Praca oryginalna *Original paper*

Effect of dietary Azolla supplementation on growth performance and histomorphometric features of the small intestine in broilers

ONUR KESER[1], FUNDA YİGİT[2], AYSE SEBNEM BİLGİN[3], ISMAİL ABAS[1], TANAY BİLAL[1], HALİL CAN KUTAY[1]

[1]Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Istanbul Univesity-Cerrahpaşa, Istanbul, Türkiye
[2]Department of Histology and Embryology, Faculty of Veterinary Medicine, Istanbul Univesity-Cerrahpaşa, Istanbul, Türkiye
[3]Pukka Veterinary Clinic, 34829, Beykoz, Istanbul, Türkiye

Received 31.10.2024 *Accepted 10.01.2025*

Keser O., Yigit F., Bilgin A. S., Abas I, Bilal T., Kutay H. C.

Effect of dietary Azolla supplementation on growth performance and histomorphometric features of the small intestine in broilers

Summary

This study was carried out to investigate the effects of *Azolla pinnata* supplementation on growth performance and intestinal histomorphometric parameters in broilers. A total of 480 day-old male broiler chicks (BW = 38.85 ± 0.16 g) were assigned to four groups for eight replicates (15 chicks for each). Chicks in the control group were fed basal diet, and experimental groups were fed diets containing Azolla (AZL) at a rate of 3% (AZL3), 4% (AZL4) and 5% (AZL5), respectively during 21 days. At the end of the experiment (day 21), the AZL3 group had significantly higher body weight than the control and other experimental groups. A significant decrease in body weight values was found when the amount of Azolla in the diets increased above 3%. No significant difference between the control and AZL3 group for average daily gain between days 0-21, but AZL4 and AZL5 groups had a significantly lower average daily gain compared to these two groups. Compared to the other experimental groups and the control group, the feed conversion rate significantly improved in the AZL3 group. According to histomorphometric data of duodenum, jejunum and ileum tissue samples obtained from the groups at the end of the experiment, it was determined that AZL3 had significantly higher villus height, villus width, villus surface area, villus height to crypt depth ratio, thickness of *Tunica muscularis* and *Tunica mucosa*, and lower crypt depth than the control and other experimental groups. As a result of this sudy, Azolla supplementation exceeding 3% to broiler diets had no significant advantage on both performance and intestinal histomorphometric parameters.

Keywords: Azolla, broiler, growth performance, intestinal histomorphology

The poultry industry, both in our country and in other countries, is one of the most important sectors in supplying valuable foods such as meat and eggs to human nutrition. Feed supply is one of the most expensive materials in poultry production, and nowadays, providing energy and protein rich ingredients for animal production is not only difficult but also costly. Therefore, the use of low-priced, safe and non-conventional feed supplements to achieve optimum animal production have been considered in recent years (22).

Azolla is a floating aquatic *macrophyte* algae that belongs to *Azollaceae* (41). Dried Azolla powder contains 22.56% crude protein, 15.08% crude fiber, 3.37% ether extract, 15.88% total ash (30). Azolla also contains flavonoids, steroids, alkaloids, phenols, triterpenoids, amino acids and fatty acids (27). In Azolla, it was reported that leucine, isoleucine lysine, arginine, phenylalanine, glycine and valine were the predominant essential amino acids, whereas sulfur-containing amino acids were found in lower amounts (15). *Azolla pinnata* can be successfully used as a feed additive in broilers and laying hens (34). Recent studies have shown different results regarding the use of Azolla in poultry diets. Kamel & Hamed (21) reported

that with Azolla supplementation up to 12% in the diet, growth performance improved in broilers; Arram et al. (9) stated that the best performance in broilers was obtained with 5 and 10% Azolla supplementation, whereas Abdelatty et al. (1) and Yadav et al. (45) reported that the best performance was obtained with 5% Azolla supplementation, while Rengma et al. (33) concluded that the addition of Azolla to the diet at 5%, 10% and 15% had no positive effect on performance.

