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Influence of level of inclusion of Azolla leaf meal on growth performance, meat quality and skeletal muscle p70S6 kinase α abundance in broiler chickens

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The interest in biodiesel production from oil-bearing seeds rather than soybean necessitates the scientific validation of other good quality protein sources that could substitute soybean meal in animal diets, particularly, broiler chickens where soybean meal constitutes a large portion of their diet. Therefore, the present study was conducted to investigate the effect of sun-dried Azolla leaf meal ***(ALM)*** *as an unconventional dietary protein source in broiler chicken diet on growth performance, meat quality, skeletal muscle cell growth and protein synthesis through regulation of ribosomal protein S6 kinase (p70S6 kinase* α*). A total of 120 male Ross 308 broiler chicks were randomly allocated to three dietary treatments. Each treatment had four cages (i.e. replicates) with 10 birds/cage. The control group was fed with a corn–soy-based diet, the* ***AZ5*** *group was supplemented with 5% ALM and the* ***AZ10*** *group was supplemented with 10% ALM for 37 days. A 5-day trial was also conducted to measure the apparent nutrient digestibility. Growth performance parameters were measured weekly. At the end of the experiment, 12 birds from each group (3/cage) were euthanized and used for samplings. Inclusion of ALM tended to improve BW gain (*$P = 0.06$*) and increased feed intake (*$P < 0.01$*). Additionally, ALM decreased the percentage of breast meat cooking loss linearly (*$P < 0.01$*). In addition, ALM at a dose of 5% increased the production of propionate in the cecum (*$P = 0.01$*). Activation of breast muscle p70S6 kinase was higher when ALM was included in a dose-dependent manner (*$P < 0.01$*). The inclusion of ALM increased breast meat redness (*$P < 0.01$*); however, the lightness was within the normal range in all groups. Findings from our study suggest that ALM could be included in a broiler chicken diet up to 5% without any major negative effect on meat quality or performance, and it regulates muscle protein synthesis through activation of mammalian target of rapamycin/6S kinase signaling.*

Keywords: dietary protein source, broiler nutrition, protein synthesis, meat quality, mammalian target of rapamycin (mTOR) signaling

Implications

The shortage in soybean meal availability for broiler chicken nutrition necessitates the finding of new dietary protein alternative like Azolla leaf meal. In the current study, inclusion of Azolla leaf meal improved body weight and reduced meat cooking loss. Therefore, inclusion of Azolla leaf meal at a rate of 5% in broiler chicken diet will be economically beneficial if the price of Azolla leaf meal is cheaper than soybean meal, for the unit of meat produced. A higher rate of Azolla leaf meal inclusion in broiler chicken diets will have a negative impact on meat color and thus consumer acceptability.

Introduction

The majority of soybean production, nearly 70%, is directed to animal feed; with poultry as the major sector that consumes soybean (USDA, 2010). The interest in biodiesel production

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from oil-bearing seeds like sunflower and rapeseeds is increasing, competing with soybean production, with negative consequences for poultry production (USDA, 2010). Therefore, it is crucial to find novel dietary protein sources that can replace, at the least partially, soybean as a plant-based protein source for poultry. Azolla is an aquatic weed rich in protein due to its ability to fix nitrogen, with an amino acid profile that is better than soybean, at the least for pigs (Brouwer *et al.*, 2018). In addition, Azolla contains a higher level of some amino acids like leucine compared to soybean meal (Sanginga and Van Hove, 1989). The application of Azolla as a dietary protein source was investigated in very few reports in broiler chickens (Rana *et al.*, 2017). However, these investigations were limited to either growth performance or meat composition, without an in-depth study on the effect of Azolla on protein synthesis and meat sensory and organoleptic parameters.

Due to its greenish color, adding Azolla leaf meal (**ALM**) to the broiler chicken diet could affect the meat color and consumer acceptability. Furthermore, the greener color of the diet could affect the bird's feed preference (Khosravinia, 2007).

Gut microbiota and their metabolites, primarily short-chain fatty acids (**SCFAs**), regulate many physiological processes in their host, including energy status, immune protection and nutrient digestibility (Yadav and Jha, 2019). Diet ingredients have a direct effect on the gut bacterial ecosystem and thus affect the type and level of SCFAs. Therefore, the effect of ALM on apparent digestibility and SCFA production was assessed in the current study. In addition, gut-muscle axis is regulated by several factors including the gut microbial ecosystem and the resulting metabolites chiefly SCFA that can affect muscle mass (Lustgarten, 2019), therefore the effect of ALM on SCFA and muscle composition and muscle fiber cross-section area was assessed in the current study.

Muscle mass is highly determined by the level of protein synthesis. The latter is dynamically regulated by the mammalian target of rapamycin (**mTOR**) pathway that plays a major role in sensing the energy level and, thus, regulates the highly energetically expensive translation process (Liu and Sabatini, 2020). The mTOR is also regulated by the level of specific amino acids, including leucine (Bar-Peled and Sabatini, 2014) Thus, the high proportion of leucine in ALM could positively influence protein synthesis via mTOR.

We hypothesized that ALM could be included in the poultry diet to partly replace soybean meal without negative effects on growth performance or meat quality. The objective of this study was to use growth performance, meat quality indices, skeletal muscle histopathological changes and protein synthesis ability as relevant parameters to evaluate the effects of using ALM as an alternative dietary protein source for broiler chicken.

Material and methods

Experimental design

Birds and housing. A total of 120 one-day-old male Ross 308 chicks were purchased from a local hatchery (El-Wadi, El-Monofeya, Egypt). Chicks were randomly allocated into three dietary treatments (40 birds/group) of four cages each (10 birds/cage). Each cage had a floor area of 0.55 m^2, and the chicks were housed on a deep litter system with sawdust and free access to feed and water.

