). Feed intake from 0 to 14 d of age was greater in chicks fed diets containing EL, while between 14 and 42 d of age, cumulative feed intakes were largely similar between dietary treatments.

Digestive Enzymes Activity

The α-amylase activity, both at 21 and 42 d of age, was greater ($P \leq 0.001$) in diets with EL, especially included at 67 g/kg (Table 6). A maximum amylase activity (close to 4-fold greater than that of control group) was observed when soybean meal was replaced by 67 g/kg EL. Trypsin activity was similar for chicks on the 4 diets at 21 d of age, while trypsin activity was greater in chick fed EL at 42 d of age ($P \leq 0.03$). Inclusion of EL in the diet of chicks enhanced ($P \leq 0.05$) lipase activity, both at 21 and 42 d of age. A lipase activity, close to 6.54 and 3.68-fold at 21 d of age and 3.29 and 7.21-fold at 42 d of age, greater than that of control group was observed when soybean meal was substituted by 67 and 203 g/kg EL, respectively. Total APA was less in chicks fed diets with EL at 21 d of age, while there APA was similar among the 4 diets at 42 d of age.

Table 4. Fourier-Transformed Infrared (FTIR) Spectroscopic Molecular Structures of Control or Graded Levels of EL (67, 135, and 203 g/kg, Respectively).

	Treatments					*P*-value and contrasts			
Molecular structure	Control	67EL	135EL	203EL	SEM	Treatment	Linear	Quadratic	Cubic
Protein secondary structures									
α-Helix height	0.305	0.310	0.293	0.351	0.046	0.858	0.746	0.541	0.618
ß-Sheet height	0.338	0.319	0.333	0.144	0.085	0.413	0.709	0.275	0.204
Ratio of α-helix to ß-sheet height	1.408	1.723	1.858	2.796	0.602	0.516	0.927	0.348	0.212
Protein, carbohydrate, and lipid FTIR molecular structures									
Amide I area	0.021	0.023	0.019	0.029	0.003	0.233	0.474	0.104	0.369
Amide II area	0.010	0.009	0.009	0.013	0.002	0.229	0.408	0.216	0.173
Ratio of amide I to II area	2.477	2.423	2.247	2.182	0.247	0.835	0.715	0.821	0.402
Carbohydrates	0.065	0.075	0.055	0.083	0.011	0.358	0.524	0.125	0.778
Carbohydrates to proteins ratio	3.364	3.140	2.997	2.935	0.307	0.822	0.560	0.665	0.486
Cellolosic compounds	0.015	0.016	0.014	0.019	0.003	0.637	0.660	0.292	0.653
Cellolosic to carbohydrates ratio	0.226	0.222	0.249	0.224	0.011	0.321	0.247	0.231	0.580
Cellolosic to proteins ratio	0.819	0.693	0.744	0.652	0.098	0.730	0.708	0.307	0.657
Lipid	0.035	0.034	0.029	0.037	0.005	0.782	0.451	0.574	0.920
Lipid to carbohydrates ratio	0.539[a]	0.470[a,b]	0.435[a]	0.431[c]	0.026	0.038	0.850	0.007	0.339
Lipid to proteins ratio	1.908	1.459	1.595	1.271	0.229	0.376	0.515	0.128	0.407

[a–c]Means within a row without a common superscript significantly differ ($P < 0.05$).

Blood Metabolites

Blood metabolites were similar among chicks fed the 4 diets, except plasma albumin concentration was lower in chicks fed 135 g/kg at 42 d of age (Table 7). Furthermore, plasma uric acid concentration tended to be lower ($P \leq 0.09$) in chicks fed the 3 diets with EL at 21 d of age.

Ileal Morphology Parameters

Villus height was greater and crypt depth lower ($P \leq 0.05$), with consequent increase in villus-to-crypt ratio, in chicks fed 135 and 203 g/kg of EL at 21 and 42 d of age (Table 8). The villus height-to-crypt depth ratio was, however, lowest in chicks fed diets containing 67 g/kg EL.