In addition to growth performance, significant histomorphological results obtained in studies are also important to evaluate the efficiency of the feed or feed additive used in the experiment. Among the experimental groups in which 5% and 10% Azolla was added to the broiler diet, jejunum villus length was significantly higher and ileum villus length was significantly lower only in the group given 5% Azolla, and it was reported that all ameliorative effects of Azolla, including performance, were the result of its positive effects on intestinal morphology and function (1). However, Ara et al. (8) observed that the addition of Azolla to the diet did not cause a significant difference between the experimental groups in terms of intestinal villus height, crypt depth and villus/crypt ratio, but there was a decline in villus heights due to the addition of Azolla to the diet compared to the control group, and they also stated that the decrease in weight gain due to high levels of Azolla may be attributed to reduced nutrient absorption as a result of decreased villus height in the small intestine.

It was noteworthy that the majority of the studies investigating the effects of Azolla were related to levels of Azolla at 5% and above in broiler diets, whereas there were few trials at levels below 5%. In some studies, although there are data on growth performance between 0-21 days, there are almost no intestinal histomorphological data for this period. In addition, the majority of the studies were 35-42 day trials. In this context, the growth performance and intestinal histomorphometric effects of Azolla levels below 5% in a short feeding program such as 21 days in broilers raised a question mark; however, considering the short feeding period, it was predicted that levels below 3% would not cause significant effects, and to investigate the effects of dietary Azolla in broilers at 3%, 4% and 5% levels were targeted in this study. Therefore, in this study, it was aimed to investigate whether the addition of low levels of Azolla, which is thought to be an economical and safe alternative feed additive in addition to traditional feed additives, to broiler diets would show its possible effects on growth performance and intestinal histomorphometric parameters in a short feeding program such as 21 days. In this context, it was thought that the results obtained would contribute both to filling the scientific gap in this direction and to sustainable animal production.

Material and methods

Azolla plant. The azolla plants (*Azolla pinnata*) were dried in air circulation dryer oven at 40°C until they reached a constant dry weight and became crispy while retaining their greenish coloration. Next, the dried material was ground to a particle size of 1 mm in a mill, packed in air tight bags and stored in a deep freezer at –18°C until further use.

Animals and diets. All experimental protocols were approved by Istanbul University Local Ethical Committee for Animal Experiments. A total of 480 day-old male broiler chicks (Cobb500) weighed individually (BW = 38.85 ± 0.16 g) and then randomly distributed into 4 treatment groups each having 8 replicates with 15 chicks per replicate. A corn-soybean meal basal diet was formulated, ensuring adequacy in all nutrients according to NRC (31). Ingredients of basal diet were presented in Table 1. Diets were supplemented with Azolla at a rate of 3%, 4% and 5% for

Tab. 1. Composition of diets and nutrient levels

	Control	AZL3	AZL4	AZL5
Ingredients,%				
Maize	56.30	54.00	53.00	52.18
Soybean meal (48% CP)	35.40	33.88	33.88	33.00
Vegetable oil	3.20	4.00	4.30	4.50
Wheat bran	1.40	1.40	1.10	1.60
Azolla	–	3.00	4.00	5.00
Dicalcium phosphate	1.70	1.70	1.70	1.70
Limestone	1.00	1.00	1.00	1.00
DL-Methionine	0.28	0.28	0.28	0.28
L-Lysine	0.12	0.12	0.12	0.12
L-Threonine	0.02	0.02	0.02	0.02
Sodium chloride	0.30	0.30	0.30	0.30
Vitamin-mineral premix*	0.30	0.30	0.30	0.30
Chemical composition (calculated)				
Metabolizable energy, Kcal kg^{-1}	3075	3085	3088	3093
Crude protein, g kg^{-1}	220.0	218.0	217.0	218.0
Crude fat, g kg^{-1}	49.6	56.8	59.4	61.3
Crude fiber, g kg^{-1}	27.3	34.5	36.9	40.1
Crude ash, g kg^{-1}	61.6	62.6	63.1	63.7
Calcium, g kg^{-1}	9.0	9.1	9.1	9.1
Available Phosphorus, g kg^{-1}	4.6	4.5	4.5	4.6
Methionine + Cysteine, g kg^{-1}	10.1	10.1	10.2	10.1
Lysine, g kg^{-1}	13.0	12.8	12.8	12.7
Threonine, g kg^{-1}	8.5	8.5	8.5	8.5

