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The Effect of Raw Full-Fat Soybean and Its Lectin on the Nutrition and Pigmentation of Broilers

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This study was aimed at determining the effect of raw full-fat soybean in the digestion/absorption of broilers and to evaluate the role of soybean agglutinin (SBA) in the pathogenesis of the runting and stunting syndrome of broilers. Six broiler groups were fed with six experimental diets for 42 days in which the defatted soybean meal of the basal diet was substituted with increasing raw full-fat soybean percentages ranging from 0 to 100%. The results suggested that SBA included in the raw full-fat soybean can play a role in the pathogenesis of the runting and stunting syndrome in broilers since binding and incorporation of SBA to enterocytes were associated with hyperplasia and dysplasia of the intestinal epithelium, as suggested by the progressive increases ($P < 0.001$) of mitosis/crypt in the duodenum and in the midintestine, as well as by intestinal villi atrophy ($P < 0.001$) in the duodenum and the midintestine. Indigestion and malabsorption of proteins induced progressive low weight gain ($P < 0.001$) up to 50% with 100% of raw full-fat soybean in the diet at 42 days. Xanthophylls in serum and skin yellowness were enhanced linearly in the broilers fed 42 days with increasing percentages of raw full-fat soybean in the diet. Increased xanthophylls absorption and decreased protein absorption can be caused by pathological changes in the epithelial intestine triggered by SBA binding and internalization.

KEYWORDS: Broiler; raw full-fat soybean; soybean agglutinin; *Glycine max*; intestine; immunohistochemistry; feces; pigmentation; growth

INTRODUCTION

The "runting and stunting syndrome" (RSS) or "malabsorption or feed passage syndrome" gives rise to considerable economic losses in aviculture worldwide. This syndrome is characterized by reduced weight gain and increased feed conversion due to indigestion and poor absorption of proteins and xanthophylls, thus increasing production costs (*1*−*3*). The pathogenesis of RSS has not been fully elucidated (*4*−*8*). Because the soybean agglutinin (SBA) is an antinutritional factor from soybean and residual concentrations of it have been found in the commercially processed defatted soybean meal (DSM) extensively used in poultry feed, it is important to assess whether, even in small doses, SBA has a detrimental effect on the intestine of broilers (*9*). It has been suggested that SBA has a deleterious effect on skin pigmentation, as occurs in RSS, by injuring the intestinal mucosa and by increasing lipids catabolism (*8*, *10*). Mucus hypersecretion caused by SBA could hinder pigment absorption since the mucus from the glycocalyx−microvilli network acts as a three-dimensional barrier that excludes all particles and restricts diffusion of large molecular weight compounds (*11*, *12*).

The aim of this study was to assess the effect of raw full-fat soybean (RFFS) and the role of SBA in the digestion/absorption function of broilers and to determine whether the latter could play a role in the pathogenesis of RSS. Weight gain, feces analyses, and serum xanthophylls concentrations were determined to assess the RFFS's effect on the digestive and absorptive intestinal function. Immunohistochemistry was used to determine SBA binding to the microvilli and to broiler

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Table 1. Calculated Analysis of the Main Nutrients (%) from the Starter and Finisher Diets for Each Experimental Group

	starter diets (0–21 days) experimental groups						finisher diets (22–42 days) experimental groups					
% raw full fat soybean in diets	0	20	40	60	80	100	0	20	40	60	80	100
CP	22	22	22	22	22	22	20	20	20	20	20	20
crude fat	6	6.1	8.5	9.3	10.1	11	7	12	12	12	12	12
calcium	1	1	1	1	1	1	0.9	0.9	0.9	0.9	0.9	0.9
available phosphorus	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4
metabolizable energy (kcal/kg)	3050	3050	3050	3050	3050	3050	3050	3050	3050	3050	3050	3050
methionine + cystine	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8
lysine	1.2	1.3	1.3	1.2	1.4	1.2	1.2	1.1	1.2	1.2	1.2	1.3
linoleic acid	2.2	2.34	3.89	4.01	4.39	4.84	2.68	5.53	5.50	5.48	5.45	5.42

enterocyte's cytoplasm, while histopathology was used to determine the deleterious effect of SBA on the intestinal mucosa and on the length and epithelial proliferation of duodenum and midintestine villi. Skin yellowness was assessed to determine the RFFS effect on the skin pigmentation.