Dietary treatments and growth performance. Sun-dried *Azolla pinnata* was purchased from Azolla farm (New Damietta, Egypt). Proximate composition analysis of dried ALM is shown in Supplementary Material S1. Three dietary treatments were fed as follows: (1) Corn–soybean meal-based diet (**CON**), (2) the CON diet supplemented with 5% ALM (**AZ5**) and (3) the CON diet supplemented with 10% ALM (**AZ10**). The experimental period was 37 days. Experimental diets and their chemical composition are presented in Supplementary Material 1. Diets were balanced to be isocaloric and isonitrogenous. Feed intake and BW were recorded weekly per cage. Daily feed intake, BW gain and feed conversion ratio (**FCR**) were calculated accordingly. Mortality was recorded on a daily basis.

Digestibility trial

At day 37, 12 birds per each group (3 birds/cage) were used in a digestibility trial. Birds were transferred to metabolic cages, with an adaptation period of 3 days and a collection period of 4 days. Feed intake was recorded and excreta were collected and weighed daily on cage basis. Excreta were pooled per each cage and were oven dried at 70°C then analyzed for chemical composition. Nutrient digestibility was then calculated as previously reported (Akinola *et al.*, 2015).

Feeding behavior

Feeding behavior of broiler chickens was assessed during the experimental period (until day 37) using an instantaneous method. Each cage was observed for 10 min, for 3 days per week (Saturday, Sunday and Wednesday). Behavior was expressed as the percentage of birds in the cage that was feeding, drinking, elimination, resting, leg scratching, leg and wing stretching and preening behavior.

Euthanasia and blood sampling

Three birds were selected from each cage (12 birds/group) and sacrificed by cervical dislocation. Blood samples were collected in empty vacutainer blood collection tubes and left to clot for 30 min at room temperature; serum was then separated as previously described (Abdelatty *et al.*, 2020) and stored in aliquots at −20°C for further analysis. The effect of ALM on liver and kidney function was assessed by measuring the serum concentration of aspartate aminotransferase, alanine aminotransferase, total protein (**TP**), creatinine, blood urea nitrogen and albumin using a Catalyst One Veterinary Chemical Analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA) loaded with Chem 10 Clip (Manufacturing # 98-11005-01), and globulin was then calculated by subtracting the albumin from TP.

Cecum short-chain fatty acids

For assessment of SCFAs, immediately after euthanasia, a piece of the cecum was ligated from both sides, then cut to avoid volatilization of SCFAs, stored in 1.5 ml Eppendorf tubes and put immediately on ice and maintained at −20°C. Samples were then analyzed using HPLC as previously described (Abdelatty *et al*., 2019). Chromatogram data were captured using Agilent ChemStation software (Agilent Technologies, Inc., Wilmington, DE, USA).

Meat quality indices

Meat composition and carcass cuts. The composition of the right side of breast and thigh meat was analyzed according to the Association of Official Analytical Chemists (AOAC, 1990), including moisture, protein, fat and ash. For determination of moisture percentage, 3 g of the sample was dried at 100°C until a constant weight was obtained. Protein percentage was determined according to the Kjeldahl method. For conversion of nitrogen into CP, a factor of 6.25 was used. Fat percentage was determined by 6-cycle extraction with petroleum ether in a Soxhlet apparatus and the difference in weight loss was calculated. Ash percentage was determined by ignition at 500°C for 5 h. Dressing percentage and carcass cut yield including thigh, breast fillet and wings were determined.

Meat pH. Immediately after euthanasia, meat pH was assessed using a portable pH meter (AD110; Adwa, Szeged, Hungary). The pH meter was calibrated for every two samples using two pH buffers 7.0 and 4.0.

Meat oxidative stability. Five grams of each meat sample were homogenized in 15 ml deionized distilled water using a stomacher (Stomacher 400 circulator; Laboratory Supply Network, Inc., Atkinson, NH, USA) for 10 s. One milliliter of the homogenate was mixed with 50 μl butylated hydroxyanisole (7.2%) and 1 ml each of 15 mM 2-thiobarbituric acid and 15% trichloroacetic acid. The mixture was vortexed, incubated in a boiling water bath for 15 min to develop color, then cooled under running water for 10 min and vortexed and centrifuged for 15 min at 100×**g**. The absorbance of the resulting supernatant was measured at 531 nm using a spectrophotometer (Unico 1200, Caledonia, WI, USA) against a blank containing 1 ml of deionized water and 2 ml of thiobarbituric acid–trichloroacetic acid solutions. The reading was multiplied by 7.8 to obtain the value of thiobarbituric acid expressed as milligram of malondialdehyde per kilogram of sample.

Meat sensory characteristics. Meat sensory characteristics of both breast and thigh meat samples were evaluated after cooking in a forced draught oven at 230°C (D-63450; Heraeus, Hanau, Germany) for a core temperature of 75°C. Eight experienced panelists (from both sexes in the age range of 30 to 45 years) were chosen from the staff members of the Department of Food Hygiene and Control at Faculty of Veterinary Medicine, Cairo University, Egypt. Panelists were selected based on their previous experience in consuming dressed chicken. After cooking, the panelists were asked to score samples for juiciness, flavor intensity, overall tenderness and off-flavor. An eight-point scale was employed for juiciness, flavor intensity and overall tenderness (1 = extreme dry, bland and tough; 2 = very dry, bland and tough; 3 = moderately dry, bland and tough; 4 = slightly dry, bland and tough; 5 = lightly juicy, intense and tender; 6 = moderately juicy, intense and tender; 7 = very juicy, intense and tender; and 8 = extremely juicy, intense and tender.). A six-point scale was employed for off-flavor (1 = extreme off-flavor; 2 = strong off-flavor; 3 = moderate off-flavor; 4 = slight off-flavor; 5 = barely detected; and 6 = not detected).