Explanations: * Provided per kg of diet: vitamin A – 1204 µg; cholecalciferol – 25 µg; vitamin E – 4.5 mg; riboflavin – 2.25 mg; niacin – 15.0 mg; d-pantothenic acid – 4.0 mg; folic acid – 0.25 mg; vitamin B12 – 5 µg; choline chloride – 200 mg; thiamine – 0.5 mg; biotin – 25 µg; ethoxyquin – 12.5 mg; menadione sodium bisulfite – 1.25 mg; pyridoxine – 0.5 mg; manganese – 24.9 mg; zinc – 22 mg; iodine – 0.2 µg; iron – 13.6 mg; copper – 1.6 mg; AZL3 – group fed diet supplemented with 3% Azolla, AZL4 – group fed diet supplemented with 4% Azolla, AZL5 – group fed diet supplemented with 5% Azolla.

experimental groups AZL3, AZL4 and AZL5, respectively. The experimental period was 21 d, and 24-h constant light and ventilation were ensured during the entire experimental period. Temperature and relative humidity of 22 to 30°C and 40 to 60%, respectively, were maintained at all times. Chicks were provided *ad libitum* access to feed and water.

Collection of data for performance assessment. Chicks were weighed at the begining of the study to record initial body weights. On d 7, 14 and 21, broilers were fasted 12 h and then weighed. Amounts of offered and refused feeds were also recorded daily during the study to calculate feed consumption. For the statistical performance analysis, data on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion rate (FCR) were used.

Sample collection and analysis procedures. Chemical analysis of diet and Azolla samples for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), crude ash (CA), calcium (Ca), phosphorus (P) and amino acid contents were performed according to 934.01, 978.04, 920.39, 962.09, 942.05, 927.02, 965.17 and 994.12 of Association of Official Analytical Chemists (5). DM determined by drying in an oven at 105°C for 10 h. For CP contents, total nitrogen (N) was determined by using semi-automatic Kjeldahl device (Gerhardt Vapodest VAP30, Germany) and the amount of N was multiplied by 6.25. EE was determined by using Soxhlet Extraction System (Soxtherm Gerhardt, Germany). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) content of Azolla were determined according to Van Soest et al. (39) by using an Ankom 2000 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA). Ca and P contents of diets and Azolla were determined as colorimetric and spectrophotometric values, respectively. Amino acid analysis was performed by using HPLC (Agilent 1100). Analyzed and calculated nutrient levels of diets were presented in Table 1. Chemical composition of dried Azolla was presented in Table 2.