MATERIALS AND METHODS

Experimental Procedure. Avian Farm × Arbor Acre 1 day old chicks ($n = 180$) of either sex, weighing 36 ± 10 g, were used in six experimental diets. Three groups of 10 chicks each ($n = 30$) were fed with each experimental diet and housed in Petersime battery cages with water and food ad libitum for 42 days. Twelve diets containing sorghum and commercial DSM were formulated as follows: starter diets for the first 21 days and finisher diets for the last 21 days, as established by Cuca et al. (*13*), using the Carlos and Carlos NUTRION III program (*14*). All diets were isoproteic with 22 and 20% of crude protein (CP) for starter and finisher diets, respectively, and isocaloric with 3050 kcal/kg of metabolizable energy. For the experimental diet, the DSM was substituted by increasing the percentages of RFFS. The negative control diet contained 0% of RFFS, and each successive diet contained 20% more RFFS, with 100% RFFS for the sixth diet (**Table 1**). The DSM was obtained from the Facultad de Veterinaria y Zootecnia (Mexico), and the soybean was dehulled, cracked, flaked, and hexane extracted. Then, the flakes were transferred to the desolventize toaster unit where the remainder of the solvent was recovered, and the flakes were subjected to stream heat to inactivate the soybean growth inhibitors. To prepare the RFFS meal, soybeans from a Mexican cultivar were ground until a 20 mesh flour size, thus retaining the full amount of fat. The finisher diets were supplemented with 70 mg of xanthophyll from *Tagetes erecta* petals (Marigolds or Cempasuchil flowers) (Hi-Gold 60 pigment, Industrial Organica, Monterrey, Mexico) per kilogram of meal.

Characteristics of DSM, RFFS, and Experimental Diets. Urease activity, hemagglutinating activity, and SBA concentration in the DSM, RFFS, and experimental diets were determined in the soybean products used. Urease activity values were determined by the Hayward's method (*15*) and following the AOAC 1990 protocol (*16*). The saline extraction of proteins from the soybean flours, used to determine the hemagglutinating activity and presence of SBA by immunodiffusion assays, was performed as described in ref *6*: dry flour was suspended in 0.15 M NaCl at 10% (w:v) and stirred for 2 h, the pH was adjusted to 4 with 4 M acetic acid, and the mixtures were allowed to stand overnight at 4 °C. The supernatant was obtained by centrifugation at 3000 rpm for 15 min. The hemagglutination titer of the saline extracts was determined with a 1% suspension of rabbit erythrocytes in PBS, pH 7.2, by the 2-fold serial dilution, and the result was reported as the reciprocal of the last 2-fold dilution showing visible agglutination. The hyperimmune anti-SBA serum was obtained from an adult New Zealand male rabbit weighing 3.5 kg, by inoculating 3 mg of SBA (Sigma Chemical, St, Louis, MO) in 6 mL of PBS and 1:1 complete Freund adjuvant. The emulsion (500 μL) was subcutaneously injected four times in the dorsal thoracic area at 7 day intervals. The rabbit was immunized two times more at 2 month intervals, and 7 days after the last inoculation, the inhibition of the hemagglutination titer was 1:32 768. The rabbit was killed by decapitation and bled; the serum was stored at −70 °C until use. The concentration of SBA in the DSM, RFFS, and experimental diets was determined by Rocket immunoelectrophoresis (*17*) in 2% agarose gel and using different SBA concentrations (Sigma) as standards. The test was validated by the standard curve of different SBA dilutions beginning with 1 mg/mL of SBA in PBS, pH 7.2.

Productivity Parameters. Mortality, averages of feed intake, and body weight gain per chick were registered weekly along the 42 days to calculate feed conversion.

Feces Analysis. Three fecal samples from each experimental diet were obtained at 38 days, to determine the SBA effect on the digestive/absorptive intestinal function. The excretion of CP was determined by the Kjeldahl method (*16*). Because CP or total nitrogen in bird feces contains only 15% of true protein (TP) that has been neither digested nor absorbed and 85% of nonprotein nitrogen (NPN), corresponding to urine products and other wastes from the intestinal tract, in the present study, the contents of TP in feces were estimated by subtracting the NPN value from CP (*18*). The lipid excretion or ether extract (EE) was determined in two samples per treatment from the same feces based on the relation existing between lipids and xanthophyll absorption. The CP content was determined by the Kjeldahl method, NPN by the trichloracetic acid method, and EE using the AOAC (*16*) and protocol of Tejada (*19*).

Histological Procedure. The effect of SBA and its presence in the intestinal mucosa was evaluated by histopathological analysis and by immunohistochemistry. At the end of the experimental period, a necropsy of nine chickens per treatment was performed after desensitization and euthanasia by means of an electric current passed from the oral cavity to the cloacae mucosa. One centimeter long transversal sections from the midduodenum in the ascendant and descendant portions of the duodenal loop and from the midintestinal region near Meckel's diverticulum were taken from each chicken. The samples were buffer−formalin-fixed for 24 h, paraffin-embedded (Paraplast X-TRA Oxford Labware, St. Louis, MO, fusion between 50 and 54 °C), and sectioned at 3−4 μm thickness (*20*). Histopathological analysis was performed on hematoxylin−eosin-stained paraffin sections from each duodenum and midintestine tissue sample. This allowed us to measure villi length and to assess the epithelial hyperplasia by the number of mitoses in the Lieberkühn crypts (*20*). These studies were performed with a photonic Leica Gallen III microscope with a 10× ocular lens and a reticular lens with 20 squares per side. By means of a micrometer slide, each subdivision was set at 185.7 μm per side, with the 4× objective lens; 75 μm with the 10× objective lens; and 18 μm with the 40× objective lens. The duodenal villi were measured with the 4× objective lens, and those of the midintestine were measured with the 10× objective lens to include, in both cases, the entire villi area. Ten villi per chick were measured. To evaluate epithelial hyperplasia, the number of mitoses in 10 crypts per chick were counted. Only the mitoses within the visual field of the 100× objective lens were counted, placing the lower border of the crypt over the optical field perimeter.