Meat cooking loss. Cooking loss is the difference in weight before and after cooking. The cooking loss percentage was calculated as the weight of raw meat sample minus the weight after cooking divided by the raw weight times 100.

Evaluation of breast meat color. Breast muscle meat color was determined using Minolta Chroma Meter (CR410; Minolta Co. Ltd, Chiyoda-ku, Tokyo, Japan) calibrated with a white plate and light trap. The color was expressed according to the Commission International de L'Eclairage (CIE, 1976) and reported as L^* (lightness), a^* (redness) and b^* (yellowness).

Breast meat histomorphometry

Muscle samples were collected from the right pectoral muscle and fixed in 10% neutral buffered formalin. Samples were processed in different grades of alcohol, xylene and embedded in paraffin, 5 μm sections were stained by hematoxylin and eosin (**H&E**) and Masson's trichrome stain. Slides were examined using an Olympus BX43 light microscope (Olympus Corporation, Center Valley, PA, USA), and images were captured using the connected Olympus DP27 camera. The cross-sectional area of 100 fibers from each sample was measured using image analysis software (Image J 1.45s; National Institute of Health, Bethesda, MD, USA) by outlining the fibers' profiles on the screen.

Immunohistochemistry

A standard tissue microarray procedure was used for the analysis of pectoral muscle samples. Briefly, H&E-stained sections were used as a guide to select the representative areas from each sample. After the selection of the paraffin blocks and marking the required area, the tissue core was removed and inserted into a recipient paraffin block; finally, the generated blocks containing the tissue cores were subjected to a gentle heat to soften the wax to fit the cores perfectly. Thick sections of 5 μm were cut into adhesive slides for immune staining. The sections were rehydrated, rinsed in phosphate buffer saline and incubated in 5% normal goat serum for 30 min at room temperature. Primary mouse monoclonal antibodies against ribosomal protein S6 kinase (p70S6 kinase-α) (H-9) (sc-8418; Santa Cruz Biotechnology, Inc., Heidelberg,

Germany) and phosphorylated p70S6 kinase-α (A-6) (sc-8416; Santa Cruz Biotechnology, Inc.) at a dilution of 1 : 200 were added to two separate series of sections for each sample and incubated overnight at 4°C in a humid chamber. After washing, the sections were incubated at room temperature for 1 h with the horseradish peroxidase-conjugated goat anti-mouse IgG H&L (ab97023; Abcam, Cambridge, UK) secondary antibody, diluted 1 : 500 and treated with avidin–biotin–peroxidase complex (Vector elite kit; Vector Laboratories, Burlingame, CA, USA). The immune reactions were detected by applying a 3,3-diaminobenzidine (**DAB**) chromogen solution (Vector DAB kit; Vector Laboratories). The sections were then counterstained with Mayer's hematoxylin. Positive immune staining was measured using Olympus BX43 light microscope (Olympus Corporation) and images were captured using the connected Olympus DP27 camera and CellSens Olympus software and expressed as a percentage of the stained area. The ratio of phosphorylated to total p70S6 kinase-α was calculated.

Statistical analysis

Data were analyzed via one-way ANOVA using the GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The model had diet as a fixed effect and pen (i.e., the experimental unit) as the random effect. Linear (L) and quadratic (Q) effects of the dose of ALM were assessed. Significance was set at $P \leq 0.05$ and tendency when $P > 0.05$ but $P \leq 0.10$.

Results

The effect of dietary inclusion of ALM on growth performance parameters is shown in Table 1. Both AZ5 and AZ10 treatments increased the feed intake by >11% compared with the CON group (P-value for $L < 0.01$ and $Q = 0.04$). Daily BW gain tended to increase ($Q = 0.06$) in birds fed with ALM compared with CON. The inclusion of ALM tended to linearly increase the FCR ($P = 0.051$). Mortality rate was similar in all groups ($P = 0.63$).

As shown in Table 2, DM digestibility tended to increase linearly with the increase in ALM included in the diet ($L = 0.09$). Additionally, there was a linear increase based on the dose of ALM in organic matter, CP and ether extract digestibility ($P < 0.05$). The effect of dietary inclusion of ALM on feeding behavior in broiler chicken is presented in Table 3. Drinking behavior tended to increase quadratically ($P = 0.09$) based on the dose of ALM.

The effect of ALM inclusion in the broiler chicken diet on serum metabolites is presented in Table 4. No significant alterations were observed in any of the measured serum indices. Inclusion of ALM in the broiler chicken diet did not alter any of the dressing percentage and carcass cuts including thigh, breast fillet and wings as shown in Table 4. As shown in Table 5, breast meat fat percentage tended to decrease linearly ($P = 0.08$) with increase in the dose of ALM fed. However, there was no change in thigh meat composition. Cooking loss percentage of breast meat linearly decreased ($P < 0.01$), whereas breast meat juiciness tends to linearly increase ($P = 0.08$) based on the dose of ALM in the diet. Breast meat color results are shown in Table 4. A significant ($P = 0.04$) decrease in lightness (L^*) was observed with 5% ALM, increase in redness (a^*) with both the ALM doses and no effect on yellowness (b^*). However, the breast meat lightness of all groups was in the normal lightness range ($50 \leq L^* \leq 56$). A quadratic increase ($P < 0.01$) in propionate level was detected with 5% but not with 10% ALM as shown in Figure 1.