For histomorphometric analysis of intestinal tissues, 16 broilers randomly selected from each main group (2 broilers from each replicated pens) were killed by decapitation and 2 cm thick tissue samples were taken from the middle regions of the duodenum, jejunum and ileum. The tissues were transferred to Istanbul University Cerrahpaşa Faculty of Veterinary Medicine, Department of Histology and Embryology Research Laboratory for histologic evaluations. The tissues were blocked in paraffin after passing through graded alcohols and xylene. 5-6 µm thick sections were taken from the blocks and stained with hematoxylin and eosin (10). The preparations were examined and visualized with a light microscope (Leica DM 4000B) with a digital camera (MBF Bioscience). Subsequently, measurements were made using the Stereo Investigator program compatible with this microscope. Histomorphometric measurements guidance for villus height (VH), villus width (VW), crypt depth (CD), thickness of *Tunica mucosa* (McT) and thickness of *Tunica muscularis* (MsT) were presented in Figure 1. Histomorphometric analyses were performed on 10 randomly determined villi and crypts of each animal section. For this purpose; VH (from the tip of the villus to the beginning of the crypts), CD (from the beginning of invagination in the region between the villi to the end of the Lieberkühn's glands), VW (from the widest part of the villi), McT (from the tip of the villus to the muscularis mucosae) and MsT (from the inner surface of the circular muscle layer at the junction with the *Tunica submucosa* to the outer surface of the longitudinal muscle layer at the junction with the *Tunica serosa*) were measured (6). The ratio of villus height to crypt depth (VH/CD) was calculated by dividing villus height by crypt depth. The villus area (mm^2) was calculated by fitting the values obtained from the measurement procedures to a geometric model [$(2\pi \times VH \times (VW/2)]$ (35).

Tab. 2. Chemical composition (g kg^{-1}) of dried Azolla

Dry matter (air dried) Dry matter (fresh)	904.9 79.6		
Dry matter basis			
Crude protein	214.6	Arginine	11.6
Ether extract	30.2	Histidine	2.9
Crude fiber	130.3	Isoleucine	6.8
NDF	396.9	Leucine	17.3
ADF	281.2	Lysine	10.3
Crude ash	138.0	Methionine	2.0
Calcium	19.7	Tryptophan	10.5
Total Phosphorus	6.1	Threonine	6.9
		Valine	7.0

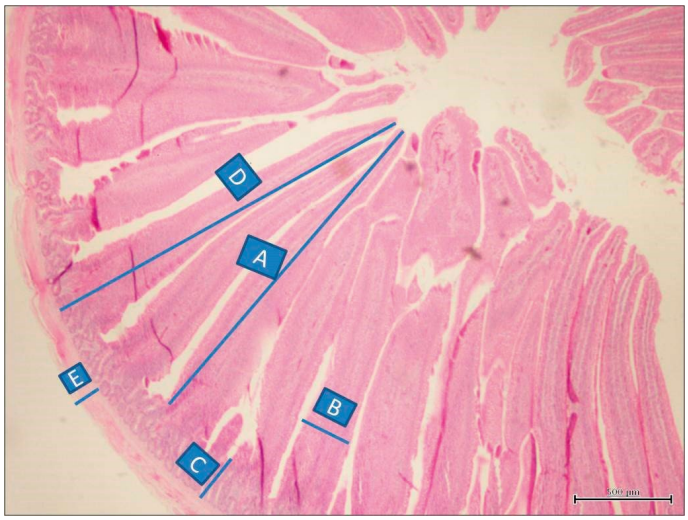

Fig. 1. Histomorphometric measurements guidance (Stained with hematoxylin and eosin, Bar: 500 µm)
Explanations: A – villus height; B – villus width; C – crypt depth; D – thickness of *Tunica mucosa*; E – thickness of *Tunica muscularis*

Statistical analysis. The data obtained were statistically analyzed by using package software (SPSS for Windows, Standard version 16.0, 2007). The normality of the data distribution was assessed by using the Shapiro-Wilk test, and the homogeneity of variances was tested by using the Levene's test. One way analysis of variance (ANOVA) was conducted for each experiment and mean differences were evaluated by Tukey's multiple comparison test. Significant differences between groups were determined at a significance level of $P \leq 0.05$, corresponding to a 95% confidence interval.