The presence of SBA was determined by immunohistochemical analysis on paraffin sections from the duodenum and midintestine of both control and RFFS-fed chickens (EnVision+ System, Peroxidase (DAB), Rabbit; Dako Corporation, Carpinteria, CA). For negative controls, paraffin sections were incubated with nonimmunized rabbit serum in place of the primary antibody. The entire immunostaining procedure was performed at room temperature. The primary antibody

was rabbit hyperimmune serum against SBA. The paraffin of the tissue sections was removed with xylene and alcohol before blocking the tissue peroxidase with 0.03% H_2O_2 for 15 min. The tissue sections were washed for 10 min with PBS and incubated with proteinase K (Dako) for 15 min, which was removed by shaking the slides. The tissue sections were incubated with a 1:800 primary antibody dilution for 30 min and washed with PBS; thereafter, the peroxidase-conjugated secondary anti-rabbit antibody (Dako) was applied for 30 min. The color was developed with 3,3′diaminobenzidine solution containing H_2O_2 (Dako) for 8 min. The samples were counterstained with hematoxylin.

Pigmentation. To evaluate the effect of SBA on the pigmentation process at the end of the experimental period, the absorption and tissue deposition of xanthophylls were measured by determining serum xanthophylls concentration and skin yellowness, respectively. Serum xanthophylls were measured in six broilers per experimental diet in a 1:10 serum and acetone solution, using a Milton Roy spectronic 20D spectrophotometer, at 480 nm of liquid absorbance, following the method described by Allen et al. (*21*). Skin pigmentation was evaluated in 10 live broilers per experimental diet, over a period of 2 h, using a Minolta CR300 chromometer at the featherless area of the body found near the most caudal rib. Serum xanthophylls and skin yellowness values were evaluated in relation to the total xanthophyll intake and total body skin area, which was estimated by means of body weight to the two-thirds power ($W^{0.67}$), according to Mc Donald et al. (*22*).

Statistical Analysis. The means of all results were compared by variance analysis and Tukey's test. Regression analysis was obtained by the minimum squares test and by Pearson's linear correlation (SAS, vers. 8 program, 1999). The model used was $Y_{ij} = \mu + R_i + S_i + \epsilon_{(i)j}$, where Y_{ij} was the effect of each (j-th) random observation for each characteristic, associated to each (i-th) soybean percentage, μ was the population mean, R_i was the effect on each experimental group of birds fed with different soybean percentages, S_i was the effect of each (i-th) soybean percentage, and $\epsilon_{(i)j}$ was the random error, NID.

RESULTS AND DISCUSSION

Characteristics of DSM and RFFS Contained in the Diets. The characteristics of the soybean flour used were satisfactory for this study. The urease activity was 0.29 for DSM vs 2.3 for RFFS (which had not been heated), indicating that the stream heat process was allowed to inactivate the urease activity, but maintained active the hemagglutinating activity of SBA (*23*, *24*). The hemagglutination titer of the DSM saline extract was 1:3 200; of RFFS, the hemagglutination titer was 1:25 600; and of the purified SBA (1 mg/1 mL), it was greater than 1:204 800. The SBA contents in RFFS and DSM used in the formulation of diets were 5658 and 884 μg/g, respectively; 756 μg/g in the 0% diet, 1034 μg/g in the 20% diet, 2447 μg/g in the 40% diet, 2747 μg/g in the 60% diet, 3196 μg/g in the 80% diet, and 3667 μg/g in the 100% diet, in relation to the peak height of antigen concentration in the standard curve used for the Rocket immunoelectrophoresis, which decreased ($P < 0.01$) following the equation $Y = -1.6553 + 0.5711\ S$, where $R^2 = 0.8023$.

Productivity Parameters and Feces Analyses. Mortality for the whole experiment was 1.1%, one from the 0% RFFS group at day 4 and another in the 100% RFFS group at day 19; thus, the death of these chicks is not attributable to a treatment effect. As increments of 20% DSM were substituted by RFFS, the food intake decreased ($P < 0.001$) according to the following equation: $Y = 67.07 + 0.036\ S + 0.002\ S^2$ with $R^2 = 0.7139$ until the 90% substitution level, and then, the values fell; the weight gain decreased ($P < 0.001$) according to the equation $Y = 1837.33 - 8.93\ S$ with $R^2 = 0.9105$, and the feed conversion increased ($P < 0.001$) according to the equation $Y = 1.4847 + 0.01\ S$ with $R^2 = 0.7454$ (**Table 2**). The linear negative effect on weight gain ($P < 0.001$) when DSM was substituted by increasing percentages of RFFS (**Table 2**) has been attributed to both the lectins and the antitrypsic effects present in the RSS (*25*). Duke (*26*) considered that the pathogenicity of SBA on the intestinal epithelium could inhibit trypsin production because it impedes secretion of enterokinases by enterochromaffin cells located in the crypts of Lieberkühn (*26*, *27*). However, Balloun (*25*), Baintner (*28*), and Douglas et al. (*29*) concluded that rather both antitrypsic factors, i.e., the Kunitz that blocks only trypsin and the Bowman−Birk that inhibits trypsin and α-chimotrypsin, are responsible for the deficient weight gain instead of SBA.