Histopathological examination of pectoral muscle sections is shown in Figure 2. The CON group revealed a normal structure of pectoral muscle; the separating connective tissue was fine with the presence of a few fibers suffering myodegeneration. The pectoral muscle of chickens fed on the diet containing 5% ALM appeared of normal histological architecture with hyalinization, vacuolation and mild mononuclear inflammatory cell infiltration frequently observed. In addition to myodegeneration, fat infiltrations were also detected. Few defective muscle fibers were detected within the examined sections of muscles from chickens fed on the diet containing 10% ALM. Perivascular edema and mild perivascular mononuclear inflammatory cell infiltration were observed in some fields as well as some fat infiltrations.

Table 1 *Effect of Azolla leaf meal (ALM) inclusion on growth performance of broiler chickens*[1]

Item	CON[2]	AZ5[3]	AZ10[4]	SEM	*P**	*L**	Q*
Initial BW (g)	40.91	40.96	41.03	0.52	0.98	–	–
BWG (g/day)[5]	54.87	59.41	57.84	1.17	0.06	0.11	0.06
FI (g/day)[6]	86.85[b]	96.75[a]	97.24[a]	1.65	0.01	0.01	0.04
FCR[7]	1.58	1.62	1.68	0.03	0.13	0.04	0.92
Mortality (%)	7.5	5	5	3.63	0.85	0.64	0.78

[1]Values are least square mean.
[2]Control group received basal soybean–corn diet.
[3]Diet includes 5% ALM.
[4]Diet includes 10% ALM.
[5]BW gain.
[6]Feed intake.
[7]Feed conversion rate.
*P value for overall (P), linear (L) or quadratic (Q) effect of ALM.
[a,b]Denote $P \leq 0.05$ between treatments.

Table 2 *Effect of Azolla leaf meal (ALM) inclusion on digestibility coefficient of broiler chickens*[1]

Item	CON[2]	AZ5[3]	AZ10[4]	SEM	*P**	*L**	*Q**
DM	77.41	78.81	81.96	1.74	0.21	0.09	0.68
Organic matter	81.22	81.76	85.21	1.22	0.09	0.04	0.36
CP	74.16	81.30	83.69	3.04	0.12	0.05	0.54
Ether extract	66.35[b]	77.09[a]	81.55[a]	2.63	0.01	0.01	0.36

[1]Values are least square mean, $n = 4$ cages /group.
[2]Control group received basal soybean–corn diet.
[3]Diet includes 5% ALM.
[4]Diet includes 10% ALM.
* *P* value for overall (*P*), linear (*L*) or quadratic (*Q*) effect of ALM.
[a,b]Denote $P \leq 0.05$ between treatments.

Table 3 *Effect of Azolla leaf meal (ALM) inclusion on broiler chickens feeding behavior*[1]

Item	CON[2]	AZ5[3]	AZ10[4]	SEM	*P**	*L**	Q*
Feeding (%)	25.00	25.71	30.71	6.77	0.81	0.56	0.80
Drinking (%)	3.21	9.29	6.79	1.78	0.10	0.19	0.09
Rest (%)	47.14	48.93	53.57	7.98	0.84	0.58	0.88
Wing stretch (%)	2.14	3.57	3.21	1.36	0.74	0.59	0.60
Leg scratch (%)	3.21	2.14	2.50	1.12	0.79	0.66	0.61
Preening (%)	11.79	10.36	11.07	1.87	0.86	0.79	0.65
Elimination (%)	3.57	2.86	2.56	1.04	0.85	0.63	0.78

[1]Values are least square mean.
[2]Control group received basal soybean–corn diet.
[3]Diet includes 5% ALM.
[4]Diet includes 10% ALM.
* *P* value for overall (*P*), linear (*L*) or quadratic (*Q*) effect of ALM.

Table 4 *Effect of Azolla leaf meal (ALM) inclusion on serum biochemical indices and carcass traits of broiler chickens*[1]

Item	CON[2]	AZ5[3]	AZ10[4]	SEM	*P**	*L**	*Q**
ALT (IU/L)[5]	197.44	207.13	200.44	4.70	0.36	0.66	0.18
AST (IU/L)[6]	14.63	13.95	14.36	0.21	0.15	0.41	0.07
TP (g/dl)[7]	4.02	4.09	3.99	0.15	0.87	0.76	0.68
Creatinine (mg/dl)	0.34	0.35	0.33	0.01	0.84	0.59	0.80
BUN (mg/dl)[8]	5.44	5.57	5.39	0.18	0.80	0.88	0.57
Albumin (g/dl)	1.54	1.48	1.66	0.07	0.18	0.25	0.13
Globulin (g/dl)	2.44	2.47	2.33	0.08	0.65	0.47	0.58
Dressing (%)	74.56	76.57	75.55	2.46	0.85	0.77	0.62
Thigh (g)[9]	619.67	631.00	635.33	58.56	0.98	0.85	0.96
Thigh without skin (g)	571.33	534.33	587.00	63.58	0.84	0.86	0.59
Thigh without bone and skin (g)	342.00	352.00	336.00	47.74	0.97	0.93	0.83
Whole wing (g)[10]	141.33	146.33	133.00	13.16	0.78	0.67	0.59
Whole breast (g)	734.33	788.00	755.67	79.23	0.89	0.85	0.67
Whole breast without skin (g)	679.67	704.67	693.67	70.40	0.97	0.89	0.84
Breast fillet (g)[11]	491.67	466.00	425.33	41.66	0.56	0.30	0.88

[1]Values are least square mean, $n = 12$ samples/group.
[2]Control group received basal soybean-corn diet.
[3]Diet supplemented with 5% ALM.
[4]Diet supplemented with 10% ALM.
[5]Alanine aminotransferase.
[6]Aspartate aminotransferase.
[7]Total protein.
[8]Blood urea nitrogen.
[9]Weight of both legs.
[10]Weight of both wings.
[11]Weight of whole breast fillet.
* *P* value for overall (*P*), linear (*L*) or quadratic (*Q*) effect of ALM.