Carcass Analysis and Gastrointestinal pH Measurements

The relative weight of abdominal fat was greater at 21 d of age and of pancreas was lower at 42 d of age when EL was included in the diets (Table 9). Furthermore, the duodenum and ileum at 21 d of age and jejunum at 42 d of age were longer ($P \leq 0.05$) in chicks fed diets with EL. The pH of contents from the gizzard and jejunum at 21 d of age were greater in chicks fed EL, while pH of duodenum content at 42 d of age was lower in chicks fed 67 g/kg LE ($P \leq 0.05$) (Table 9).

DISCUSSION

The main finding of current study was that the expansion of linseed reduced or eliminated the anti-nutritional factors (e.g., trypsin inhibitor) and improved linseed nutritional value resulting in an increased final live weight, and improved FCR in broiler chicks at 42 d of age (control vs. 67 and 135 g/kg EL). Inclusion of 203 g/kg of EL in the diet, substituting of soy bean meal, resulted, however, in the lowest FCR at all growth stages. The magnitude of growth depression and reduction in live weight gain in the current study was, however, negligible in comparison to previous studies with broilers fed diets with whole linseed or demucilaged linseed [3, 5].

Table 5. Correlation Between FTIR Molecular Structures and Chick Parameters Measured at 21 and 42 d of Age.

Item	α-Helix height		ß-Sheet height		Ratio of α-helix to ß-sheet		Carbohydrates to proteins ratio		Lipid to proteins ratio		Lipid to carbohydrates ratio		Ratio of amide I to II area	
21 d of age	R	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	R	*P*-value			r	*P*-value
FCR (g of feed/g of live weight)	0.65	0.34	–0.80	0.19	0.94	0.05	0.97	0.03	0.88	0.12	–	–	0.96	0.03
Total protein (mg/dL)	–	–	–0.68	0.31	0.66	0.33	–0.51	0.50	–0.87	0.13	–0.97	0.03	–	–
Plasma Uric acid (mg/dL)	–	–	0.49	0.51	–0.72	0.28	0.97	0.03	0.88	0.12	–	–	0.96	0.03
Cholesterol (mg/dL)	–	–	–0.95	0.04	0.97	0.03	–0.73	0.26	–0.71	0.29	–0.68	0.32	–	–
Triglyceride (mg/dL)	–	–	–0.75	0.25	0.83	0.16	–0.82	0.17	–0.98	0.03	–0.91	0.08	–	–
Low density lipids (mg/dL)	–	–	–0.55	0.44	0.30	0.70	0.27	0.73	–0.14	0.85	–0.56	0.43	–	–
42 d of age														
Feed Intake (kg)	0.64	0.36	–0.64	0.35	0.71	0.28	–0.71	0.29	–0.96	0.04	–	–	–0.5	0.49
FCR (g of feed/g of live weight)	0.42	0.57	–0.63	0.36	0.81	0.19	–0.93	0.07	–0.66	0.33	–	–	–0.99	0.01
Plasma Uric acid (mg/dL)	–	–	0.37	0.62	–0.11	0.90	–0.45	0.55	–0.02	0.98	0.43	0.56	–	–
Trypsin activity (nmol/mg of protein)	0.63	0.37	–	–	–	–	–0.88	0.12	–0.98	0.04	–	–	–0.72	0.28
Total protease activity (nmol/mg of protein)	–0.45	0.54	–	–	–	–	0.95	0.05	0.71	0.29	–	–	0.98	0.01
Lipase activity (nmol/mg of protein)	–	–	–0.95	0.04	0.97	0.02	–	–	–	–	–	–	–	–
High density lipids (mg/dL)	–	–	–0.23	0.77	–0.05	0.95	0.54	0.45	0.09	0.90	–0.36	0.63	–	–

Table 6. Effects Feeding Control or Graded Levels of EL (67, 135, and 203 g/kg) to Broiler Chicks on Digestive Enzymes Activities at 21 and 42 d of Age.