Results and discussion

As shown in Table 2, the DM ratio of the Azolla was determined as 904.9 g $kg^{-1}$and this value was comparable to the values reported by Khursheed et al. (23) i.e. 90.03%, Mishra et al. (29) i.e. 90.00%, Munnarwar et al. (30) i.e. 89.91% for Azolla. In this study, the CP content of Azolla was determined as 214.6 g kg^{-1}, and it was observed that this CP content was similar to the CP contents reported by Alalade & Iyayi (4), Alagbe & Soares (3) and Munnarwar et al. (30), and lower than the CP content reported by Bhattacharyya et al. (12), Joysowal et al. (20), Hassen et al. (17), Mishra et al. (29), Shambhvi et al. (37) Yadav et al. (45) and Munnarwar et al. (30). The EE content detected was 30.2 g kg^{-1}, which was higher than the EE contents reported by Kumar et al. (24), Lakshmi et al. (28), Mishra et al. (29), Shambhvi et al. (37) and Yadav et al. (45), lower than the EE contents reported by Ara et al. (7), Joysowal et al. (20), Hassen et al. (17), and comparable to the EE contents reported by Bhattacharyya et al. (12), Alagbe & Soares (3), Gupta et al. (16), Sharma et al. (38), Munnarwar et al. (30). The CF content (130.3 g kg^{-1}) was similar to that reported by Alalade & Iyayi (4), Alagbe & Soares (3) and Sharma et al. (38) but lower than that reported by Joysowal et al. (20), Khursheed et al. (23), Hassen et al. (17), Shambhvi et al. (37) and Munnarwar et al. (30), and higher than that reported by Kumar et al. (24) and Kumar et al., (25), Mishra et al. (29) and Yadav et al. (45). In this study, NDF and ADF contents of Azolla were determined as 396.9 g $kg^{-1}$and 281.2 g kg^{-1}, respectively. These levels were close to the levels determined by Kumari et al. (26) and Sharma et al. (38), and lower than the levels reported by Bhattacharyya et al. (12) and Gupta et al. (16). The CA content obtained in this study (138.0 g kg^{-1}) was comparable to the ash level determined by Sharma et al. (38) and Adzman et al. (2). The detected Ca and P contents for Azolla used in this sudy were 19.7 g kg^{-1} and 6.1 g kg^{-1}, respectively, and these values were comparable to the contents reported by Ara et al. (7), Joysowal et al. (20), Alagbe & Soares (3) and Lakshmi et al. (28). Regarding the amino acid composition, it was reported that all Azolla strains contained essential and non-essential amino acids, and that leucine, isoleucine lysine, arginine, phenylalanine, glycine and valine were the predominant essential amino acids, whereas sulfur-containing amino acids (methionine and cystine) were found in lower amounts (15). Similarly, it is noteworthy that arginine, leucine, lysine and tryptophan amino acids are predominant in the amino acid contents detected in the Azolla used in this study. Except for tryptophan, this dominant situation for arginine, lysine and leucine was similar to the situation reported by Alalade & Iyayi (4) for the same amino acids and the values obtained for the other amino acids were almost at similar levels. These differences in the nutrient content of Azolla are most likely due to differences in Azolla strains as reported by Sharma et al. (38) and Mishra et al. (29), cultivation and methods, collection and processing methods, environmental conditions such as temperature, humidity, wind speed and light intensity, as well as soil and water composition, which can affect the growth morphology and nutrient composition of Azolla plants.

The effects of dietary Azolla supplementation on body weight (BW), daily body weight gain (ADG), feed intake (FI) and feed conversion ratio (FCR) are presented in Table 3. In this study, as an indication that the animals were homogeneously distributed among the groups, no statistical difference was found between the groups in terms of initial BWs. On d 7, 14 and 21, the mean BW in AZL4 and AZL5 groups was significantly lower than the control and AZL3 group ($P < 0.05$). Although BW was significantly lower in AZL3 group on d 7 compared to the control group, it increased by about 3.4% on d 14 compared to the control group, but the difference between these two groups was statistically insignificant. However,

Tab. 3. Performance values of broilers (n = 120)