In this study, despite a lack of significant ($P > 0.05$) findings in feces in regard to CP or total nitrogen, the deficient weight gain with increased RFFS in the diet was associated with an increase of TP in feces ($P < 0.001$) according to the equation $Y = 182.04 + 0.41\ S$ with $R^2 = 0.5099$ (**Table 3**). This result suggests that protein digestion was worst as the RFFS percentage increased, as had been observed by Bielorai et al. (*18*). Coates et al. (*30*) point out that at greater weight gain, the food intake is greater, such that there is greater excretion of NPN if all of the protein contained in the diets is absorbed and metabolized. The decreased content of NPN in feces observed in the present study could be due to the decreased food intake ($P < 0.001$) observed when increased RFFS quantities were added to the diet (**Table 3**). These authors attributed the decreased weight gain largely to SBA, based on their observation that the addition of a Kunitz inhibitor reduced broiler growth by 6% in average, whereas weight gain was reduced in 43.5% by RFFS addition, when compared to broilers receiving adequately cooked DSM. Consequently, these authors concluded that the proteases in

Table 2. Productive Parameters (g) from Broilers at Day 42[a]

experimental diets	productive parameters (mean ± SE)[b]		
	feed intake (g)[c]	body weight gain (g)[d]	feed conversion (g)[e]
0%[f]	67.33 ± 2.06[ab]	1787.33 ± 56.74[a]	1.54 ± 0.11[e]
20%	68.27 ± 2.06[a]	1732.70 ± 56.74[a]	1.61 ± 0.11[de]
40%	61.80 ± 2.06[bc]	1428.33 ± 56.74[b]	1.74 ± 0.11[cd]
60%	63.25 ± 2.06[abc]	1332.70 ± 56.74[b]	1.96 ± 0.11[bc]
80%	60.51 ± 2.06[c]	1169.00 ± 56.74[c]	2.13 ± 0.11[ab]
100%	49.80 ± 2.06[d]	894.00 ± 56.74[d]	2.30 ± 0.11[a]
$P \leq$[g]	***	***	**

[a] Superscript letters a–e: means in the same column with different superscripts were significantly different. [b] Means ± standard error. [c] Daily average of feed intake (g) per chick, in each treatment, over 42 days ($P < 0.001$). [d] Body weight gain per chick with each treatment, over 42 days, minus the mean body weight at day 1 (g) ($P < 0.001$). [e] Relation of feed intake per chick/body weight gain per chick at day 42 ($P < 0.01$). [f] Experimental diets with increasing percentage of RFFS. [g] Significance of effects: ***$P \leq 0.001$; **$P \leq 0.01$; without * corresponds to $P \geq 0.05$.

Table 3. Chemical Analytes of Feces (g/kg) from Broilers at Day 38[a]

experimental diets	chemical analytes of feces[b] (mean ± SE)[c]			
	CP[d]	TP[e]	NPN[f]	EE[g]
0%	314.7 ± 11.6	179 ± 9.4[c]	135.7 ± 8.3[a]	116.8 ± 9.7[b]
20%	306.7 ± 11.6	192.6 ± 9.4[bc]	114.1 ± 8.3[abc]	128.8 ± 9.7[ab]
40%	290.8 ± 11.6	200.2 ± 9.4[bc]	90.4 ± 8.3[c]	144.9 ± 9.7[ab]
60%	332.7 ± 11.6	216.0 ± 9.4[ab]	116.7 ± 8.3[ab]	131.5 ± 9.7[ab]
80%	345.6 ± 11.6	238.8 ± 9.4[a]	106.8 ± 8.3[bc]	167.4 ± 9.7[a]
100%	314.2 ± 11.6	212.7 ± 9.4[ab]	101.5 ± 8.3[bc]	171.0 ± 9.7[a]
$P \leq$[h]		**	*	*

[a] Superscript letters a–c: means in same column with different superscripts were significantly different. [b] Chemical analytes of three samples of feces from each treatment taken at day 38 (g/kg). [c] Mean ± standard error. [d] CP $P > 0.05$. [e] TP ($P < 0.01$). [f] NPN ($P < 0.05$). [g] EE of two samples of feces from each treatment taken at day 38 (g/kg). [h] Significance of effects: **$P \leq 0.01$; *$P \leq 0.05$; without * corresponds to $P \geq 0.05$.