Table 5 *Effect of Azolla leaf meal (ALM) inclusion on broiler chicken breast and thigh meat quality*[1]

Item	CON[2]	AZ5[3]	AZ10[4]	SEM	*P**	*L**	*Q**
Breast meat							
Moisture (%)	72.76	73.60	74.11	0.61	0.36	0.17	0.82
Fat (%)	2.20	1.58	1.14	0.36	0.19	0.08	0.83
Protein (%)	23.77	23.57	23.50	0.71	0.96	0.80	0.94
Ash (%)	1.12	1.20	1.17	0.07	0.67	0.59	0.49
Meat pH[5]	6.23	6.30	6.28	0.04	0.42	0.30	0.36
TBARS[6]	0.22	0.16	0.18	0.04	0.61	0.50	0.48
Cooking loss (%)	44.4[a]	37.74[b]	32.26[c]	1.67	<0.01	0.01	0.53
Juiciness	6.00	6.17	7.33	0.46	0.16	0.08	0.41
Flavor intensity	6.83	6.50	6.66	0.75	0.93	0.88	0.79
Tenderness	6.67	7.00	7.67	0.64	0.56	0.31	0.83
*L**[7]	55.2[a]	51.99[b]	54.41[ab]	0.71	0.04	0.43	0.02
*a**[8]	10.9[c]	13.00[a]	12.06[b]	0.19	< 0.01	< 0.01	< 0.01
*b**[9]	14.63	13.36	12.50	0.84	0.27	0.12	0.85
Thigh meat							
Moisture (%)	75.98	75.75	75.40	0.53	0.75	0.47	0.93
Fat (%)	3.77	3.52	2.92	0.55	0.56	0.31	0.79
Protein (%)	19.13	19.23	20.60	0.72	0.34	0.19	0.49
Ash (%)	1.09	1.49	1.01	0.16	0.17	0.76	0.07
Meat pH	6.43	6.42	6.40	0.04	0.96	0.79	0.92
TBARS	0.30	0.35	0.24	0.05	0.36	0.39	0.24
Cooking loss	43.66	43.19	42.54	0.71	0.57	0.31	0.91
Juiciness	6.67	7.00	6.83	0.46	0.88	0.80	0.67
Flavor intensity	6.33	7.33	7.00	0.54	0.46	0.41	0.36
Tenderness	7.33	7.67	7.34	0.33	0.73	1.00	0.45

[1]Values are least square mean, $n = 12$ samples/group.
[2]Control group received basal soybean–corn diet.
[3]Diet supplemented with 5% ALM.
[4]Diet supplemented with 10% ALM.
[5]Meat zero time pH immediately after euthanasia.
[6]Thiobarbituric acid reactive substances.
[7]Lightness.
[8]Redness.
[9]Yellowness.
*P value for overall (P), linear (L) or quadratic (Q) effect of ALM.
[a,b]Denote $P \leq 0.05$ between treatments.

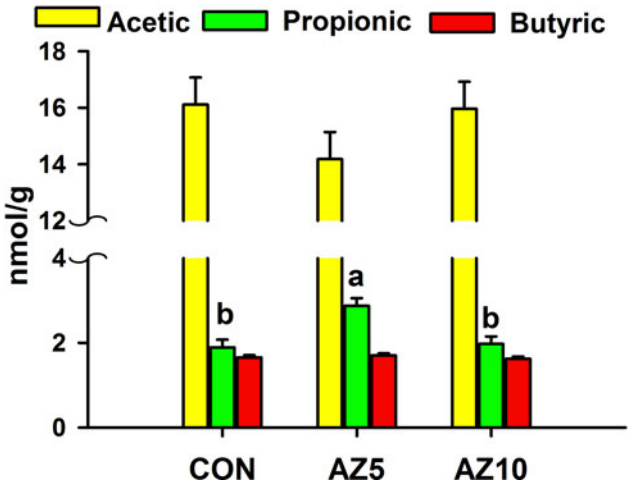

Figure 1 (colour online) Effect of Azolla leaf meal (ALM) on cecal short chain fatty acids of broiler chicken. The ALM at 5% level increased the propionate level ($Q < 0.01$), where CON is the control group, AZ5 is the group fed on diet containing 5% ALM and AZ10 is the group fed on diet containing 10% ALM.

There was only a mild tendency to a quadratic increase in muscle fiber cross-section area in AZ5 group ($Q = 0.10$), and the cross-section area was 490.3, 462.5 and 449 μm^2 in AZ5, CON, and AZ10 groups, respectively.

Nuclear and cytoplasmic expressions of total p70S6 kinase-α and phosphorylated (P-p70S6 kinase-α) are shown in Figure 3. In pectoral muscle samples of chicken, the phosphorylated p70S6 kinase-α had a linear increase with increasing dose of ALM in the diet ($P < 0.01$). The total p70S6 tended to quadratically decrease ($Q = 0.08$) in the group fed on the diet containing 5% ALM. The ratio of phosphorylated p70S6 kinase-α over total p70S6 kinase-α was positively affected by the ALM regardless of the dose ($L < 0.01$ and $Q = 0.02$).