Parameters/days	Control	AZL3	AZL4	AZL5	SEM	P-values
BW (g)						
Initial	38.73	38.75	39.11	38.81	0.16	0.820
d 7	136.56^{a}	131.81^{b}	126.06^{c}	122.97^{c}	0.88	0.001
d 14	309.81^{a}	320.38^{a}	290.73^{b}	285.30^{b}	2.15	0.001
d 21	690.76^{b}	720.28^{a}	645.60^{c}	606.21^{d}	4.99	0.001
ADG (g/d)						
d 0-7	13.98^{a}	13.30^{a}	12.43^{b}	12.02^{b}	0.19	0.001
d 7-14	24.75^{b}	26.93^{a}	23.52^{b}	23.19^{b}	0.36	0.001
d 14-21	54.42^{ab}	57.13^{a}	50.69^{b}	45.84^{c}	1.04	0.001
d 0-21	31.05^{a}	32.45^{a}	28.88^{b}	27.02^{c}	0.45	0.001
ADFI (g/d)						
d 0-7	18.71	18.95	18.19	19.03	0.17	0.329
d 7-14	33.46	33.42	33.69	34.59	0.19	0.121
d 14-21	114.92	109.86	108.53	115.00	1.16	0.230
d 0-21	55.70	54.08	53.47	56.21	0.45	0.170
FCR (g/g)						
d 0-7	1.34^{c}	1.42^{b}	1.46^{b}	1.58^{a}	0.02	0.001
d 7-14	1.35^{b}	1.24^{c}	1.43^{ab}	1.49^{a}	0.03	0.001
d 14-21	2.11^{b}	1.92^{c}	2.14^{b}	2.51^{a}	0.05	0.001
d 0-21	1.79^{b}	1.67^{c}	1.85^{b}	2.08^{a}	0.04	0.001

Explanations: BW – average body weight; ADG – average daily gain; ADFI – average daily feed intake; FCR – feed conversion ratio; AZL3 – group fed diet supplemented with 3% Azolla; AZL4 – group fed diet supplemented with 4% Azolla; AZL5 – group fed diet supplemented with 5% Azolla; SEM – standard error of mean; a, b, c, d – means within a row with different superscript letters indicate significant differences ($P < 0.05$).