	21 d of age				42 d of age			
Type of diet	α-amylase (UA/mg of protein)	Lipase activity (mU/mg of protein)	Trypsin (nmol/mg of protein)	APA[1] (nmol/mg of protein)	α-amylase (UA/mg of protein)	Lipase activity (mU/mg of protein)	Trypsin (nmol/mg of protein)	APA[1] (nmol/mg of protein)
Control	54.47^{c}	5.72^{b}	2.41	7,039^{a}	0.81^{b}	5.28^{b}	2.18^{b}	5,519
67EL	205.65^{a}	37.46^{a}	2.82	5,664^{a,b}	3.96^{a}	17.41^{a,b}	2.80^{a}	5,248
135EL	154.31^{a,b}	5.26^{b}	2.69	4,623^{b}	2.42^{a,b}	13.72^{a,b}	2.64^{a,b}	4,457
203EL	67.25^{b,c}	21.08^{a,b}	2.54	7,914^{a}	2.49^{a,b}	37.80^{a}	2.99^{a}	4,290
SEM	33.08	8.43	0.13	715	0.69	7.91	0.17	1,445
P-value	0.004	0.05	0.20	0.03	0.05	0.05	0.03	0.91

[a–c]Means within a column without a common superscript significantly differ ($P < 0.05$).

[1]Total alkaline protease activity.

Various anti-nutritional factors, such as mucilage, linatine, cyanogenic glycosides, trypsin inhibitors, phytic acid, and water-soluble non-starch polysaccharides, are present in linseed [3, 4, 5], which can markedly increase ileal digesta viscosity and can reduce nutrient digestibility and utilization with consequent reduced growth when fed to broiler chicks. Although incorporation of EL in diets linearly increased crude fiber and non-starch polysaccharides concentration in the current study, expansion of linseed before incorporation in the diet of broilers ameliorated its negative effects in current trail. Alzueta et al. [4] found that the removal of most of the mucilaginous material from linseed (82%) by hot water extraction markedly improved weight gain and food utilization in broiler chicks. Mucilaginous material might have been broken down by extrusion in the current study.

Several other studies have demonstrated that extruded expanding of the diet increases protein and amino acid digestibility [39], and also fiber solubility and digestibility [11, 12]. Trypsin inhibitor activity was reduced in EL and chicks fed EL had enhanced digestive trypsin activity, which might have contributed to an enhancement of protein and amino acids digestibility. α-amylase activity, especially at 21 d of age, was also enhanced in chicks fed LE. Björck et al. [11] reported that wheat flour extruding increased soluble fiber by up to 75%. Increasing soluble fiber content may improve fiber digestibility, and likely increase digestible energy content of the diet [12]. Singh et al. [40] reported that starch gelatinization and increase in fibers solubility can increase diet digestibility in gastrointestinal tract. Therefore, modification of fiber and non-starch polysaccharides by expansion of linseed might be the reason for the enhanced α-amylase activity in chicks fed EL.

Alzueta et al. [4] mentioned that reduced fat and fatty acids digestibility reduced dietary metabolizable energy content of linseed, with consequent growth depression in chicks fed diets containing linseed. Interestingly, lipase activity was greater in chicks fed diets containing EL at 21 and 42 d of age. Furthermore, there was a significant reduction in lipid to carbohydrate and lipid to protein molecular spectral ratio as the amount of EL in the basal diets increased. High negative and positive correlation

Table 7. Effects Feeding Control or Graded Levels of EL[1] (67, 135, and 203 g/kg) to Broiler Chicks on Blood Characteristics at 21 and 42 d of Age.