Discussion

Relevant research evaluating the effect of Azolla on broiler chicken digestibility, gastrointestinal bacterial products (i.e.,

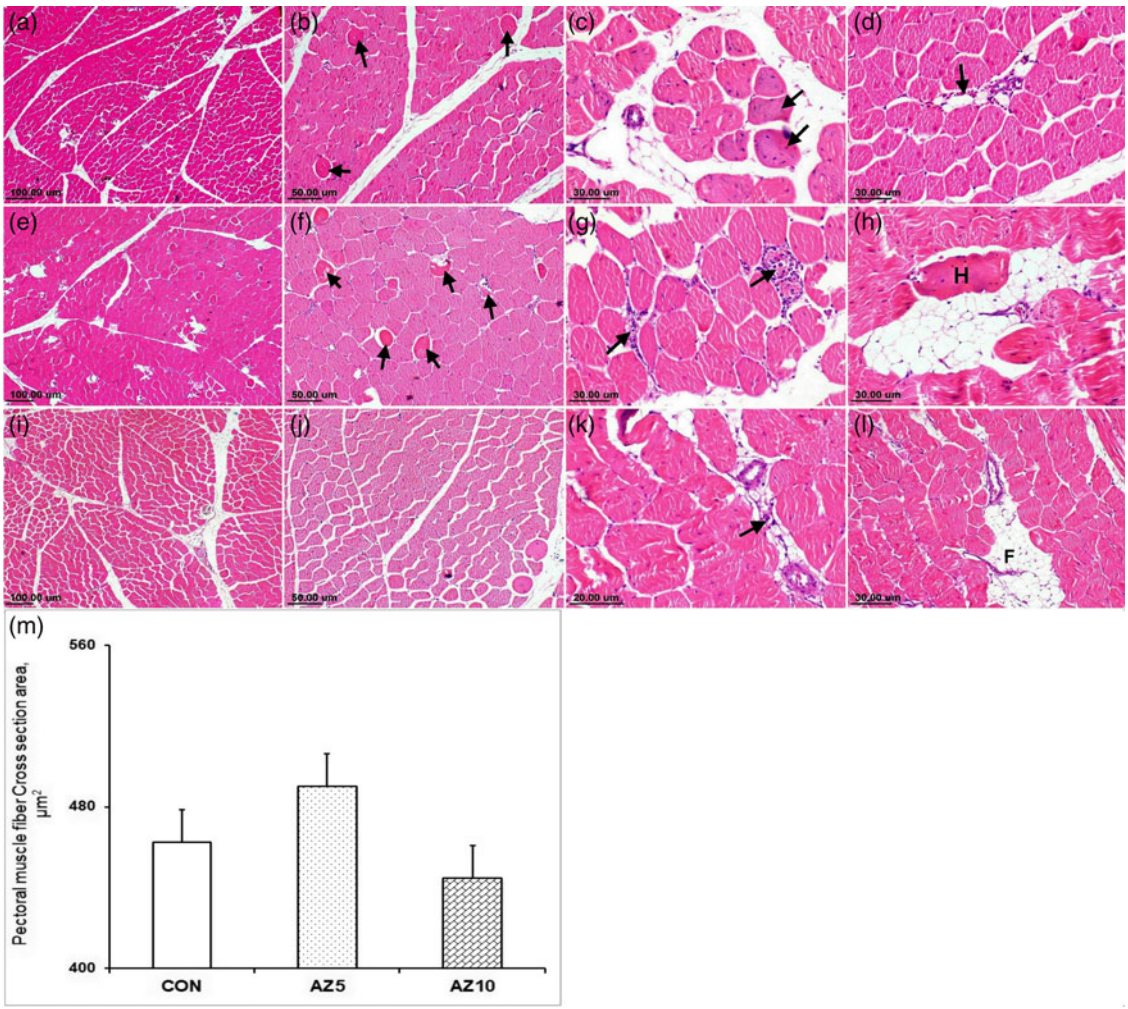

Figure 2 (colour online) Pectoral muscle histopathology (H&E stain), cross-sectional area of muscle fibers (μm^2) in different groups, where CON is the control group, AZ5 is the group fed on diet containing 5% ALM and AZ10 is the group fed on diet containing 10% ALM (a) CON group, showing normal architecture of muscle, (b) CON, higher magnification, showing few hyalinized muscle fibers, (c) CON, higher magnification, showing homogenous eosinophilic glassy sarcoplasm of degenerated muscle fiber, (d) CON, showing minute perivascular hemorrhage, (e) AZ5 group, normal primary muscle fascicle outlines, (f) AZ5, hyalinized myofibers, (g) AZ5, degenerated myofiber with mononuclear inflammatory cells infiltration, (h) AZ5 group, fatty infiltration within the muscle bundle with the presence of hyalinized myofibers (H), (i) AZ10 group, normal muscle architecture, (j) AZ10, higher magnification showing few abnormal muscle fibers, (k) AZ10, perivascular edema, (l) AZ10, fatty infiltration (F) of muscle bundles and (m) cross-sectional area of muscle fibers (µm).

SCFA), breast muscle histopathological changes and protein synthesis ability is still not well-documented.