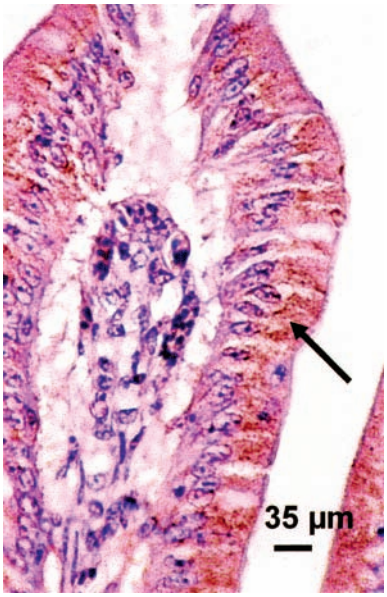

Figure 1. Tip of duodenum villi from a chicken fed with 100% RFFS in the diet. The SBA is shown inside the cellular cytoplasm (arrow); immunostain; bar = 35 μm.

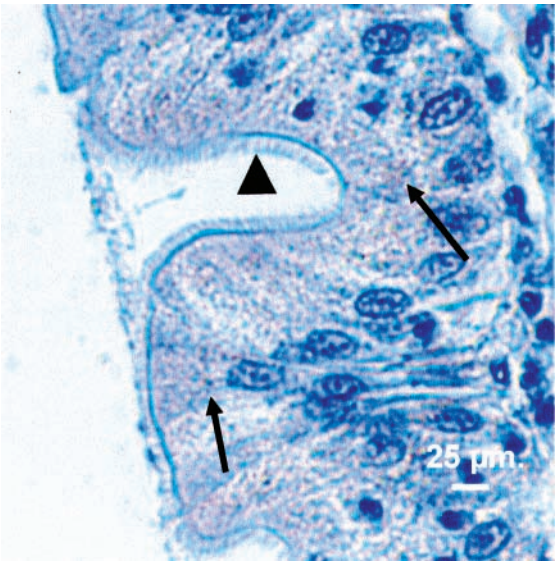

Figure 2. Epithelium of midgut villi from a chicken fed 60% RFFS. The epithelial fold with superimposed cells due to hyperplasia shows brown SBA particles in the cellular cytoplasm (arrow) but not in the microvilli (arrowhead); immunostain; bar = 25 μm.

soybean play a minor role in the deficient growth of broilers. Physiologically, the broiler's intestine secretes enterokinases that then convert the trypsinogen secreted by the pancreas to trypsin, which, in turn, stimulates intestinal pancreatic secretions to secrete intestinal enterokinases.

On the other hand, the NPN diminished ($P > 0.05$) according to $Y = 119.74 - 0.23\ S$ with $R^2 = 0.1556$ (**Table 3**). In fowl feces, the quantity of CP or total nitrogen obtained by the Kjeldahl method is made up of two fractions: TP, corresponding to the not used protein, be it through indigestion or malabsorption; and NPN, the fraction made up of uric acid, ammonium, ammonium acids, urea, and creatinine, which are the products of protein metabolism and bacterial fermentation (*31*). The increased NPN values in rats fed with *Phaseolus vulgaris* meal, containing *P. vulgaris* agglutinin, suggested increased muscular catabolism (*32*). In contrast, in our study, muscular catabolism seems to be improbable since NPN values decreased with increasing RFFS intake.

Histological Results. The enterocytes membrane of broilers seems to possess oligosaccharides containing GalNAc/Gal, which are receptors specifically recognized by SBA. The immunohistochemical analysis showed positive SBA recognition in the 0% RFFS control group, as well as in all broilers from the experimental groups at 42 days. The presence of SBA in the 0% RFFS control group indicates a residual SBA content in the commercial DSM (*9*). SBA identified in the 42 days old chicks was homogeneously distributed over the entire villi epithelium, except in the Lieberkühn crypts. These results show that the glycoprotein receptors recognized by SBA are present only in mature cells since the Lieberkühn crypts are made up of progenitor, undifferentiated, or embryonic cells (*11*). The positive SBA reaction was observed in the cellular cytoplasm in all cases and in the microvilli in 50% of the experimental chicks (**Figures 1**, **2** and **3**). As demonstrated in other animal lectin models, SBA adheres to the enterocyte membrane and is internalized by pinocytosis (*7*, *8*, *10*). The presence of SBA within the cytoplasm of enterocytes induced hyperplasia of the intestinal epithelium confirmed upon finding a linear increase ($P < 0.001$) of 6.8 mitoses per crypt in the duodenum for each 20% of DSM substitution, as described by the equation $Y = 19.91 + 0.34\ S$, where $R^2 = 0.5876$, and 1.3 mitoses per crypt in the midintestine, as described by the equation $Y = 15.58 + 0.065\ S$, where $R^2 = 0.0981$ (**Table 4**). A similar effect has been documented in rats with other lectins (*4*, *6*−*8*) where histology of the intestines showed that the hyperplasia was affecting not only the enterocytes but also the mucus-producing cells. Furthermore, Oliveira et al. observed, in rats, that the continuity of the enterocyte brush border is not altered and neither ulceration nor villi collapse occurs with exposure of the lamina propia (*10*). Whereas the normal intestinal epithelium presented as a continuous ribbon with uniform parallel cells in the histological sections of this study, epithelial hyperplasia was evidenced by the discontinuity of the villi epithelium with severalfolds of heaped cells conferring a fernlike shape (**Figure 4**). This phenomenon can be explained by the mitogenic effect of SBA causing an unbalance in the physiological renovation process of the villi epithelium determined by antagonic enzymes that could stimulate or inhibit the proliferation of undifferentiated cells located in the Lieberkühn crypts (*8*). Even though mitosis