Measurement age	Treatments	SUN,[2] (mg/dl)	Uric acid, (mg/dl)	Albumin, (mg/dl)	Total protein (mg/dl)	Cholesterol, (mg/dl)	Triglyceride, (mg/dl)	HDL,[3] (mg/dl)	LDL,[4] (mg/dl)
	Control	5.42	5.15	1.66	3.08	114.75	69	86.50	14.25
21 d	67EL	4.67	3.89	1.77	3.34	114.50	73.50	85.25	13.50
	135EL	4.15	3.03	1.66	3.10	116.25	71.75	92.25	11
	203EL	5.37	3.12	1.68	3.36	121	75	92	14.75
	SEM	0.48	0.60	0.08	0.12	5.92	8.04	4.33	2.44
	Control	4.50	3.16	2.02[a]	3.95	134.50	42.50	92.25	30.50
42 d	67EL	6.45	3.39	1.89[a]	3.87	115.75	54.50	86.50	16.75
	135EL	4.07	4.16	1.49[b]	3.17	106	55.75	53.75	11.75
	203EL	4.75	3.23	1.87[a]	4.08	127.25	47	85	30.50
	SEM	1.29	0.62	0.09	0.24	11.61	9.12	5.50	6.88
Effect									
21 d		0.24	0.09	0.78	0.25	0.85	0.95	0.56	0.71
42 d		0.59	0.65	0.01	0.09	0.36	0.70	0.71	0.17

[a,b]Means within a column without a common superscript significantly differ ($P < 0.05$).

[1]EL = expanded linseed.

[2]SUN = serum urea nitrogen.

[3]HDL = high density lipoprotein.

[4]LDL = low density lipoprotein.

Table 8. Effects Feeding Control or Graded Levels of EL (67, 135, and 203 g/kg) to Broiler Chicks on Ileum Morphological Characteristics at 21 and 42 d of Age.

	21 d of age			42 d of age		
Treatments	Villus height, μm	Crypt depth, μm	Villus:crypt ratio	Villus height, μm	Crypt depth, μm	Villus:crypt ratio
Control	634.62[b]	166.35[a,b]	3.98[b]	752.89[b]	177.41	4.48[b]
67EL	558.41[c]	174.16[a]	3.31[c]	646.93[c]	183.29	3.77[b]
135EL	674.88[a,b]	158.77[a,b]	4.33[a,b]	754.57[b]	188.34	4.19[b]
203EL	682.57[a]	153.48[b]	4.65[a]	819.23[a]	160.70	5.21[a]
SEM	15.93	6.55	0.21	19.02	8.73	0.24
P-value	0.001	0.05	0.002	0.001	0.14	0.001

[a–c]Means within a column without a common superscript significantly differ ($P < 0.05$).

between the lipase activity and ß-sheets area and the ratio of α-helix to ß-sheet, respectively, explained that the expansion processing can reduce the ß-sheet area and increase α-helix to ß-sheet ratio (from 1.4 in control to 2.8 in 203 g/kg EL diet), which leads to an enhancement of lipase activity and finally improves the digestible energy content of linseed containing diets.

Data of FTIR spectroscopy revealed a little difference in lipid, protein and carbohydrate structure between unprocessed linseed, and EL-incorporated diets. Lipid to carbohydrate molecular structure ratio was the only parameter that significantly reduced by inclusion of EL in the diet. This was the first study, to our knowledge, that determined the relationship between FTIR carbohydrate and lipid molecular structures and nutrient digestibility of diets with linseed. Expanded extruding cooking might change the molecular structures within the raw materials, such as molecular fragmentation and covalent or non-covalent cross linking [41, 42]. It has been shown that extrusion of plant protein sources can change the protein molecular weight distribution [43] and protein secondary structures [44]. During the expanded extruding cooking under high moisture condition, disruption in hydrogen bonds may occur and new bonds such as disulfide bonds might develop between

Table 9. Effects Feeding Control or Graded Levels of EL (67, 135, and 203 g/kg) to Broiler Chicks on Carcass Analysis and Gastrointestinal pH at 21 and 42 d of Age.