at the end of the experiment, AZL3 group was significantly higher than the control group in terms of BW ($P < 0.05$), and the addition of 3% Azolla to diet caused an increase of approximately 4.3% in BW compared to the control group. A significant decrease in BW values was found when the amount of Azolla in the diets increased above 3%. Contrary to this study, in a study conducted by Samad et al. (36) in broilers in which Azolla supplementations were 0, 5, 10 and 15%, no significant difference was found between the groups for BWs at week 3. In the broiler study conducted by Bhattacharyya et al. (12), no significant difference was found between the groups in terms of BWs at week 3 between the groups supplemented with 0, 4.5 and 5.5% Azolla. However, when compared AZL4 and AZL5 groups in the present study, which were close to the Azolla supplementation by Bhattacharyya et al. (12), it was noteworthy that the AZL4 group had significantly higher BW than the AZL5 group, but both experimental groups had significantly lower BW than the control group. In a broiler study in which 5% and 10% Azolla was suplemented to diets by Islam (19), the best result for BW was recorded in the control group and the addition of 5% Azolla resulted in significantly lower BW than the control group. This result was in line with the finding in the present study that the AZL5 group had significantly lower BW than the control group. ADG was significantly lower in AZL4 and AZL5 groups than the AZL3 group during d 0-7, 7-14, 14-21 and the entire duration of the experiment ($P < 0.05$). Compared to the control group, the AZL3 group was significantly higher in terms of ADG only between d 7-14 ($P < 0.05$), and no significant difference was found between the control and AZL3 group in terms of ADG between the other days and during the whole experiment. However, while the addition of 3% Azolla to diet significantly increased ADG compared to the other experimental groups ($P < 0.05$), only 5% and 4.5% numerical increase was observed between d 14-21 and during the whole experimental period compared to the control group, respectively. The fact that the lowest ADG was observed in the AZL5 group indicates that the addition of Azolla caused a decrease in ADG in parallel with the increase above 3%. In contrast to these findings, in the study conducted by Samad et al. (36), no significant difference was found between the groups supplemented 0, 5, 10 and 15% Azolla for BWG between d 1-21. In the study by Kumar et al. (24) in which 2.5, 5.0, 7.5 and 10% Azolla was added to broilers, there was no significant difference in terms of ADG between the control and 2.5% Azolla group between 0-3 weeks, which is similar to the insignificant difference in the relevant parameter between the control and the AZL3 group in the present study. However, the significant difference between AZL4 and AZL5 groups in the present study and the fact that this difference occurred due to the increase in Azolla percentage draws attention as a different finding compared to the reports that there was no significant difference between all groups in the study conducted by Kumar et al. (24) and between 0%, 1.5%, 2.5%, 3.5%, 4.5% and 5.5% Azolla groups in the study conducted by Sharma et al. (38) for the same period. Although the AZL3 group, which had significantly higher values than the other experimental groups (AZL4 and AZL5) in terms of both BW and ADG, had significantly higher BW than the control group, the statistically insignificant difference for ADG suggests that it may also be due to the positive effects on carcass characteristics that were not evaluated in the present study. As a matter of fact, in a study conducted by Varadharajan et al. (42) in which 0, 3 and 6% Azolla was added to quails, the fact that the 3% Azolla group was significantly higher in terms of giblet, back and wing weights than the control and 6% Azolla groups is a finding that strengthens this possibility. On a weekly basis, in the present study, the AZL3 group had significantly higher ADG than the other experimental groups in week I (d 0-7), week II (d 7-14) and week III (d 14-21). Compared to the control group, except for week II, the AZL3 group had similar ADG with the control group in other weeks, and ADG was significantly lower in the other experimental groups than the control group ($P < 0.05$). In the present study, the insignificant difference between the control and AZL3 group for ADG is similar to the stastitically insignificant difference between the control and Azolla group for days 1-14 and days 15-28 in the study conducted by Shambhvi et al. (37).

No significant difference was found for ADFI. Varadharajan et al. (42), Shambhvi et al. (37) and Samad et al. (36), reported that the addition of Azolla to diets did not cause a significant difference in feed intake, which was similar to the result of the present study. In this study, except for the period between d 0-7 and during the whole experimental period, FCR significantly improved in AZL3 group compared to the other experimental groups and the control group ($P < 0.05$). Increasing the rate of Azolla to 5% resulted in a significant increase in FCR compared to the other groups ($P < 0.05$) and caused deterioration in feed utilization. FCR is an indicator of how efficiently the feed is used by the animal for production. In this study, FCR, which shows the ratio of the average daily feed intake to the average daily body weight gain, was lowest in the AZL3 group ($P < 0.05$). An increase in FCR was also observed with the increase in the dietary Azolla and AZL5 group had the highest FCR ($P < 0.05$). FCR, which is known as the amount of feed consumed per unit body weight gain, was lowest in the AZL3 group ($P < 0.05$), indicated that the animals in this group exhibited higher feed utilization efficiency compared to other groups. Considering that there was no significant difference in ADFI between the groups in this study, it is obvious that the high FCR and therefore the

decrease in feed efficiency in the other experimental groups given more than 3% Azolla was due to the decrease in BW and ADG due to the increase in the amount of Azolla in the diet. It is also highly probable that the decrease in BW, ADG and hence feed efficiency with increasing Azolla content may be due to NDF (13) and high CF (11) in Azolla, which are limiting factors that negatively affect the effective feed utilization by monogastric animals.

The effects of dietary Azolla supplementation on histomorphometric features of duodenum, jejunum and ileum tissue samples obtained from the groups at the end of the experiment are presented in Table 4. Representative photomicrographs of transverse section of the duodenum, jejunum and ileum are presented in Figure 2.