Table 4. Mitoses Per Crypt and Length of Villi in the Duodenum and Midintestine of Broilers at Day 42[a]

experimental diets	amount of mitoses per crypt (mean ± SE)[b]		length of villi (mean ± SE)[c]	
	duodenum[d]	midintestine	duodenum[e]	midintestine
0%	20.95 ± 2.17[e]	13.94 ± 1.60[c]	2053.48 ± 30.67[a]	1028.87 ± 14.60[a]
20%	28.05 ± 2.61[de]	19.33 ± 1.60[b]	1983.90 ± 28.91[a]	1072.88 ± 13.75[b]
40%	33.17 ± 2.61[cd]	18.72 ± 1.60[b]	1907.46 ± 25.44[b]	928.75 ± 14.20[c]
60%	36.39 ± 2.61[c]	17.55 ± 1.60[b]	2005.16 ± 31.19[a]	892.28 ± 13.40[cd]
80%	46.40 ± 2.71[b]	21.44 ± 1.60[ab]	1829.39 ± 29.03[c]	859.28 ± 15.00[d]
100%	60.24 ± 2.91[a]	22.05 ± 1.60[a]	2046.50 ± 37.93[a]	797.22 ± 14.10[e]
$P \leq$[f]	***	***	***	***

[a] Superscript letters a–e: means in same column with different superscripts were significantly different. [b] Mean of amount of mitoses per crypt in the duodenum and midintestine ± standard error. [c] Mean of villous length in duodenum and midintestine ± standard error. [d] Amount of mitoses in 10 crypts per chicken from nine chickens per treatment at 42 days in the duodenum and midintestine. [e] Length of 10 villi in the duodenum and midintestine from nine chickens per treatment at 42 days. [f] Significance of effects: ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; without * corresponds to $P \geq 0.0$.

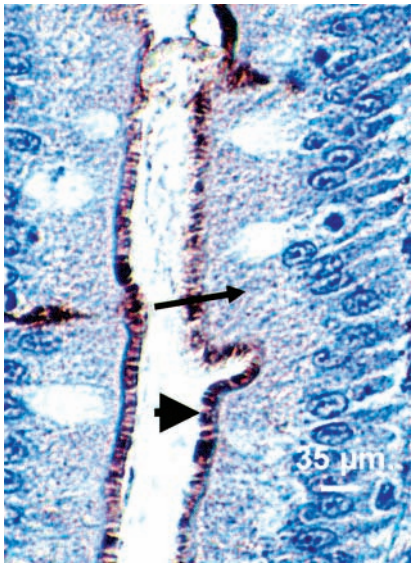

Figure 3. Epithelium of midgut villi from a chicken fed 60% RFFS in the diet. The epithelium does not show hyperplasia. There are brown SBA particles in both the cellular cytoplasm (arrow) and the microvilli (arrowhead); immunostain; bar = 35 μm.

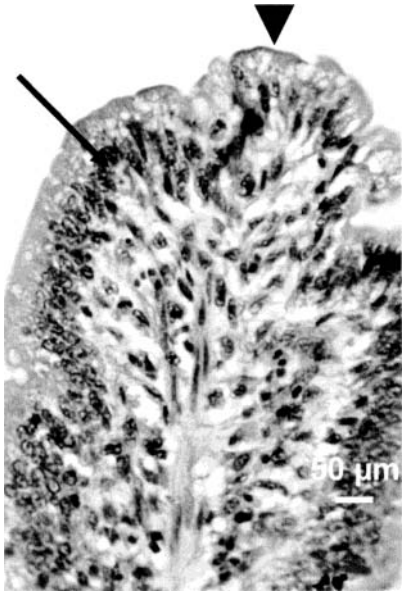

Figure 5. Midgut villi from a chicken fed 100% RFFS in the diet. The upper third of villi shows epithelial dysplasia (arrow). The villi tip does not show desquamation (arrowhead); hematoxylin and eosin; bar = 50 μm.