Measurement Time	Treatments	Carcass	Breast	Thigh	Abdominal fat	Gizzard	Liver	Bursa of Fabricius	pancreas	Duodenum	Jejunum	Ileum	Gizzard	Duodenum	Jejunum	Ileum
		Weight (g/100 g BW)								Length (cm/g BW)			pH			
21 d of age	Control	73.46	30.40	22.87	0.55^{b}	2.16	3.04	0.39	0.46	0.035^{a,b}	0.082	0.072^{b}	4.60^{b}	6.05	6.05^{b}	6.30
	67EL	72.01	30.13	24.46	0.97^{a,b}	2.19	3.22	0.41	0.44	0.035^{a,b}	0.081	0.085^{a,b}	4.72^{a,b}	6.31	6.31^{a}	6.21
	135EL	73.14	31.30	24.37	1.03^{a}	1.99	3.11	0.42	0.42	0.033^{b}	0.076	0.082^{a,b}	5.17^{a}	6.29	6.27^{a}	6.37
	203EL	73.86	30.98	23.58	0.75^{a,b}	2.17	2.98	0.34	0.39	0.038^{a}	0.088	0.092^{a}	5.10^{a}	6.15	6.29^{a}	6.51
	SEM	0.61	0.89	0.59	0.13	0.13	0.21	0.06	0.03	0.001	0.005	0.004	0.13	0.09	0.06	0.08
42 d of age	Control	77.52	34.29	23.35	0.85	1.87	2.40	0.213	0.327^{a}	0.015	0.042^{a,b}	0.041	4.52	5.95^{a}	5.93	6.61
	67EL	76.27	33.28	23.74	0.84	1.55	2.95	0.182	0.267^{b}	0.015	0.038^{b}	0.039	4.45	5.51^{b}	5.94	6.37
	135EL	77.52	32.85	24.33	1.04	1.51	2.34	0.275	0.266^{b}	0.014	0.043^{a,b}	0.044	4.62	5.79^{a,b}	6.06	6.72
	203EL	75.15	31.52	23.52	0.99	1.75	2.63	0.225	0.331^{a}	0.016	0.047^{a}	0.049	4.61	5.81^{a,b}	6.12	6.49
	SEM	1.02	0.85	0.52	0.16	0.16	0.23	0.028	0.018	0.001	0.002	0.003	0.11	0.11	0.12	0.34
Effect																
21 d of age		0.21	0.76	0.24	0.04	0.71	0.86	0.71	0.47	0.05	0.44	0.03	0.04	0.26	0.05	0.41
42 d of age		0.32	0.19	0.57	0.75	0.41	0.32	0.20	0.03	0.56	0.05	0.16	0.65	0.05	0.67	0.91

^{a,b}Means within a column without a common superscript significantly differ ($P < 0.05$).

the proteins, leading to protein aggregation [9, 34, 45]. Beck et al. [46] evaluated the effects of heat treatment and applied shear force of pea protein isolate and found that in the raw material the ß-sheet structures were involved in aggregates and interactions, and the bonds were heat sensitive and disrupted during extrusion.

FTIR spectroscopy revealed considerable reduction in carbohydrate to protein, cellulosic compound to protein, and lipid-to-protein ratio by increasing the EL incorporation in diets. Zhang and Yu [47] reported that the changes of the carbohydrate and lipid molecular spectroscopic features in the high-protein, high-fiber, high-fat, and low-starch ingredients were highly correlated with carbohydrate chemical profiles, carbohydrate sub-fraction degradation, and fermentation in ruminants. However, physiological and mechanical degradation kinetics of carbohydrate and fat are different in poultry than in ruminants. On the other hand, the results of the correlation analysis of protein second structure in this trail showed high negative correlation between lipase activity and ß-sheets area, where by inclusion of EL in diets ß-sheets area is reduced and α-helix to ß-sheet ratio was increased and a 7.15 and 2.68-fold increase in lipase activity was observed. These might be as a result of increased sensitivity and disruption of protein by expansion.

The results showed a tremendous increase in the α-amylase, lipase, trypsin, and total alkaline protease by incorporation of EL in diets. Modification of carbohydrate and cellulosic compounds along with increase in fiber and non-starch polysaccharides solubility and starch gelatinization maybe an important reason for enhancement of α-amylase activity by inclusion of EL in diets. Alzueta et al. [4] reported a reduction in fat and single fatty acids digestibility by incorporation of linseed to broiler diet. On the other hand, these researchers reported that the adverse effects of linseed on fat digestion could be partially overcome by substituting the demucilaged linseed for linseed, and such changes have led to an increase in digestibility of fat and total fatty acids, respectively [4]. Therefore, in the current study, expansion may increase lipase activity by reducing and modifying mucilage and the other anti-nutritional factors of linseed. Liener [48] noted that trypsin inhibitors and lectin in inad-

equately processed soybean meal lead to a reduction in protein digestibility and in pancreatic hypertrophy. A high level of trypsin inhibitor in inadequately processed linseed caused a marked reduction in the total proteolytic activity in the small intestine of chicks, rats, and mice [49, 50]. The higher greater digestive enzyme activities observed in birds fed EL in the current study may have contributed to the improved performance by those animals because it is known that enzymes play a rate-limiting role in providing great amounts of readily available nutrients for growth [51].