In the current study, the greenish color of diets containing sun-dried ALM did not interfere with feeding behavior of broiler chickens and feed intake was higher in ALM fed groups similar to a prior study on feed color preference for broiler chicken that concluded green-colored feed was preferred over the other feed colors (Khosravinia, 2007). Adding ALM to broiler chicken diet increased feed intake, tended to increase the BW gain and did not negatively impact the dressing percentage, which are all similar to a prior study (Rana *et al.*, 2017). The effect of ALM supplementation on nutrient absorptive surface was not formerly reported; however, the noticeable increase in feed intake in ALM fed groups could be due to the effect of ALM on intestinal villi and bacterial ecosystem as noticed by its effect on bacterial products (SCFA). Additionally, a former study reported that ALM affected blood glucose level (Anitha *et al.*, 2016), indicating a likely increase in hepatic gluconeogenesis, which is chiefly controlled by adenosine monophosphate-activated protein kinase that plays an important role in controlling hepatic gluconeogenesis (Li *et al.*, 2020).

The increase in efficiency of protein digestibility of ALM fed group is similar to a former study when sun-dried ALM was fed to pigs (Dominguez *et al.*, 1996). Dietary protein source and amino acid profile impacted protein digestibility, according to a former report (Soomro *et al.*, 2017). Based on our findings, further study on the interaction of ALM and intestinal tract histopathology and morphometry could explain the effect of ALM on the efficiency of nutrient digestibility. The higher digestibility of fat present in ALM observed in our study is similar to what has been observed in fish (Gangadhar *et al.*, 2017). The increased crude fat fraction in the cecum contents could be due in part to the

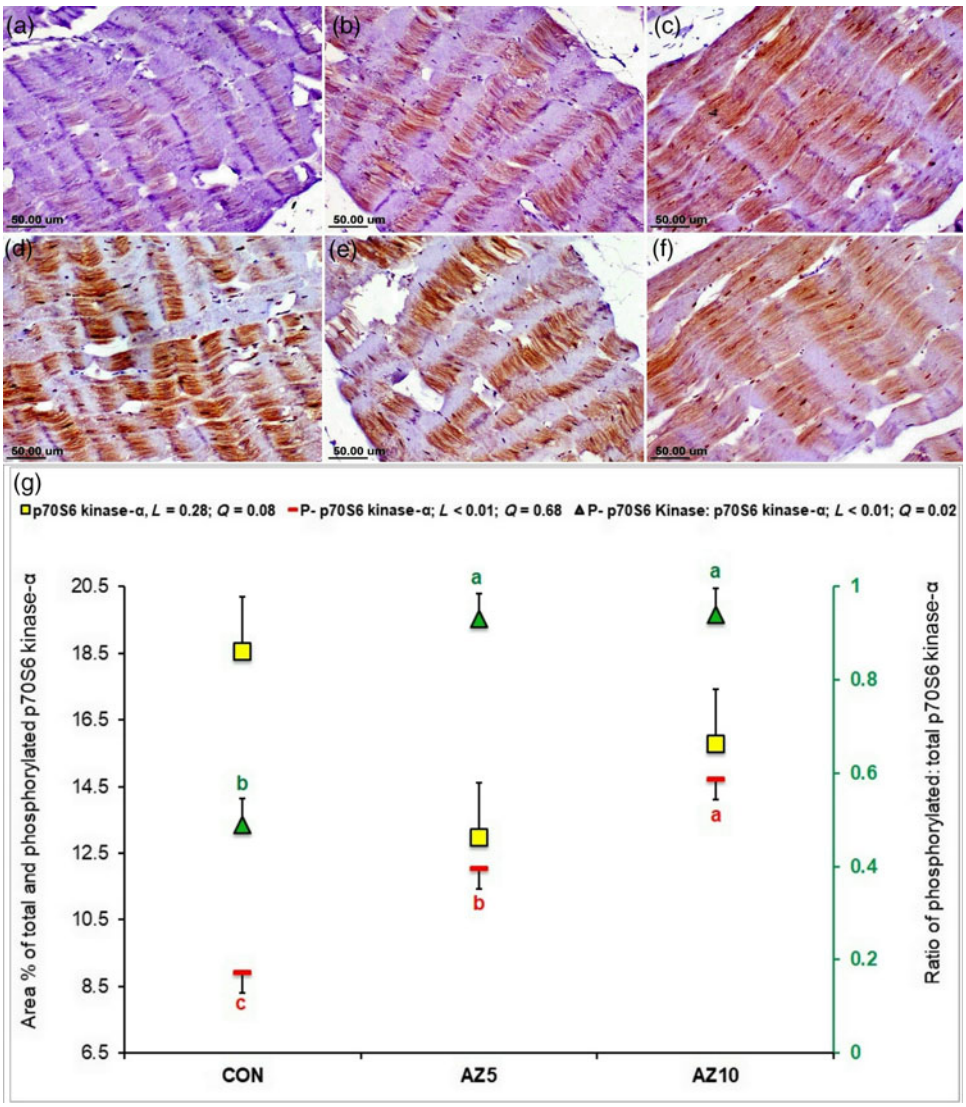

Figure 3 (colour online) Expression of total p70S6 kinase α and phosphorylated p70S6 kinase-α in pectoral muscle of broiler chicken), where CON is the control group, AZ5 is the group fed on diet containing 5% ALM and AZ10 is the group fed on diet containing 10% ALM. (a) CON group, (b) AZ5% group and (c) AZ10% group. Phosphorylated (P-p70 S6 kinase-α) expression in (d) CON group, (e) AZ5% group and (f) AZ10% group and (g) area percentage (%) of total (p70S6 kinase) and phosphorylated (P-p70 S6 kinase-α) in chicken pectoral muscles.

relatively high lipid content of Azolla (8%) plant (Brouwer *et al.*, 2019).