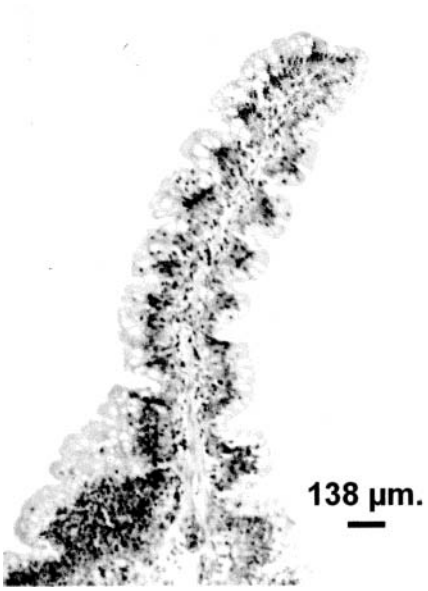

Figure 4. Villi of duodenum from a chicken fed 60% RFFS. Hyperplasia of the epithelium with several folds confers a fernlike shape to the villi; hematoxylin and eosin; bar = 138 μm.

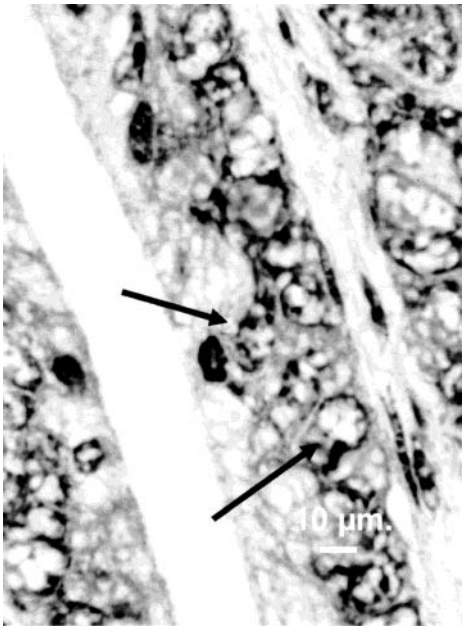

Figure 6. Mid-third of the midgut villi from a chicken fed 100% RFFS. Note the epithelial dysplasia with oval or round nuclei of variable sizes, dispersed and hyperchromatic chromatin (arrow), and an increase in the nucleus-to-cytoplasm ratio; hematoxylin and eosin; bar = 10 μm.

in the crypt increased linearly, the hyperplasia viewed in the histological sections was only seen with SBA, contained in RFFS at concentrations between 884 and 2 747 μg/g (in treatments ranging from 0 to 60%). Conversely, the 80 and 100% treatments, in which the SBA concentration ranged from 3196 to 3667 μg/g, hyperplasia was substituted by dysplasia along the entire epithelium, even at the tip of the villi, which did not show desquamation (extrusion) of the cells. This dysplasia was characterized by a continuous epithelium made up of disordered layering of numerous cells with morphological characteristics that are true of immature embryonic cells normally found in the crypts of Lieberkühn. These cells presented oval or rounded nuclei of variable size, although very large occasionally, dispersed and hyperchromatic chromatin, one or two large and very apparent nucleoli, and an increase in the nucleus-to-cytoplasm ratio (**Figures 5** and **6**). Dysplasia is considered to be reversible, yet has sometimes been seen to be a precursor of neoplastic processes (*33*).

As the RFFS increased in the diet, the villi length decreased linearly ($P < 0.001$) in the duodenum, by 105.4 μm for each 20% of SBM substitution, as described by the equation $Y = 2064.35 - 5.27\ S + 0.04\ S^2$, where $R^2 = 0.0632$, whereas in the midintestine it decreased ($P < 0.001$) 53.2 μm, as described by the equation $Y = 1063.45 - 2.66\ S$, where $R^2 = 0.2054$ (**Table 4**). Thus, villi atrophy of chickens increased with increasing SBA content in RFFS. Similar to other lectins and animal models, villi atrophy affected the digestive and absorptive processes, as reflected in the chemical characteristics of the feces, food intake, weight gain, and, thus, in feed conversion (*4*, *6*−*8*).

Table 5. Total Pigment Intake (mg) Per Chick, Skin Area, Optical Density of Serum Xanthophylls, and Skin Pigmentation (CIE Lab Scale) at 42 Days[a]

	mean ± SE			skin pigmentation[b]		
diets[c]	pigment intake[d]	body area[e]	optical density of serum xanthophylls[f]	L^g ± SE	a^h ± SE	b^i ± SE
0%	170 ± 4.51[a]	153 ± 3.67[a]	0.12 ± 0.029[bc]	67 ± 0.96	10 ± 0.78	32 ± 2.13[c]
20%	175 ± 1.45[a]	150 ± 3.67[a]	0.10 ± 0.029[c]	69 ± 0.88	8 ± 0.71	39 ± 1.94[ab]
40%	151 ± 3.51[ab]	132 ± 3.67[b]	0.18 ± 0.039[abc]	68 ± 0.92	9 ± 0.74	35 ± 2.03[bc]
60%	159 ± 8.45[ab]	126 ± 3.67[b]	0.16 ± 0.034[bc]	69 ± 0.88	9 ± 0.71	42 ± 1.94[ac]
80%	150 ± 6.11[ab]	116 ± 3.67[bc]	0.19 ± 0.031[ab]	69 ± 0.88	8 ± 0.71	37 ± 1.94[abc]
100%	127 ± 5.45[b]	97 ± 3.67 [c]	0.27 ± 0.031[a]	69 ± 0.92	7 ± 0.74	38 ± 2.03[abc]
$P \leq$[j]	**	**	**			*