The effect of expansion of linseed on plasma lipid profile was determined because of beneficial health effects of linseed oil, especially omega-3 fatty acids [52]. Despite the increased in lipase activity and abdominal fat content, no alteration blood concentrations of cholesterol, triglyceride, HDL and LDL occurred in birds fed EL. However, the 2-fold increase in abdominal fat pad might indicate increased digestion and absorption of healthy fatty acids from extruded linseed. Apperson and Cherian [7] reported that the addition of carbohydrase enzymes to flax-based broiler diets decreased total lipids content in broiler breast muscle. Further studies are needed to investigate the possibility of enhancing carcass lipid profile with EL incorporation in the broiler diets. Kristensen et al. [53] reported that linseeds could form highly viscous digesta, which might interfere with bile acid recycling and reduce fat digestibility, with consequent reduction in serum cholesterol and triglyceride. Many trails have demonstrated that the consumption of soluble fibers can reduce total and LDL cholesterol and triglycerides by enhancing the losses of bile acids in the faeces [54].

Increased villus height, decreased crypt depth, and increased villus-to-crypt ratio in the ileum especially of chicks fed diets containing 203 g/kg of EL were apparent. Apperson and Cherian [7] found that feeding a linseed containing diet to broilers reduced villus height and thickened crypt depth, while addition of enzymes to the linseed containing diet led to a large increase in villi height and villi width in the jejunum of birds fed 10% linseed, and an increase in crypt depth in the jejunum of birds fed 15% linseed. Montagne et al. [55] indicated that an increase in villus-to-crypt ratio will increase the differentiation of the intestinal mucosa leading to an increase in nutrient digestion and absorption. Leeuwen et al. [56] reported that villi length and crypt depth are principal factors to evaluate the intestinal capacity for nutrient digestion and absorption. Apperson and Cherian [7] suggested that long villi are responsible for improvements in absorptive capacity of the intestine, and addition of enzymes in linseed-based diets improved the gut health status of the birds. Anti-nutritional factors in raw linseed can cause gastrointestinal wall thickening and decrease villi growth in the gastrointestinal tract of chicks. The improved ileal morphology in chicks fed EL suggests that linseed anti-nutritional factors were ameliorated, especially soluble non-starch polysaccharides.

Duke [57] reported that high fibrous diets enhances digesta retention time and promotes HCl production by stimulating the mechanical receptors of the proventriculus and reduces gizzard pH. Despite the increase in fiber content of EL containing diets, gizzard and jejunum pH was greater with EL diets, which might be due to an increased fiber solubility and digestibility as a result of expansion. Hetland and Svihus [58] showed that low-fiber diets resulted in less development of the gizzard, and addition of fiber to these diets increased relative weight of the gizzard. In the current study, differences in dietary fiber content between treatments did not result in differences gizzard weight.

Therefore, expansion may be change the fiber digestibly and modify soluble fiber content of linseed. Further studies is necessary by relying on the effect of expansion on fiber and non-starch polysaccharide structural alteration and fiber digestibility of linseed to support the effect of expansion processing on linseed fiber modification.

CONCLUSIONS AND APPLICATIONS

1. The results of the current study indicated that expansion of linseed had a great impact on its nutritional value by ameliorating the anti-nutritional factors.

2. Modification of fiber and non-starch polysaccharides in linseed by expansion processing reduced the anti-nutritional effects found in raw linseed, which resulted in improved gut health, greater intestinal enzymes activity, enhanced growth of villi, and finally improved broiler chicks' performance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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