To our knowledge, this is the first study to report the effect of ALM on SCFA production in broiler chickens. The interesting increase in propionate level when ALM was introduced to the diet at a rate of 5% could support the selective effect of ALM on intestinal microbiota. *In vitro* fermentation of *Azolla filiculoides* by *Enterobacter cloacae* resulted in the production of acetate and butyrate (no propionate) (Brouwer *et al.*, 2019), which is different from our findings. We do not have a clear explanation for the discrepancy between the results of our study *v.* the above *in vitro* study; however, the gut of chickens contains a complex and dynamic microbiota and the species of Azolla used by us (*Azolla pinnata*) is different than the one used for the *in vitro* study. Additionally, propionate synthesis requires the availability of Vitamin B_{12} which is present at a sufficient level in Azolla (0.6 μg/g of fresh Azolla) (Johnson *et al.*, 1966).

The quadratic increase in propionate proportion by the increased dose of ALM is difficult to explain; however, we have two speculations based on the former reports. One explanation is based on the role of SCFA as signaling molecules that activate certain G protein-coupled receptors, such as Free fatty acid receptor 2 (**FFAR2**) and Free fatty acid receptor 3 (**FFAR3**), that are involved in glucose and lipid metabolism (Zhang *et al.*, 2019). Each of these receptors has an affinity to specific SCFA molecule, and the intraluminal level of these SCFAs also affects the affinity of these receptors (Larraufie *et al.*, 2018). This could explain that the increased level of propionate was subjected to fast clearance from the intestine to circulation (Boets *et al.*, 2017), which might explain the decline in cecum propionate levels

in the group that received 10% ALM in their diet, which was still numerically higher relative to the control group. Another possible cause for the dose-dependent effect of ALM on the propionate level is the bioactive compounds in ALM. For instance, aqueous and organic extracts of Azolla possess different anti-microbial activity (Pereira *et al.*, 2015) that could induce dose-dependent effects on the microbiota.

The inclusion of ALM was associated with improved meat quality in terms of decreased cooking loss percentage and increased breast meat juiciness. These findings support previous findings (Rana *et al.*, 2017). Cooking loss is regulated by different compounds, for instance, dietary amino acid level and interaction were reported to interfere with the water-holding capacity (Peng *et al.*, 2010). Amino acids like lysine were found to increase the water-holding capacity in broiler breast meat (Berri *et al.*, 2008). On the other side, lysine level was negatively related to intramuscular fat level in *longissimus dorsi* muscle of pigs (Wang *et al.*, 2017). This is supported in our study by the tendency for a negative effect of ALM dose on fat content of breast muscle. The level of lysine is high in Azolla (Sanginga and Van Hove, 1989). Further studies on the effect of ALM on muscle amino acid composition could give more detailed explanation for this finding.

Despite the statistically significant effect of ALM inclusion in the diet of broiler chickens on breast meat redness and lightness, the values were still in the normal ranges, for example, for breast meat L^* is between 50 and 56 (Petracci *et al.*, 2004). Therefore, all groups fell into the normal lightness range. Thus, the addition of ALM in the diet of broiler chickens should not affect the consumer preference for lighter breast meat color.

The ribosomal protein p70S6 kinase plays a key role in the regulation of cell survival, growth and proliferation as well as in protein synthesis (Duchêne *et al.*, 2008) through phosphorylation of ribosomal protein S6, a downstream target of mTOR signaling. To our knowledge, very few studies have investigated the activation of p70S6 kinase in broiler chickens (Duchêne *et al.*, 2008). In the current study, an increase in activated p70S6 kinase-α was observed in breast muscle of ALM fed broiler chickens. This effect is likely due to higher availability of leucine by ALM, and this amino acid being a known activator of mTOR in mammals and also in birds as observed in the muscle of neonatal chicks (Dyachok *et al.*, 2016). Additionally, feed intake positively regulates S6 kinase in chicken skeletal muscle (Dyachok *et al.*, 2016) which could be another mechanism of ALM regulation of p70S6 kinase activation. Increased activation of mTOR should induce muscle mass, by controlling both anabolism and catabolism (Yoon, 2017). None of our data indicated muscle hypertrophy, including the lack of effect on muscle fiber cross-section area. The reason for the detected lack of muscle hypertrophy despite the activation of p70S6 in our study is still unclear. Nevertheless, Azolla is rich in several bioactive compounds including tannins, phenolic compounds and flavonoids that have antioxidant activity (Selvi *et al.*, 2017), and to what extent such bioactive metabolites could affect the intestinal microbiota or the biology of the chicken, affecting the carcass quality and growth performance, remains unknown.

Azolla is known to have a high amount of ash, almost 20% of the DM (Bhaskaran and Kannapan, 2015). Although this could be an issue in inserting Azolla into the diet of poultry or any other livestock species, our data do not indicate any negative consequence in feed intake when provided with up to 5% of the diet. The high amount of ash could be one of the reasons for the lack of additional benefit or even a decrease in benefit when provided with higher doses, as observed in our study in most of the measured parameters. However, the high ash content of ALM did not affect the amount of ash in the meat of broilers.

Conclusion

Inclusion of ALM in broiler chicken diets activated the key muscle protein synthesis regulator p70S6 kinase. The effect of ALM on propionate production was only limited to a dose of 5%. Inclusion of ALM in the broiler chicken diet of up to 5% could be beneficial to partially replace soybean meal in the diet of broiler chickens without any negative impact on the growth performance but with a positive effect on meat quality. Part of the reason for the positive effect of ALM is due to the stimulation of the muscle protein synthesis through activation of the mTOR/S6 kinase signaling pathway.

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Conflict of interest

There is not conflict of interest to declare.

Ethical statement

All the experimental procedures of this study approved by Cairo University Institutional Animal Care and Use Committee (CU- IACUC; Approval# CU/II/F/70/18).

Software and data repository resources

The model was not deposited in an official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731120001421

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