[a] Superscript letters a–c: means in same column with different superscripts were significantly different. [b] Skin pigmentation evaluated from 10 chicks per treatment at 42 days, with a Minolta CR300 chromometer (CIE lab scale). [c] Experimental diets with increasing percentage of raw full fat soybean. [d] Total pigment intake per chick along the 21 days with finisher diet (mg). [e] Body area = $W^{0.67}$. [f] Serum xanthophylls concentration from six chicks per treatment, at day 42, liquid spectrophotometry with 480 nm absorbance. [g] Brightness. [h] Redness. [i] Yellowness. [j] Significance of effects: $^{**}P \leq 0.01$; $^{*}P \leq 0.05$; without * corresponds to $P \geq 0.05$. Values represent the means ± standard error of the mean.

Pigmentation. In the present study, increasing RFFS substitution in the diet increased xanthophylls absorption and decreased protein absorption that could have been caused by the pathological changes of epithelial intestine triggered by SBA binding and internalization. The xanthophyll serum concentration increased linearly ($P < 0.01$), as described by $Y = 0.1008 + 0.0013\ S$, where $R^2 = 0.2666$, despite the linear reduction ($P < 0.01$) in feed intake and, thus, in total xanthophyll intake, as described by $Y = 27.85 + 5.6\ S - 0.677\ S^2$, where $R^2 = 0.0919$. This result suggests that the pigment absorption process became more efficient as RFFS inclusion in the diet increased (**Table 5**). This efficiency could be due to sufficient maturity of the hyperplasic cells during their migration to the tip of the villi with a consequent increase in absorptive surface area (*8*) and also to markedly enhanced absorption of lipids and bile acids between the duodenum and the upper jejunum with RFFS (*34*, *35*), since xanthophylls are liposoluble compounds and their absorption is intimately associated to the digestion and absorption of fatty acids found in the diet (*36*, *37*). Tyczkowski et al. (*38*) found that lutein, the most abundant pigmenter of Marigold petals, is absorbed mainly in the duodenum and jejunum. This increased absorption of pigment was reflected in the greater pigment deposition in the skin ($P < 0.05$) with increasing RFFS substitution that caused more yellowness. However, the linear increase in cutaneous yellowness was observed only with up to 60% RFFS substitution in place of DSM, whereas with greater levels of substitution the yellowness decreased, as described by the equation $Y = 27.85 + 5.6\ S - 0.677\ S^2$, where $R^2 = 0.0919$. This effect could be related to the increased fatty acid excretion (**Table 3**).

In the chemical analysis of the feces, the EE values, which correspond to fatty acid excretion, increased ($P < 0.05$) with increasing RFFS substitution, as described by the equation $Y = 116.75 + 0.53\ S$, where $R^2 = 0.8111$, as observed in Table 3. These results concur with observations by Sklan et al. (*34*, *35*) who showed that in spite of the fact that RFFS increases absorption of lipids and bile acids between the duodenum and the upper jejunum, it also reduces the rate of disappearance of residual amounts of these compounds in the ileum and colon increasing fecal excretion of fatty acids and bile acids. On the other hand, the increase in fatty acid excretion in rats fed with the lectin from *P. vulgaris* corresponded to triglycerides produced during the increased catabolism of lipids in the subcutaneous tissue (*8*). The skin pigmentation is related to the quantity of pigment deposited in subcutaneous adipose tissue. Perhaps, for this reason, the yellowness of skin values decreased when broilers were fed with 80−100% RFFS meal, which contains more than 2747 μg/g, as compared to broilers fed with 60% RFFS (**Table 5**).

It is inferred that the damaging effects of SBA in the broiler intestine do not affect either absorption or xanthophyll deposition in the adipose tissue of the skin. On the basis of these observations it might be advisable to verify the SBA concentrations in commercial soybean meal used for broiler feed. Broilers seem to have specific glycoprotein receptors for SBA in the intestinal epithelium. When SBA adheres to the microvilli and penetrates into the cytoplasm of the enterocytes, in concentrations ranging from 884 to 2747 μg/g, there is epithelial hyperplasia and intestinal villi atrophy, but at concentrations greater than 3196 μg/g, the villi atrophy is severe and the epithelial cells that cover the villi are immature due to dysplasia. From these considerations, it is inferred that these lesions could be responsible for indigestion and malabsorption of nutrients and, as such, induce deficient weight gain similar to the events occurring during the RSS of broilers.

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