The digestive system of poultry is formed anatomically in the embryonic period, and after hatching, villus development is completed on day 7 in the duodenum and continues until day 14 in the jejunum and ileum (40). Therefore, in the present study, a 21-day experimental period was thought to be sufficient in terms of intestinal histomorphology. In Table 4, where intestinal histomorphometric data are presented, the first thing that draws attention is that the VH values of the duodenum are significantly higher than those of the jejunum and ileum regardless of the feeding method. Indeed, Iji et al. (18) also reported that the length of villi increased in all small intestinal parts between days 0-21 in broiler chicks and the longest villi were found in the duodenum region and therefore had a larger absorption surface

Tab. 4. Histomorphometric values of duodenum, jejunum and ileum samples (n = 16)

Parts	Control	AZL3	AZL4	AZL5	SEM	P-values
Duodenum						
VH (µm)	1756.74^{b}	1844.48^{a}	1782.40^{b}	1789.57^{b}	8.40	0.003
VW (µm)	183.04^{b}	212.25^{a}	192.21^{b}	193.68^{b}	1.78	0.001
VSA (mm^2)	1.00^{c}	1.23^{a}	1.07^{b}	1.08^{b}	0.01	0.001
CD (µm)	231.25^{a}	197.67^{c}	227.61^{a}	214.43^{b}	1.87	0.001
VH/CD	7.86^{c}	9.22^{a}	7.95^{c}	8.44^{b}	0.06	0.001
MsT (µm)	184.37^{b}	195.56^{a}	174.97^{b}	177.37^{b}	1.68	0.002
McT (µm)	1964.94^{b}	2053.26^{a}	1979.31^{b}	1968.18^{b}	9.43	0.005
Jejunum						
VH (µm)	750.89^{b}	820.62^{a}	772.63^{b}	738.76^{b}	6.37	0.001
VW (µm)	161.52^{c}	171.83^{a}	168.25^{b}	165.85^{bc}	1.55	0.001
VSA (mm^2)	0.38^{b}	0.43^{a}	0.39^{b}	0.38^{b}	0.01	0.001
CD (µm)	171.01^{a}	154.97^{c}	163.71^{b}	168.92^{a}	1.77	0.011
VH/CD	4.60^{b}	5.38^{a}	4.74^{b}	4.66^{b}	0.05	0.009
MsT (µm)	151.80^{b}	168.83^{a}	150.30^{b}	150.15^{b}	1.82	0.029
McT (µm)	916.31^{bc}	998.26^{a}	937.11^{b}	881.60^{c}	6.97	0.001
Ileum						
VH (µm)	608.64^{bc}	646.96^{a}	638.96^{ab}	587.08^{c}	5.06	0.001
VW (µm)	164.37^{b}	177.26^{a}	167.03^{b}	163.48^{b}	1.58	0.027
VSA (mm^2)	0.31^{b}	0.35^{a}	0.31^{b}	0.30^{b}	0.01	0.001
CD (µm)	173.20^{a}	141.88^{b}	170.01^{a}	174.58^{a}	1.92	0.001
VH/CD	3.71^{b}	4.61^{a}	3.98^{b}	3.51^{b}	0.05	0.001
MsT (µm)	187.28^{b}	206.15^{a}	177.65^{b}	182.06^{b}	2.01	0.001
McT (µm)	723.49^{b}	820.57^{a}	731.42^{b}	725.76^{b}	5.90	0.001

Explanations: VH – villus height; VW – villus width; VSA – villus surface area; CD – crypt depth; MsT – thickness of *Tunica muscularis*; McT – thickness of *Tuninca mucosa*; AZL3 – group fed diet supplemented with 3% Azolla; AZL4 – group fed diet supplemented with 4% Azolla; AZL5 – group fed diet supplemented with 5% Azolla; SEM – standard error of mean; a, b, c – means within a row with different superscript letters indicate significant differences ($P < 0.05$)

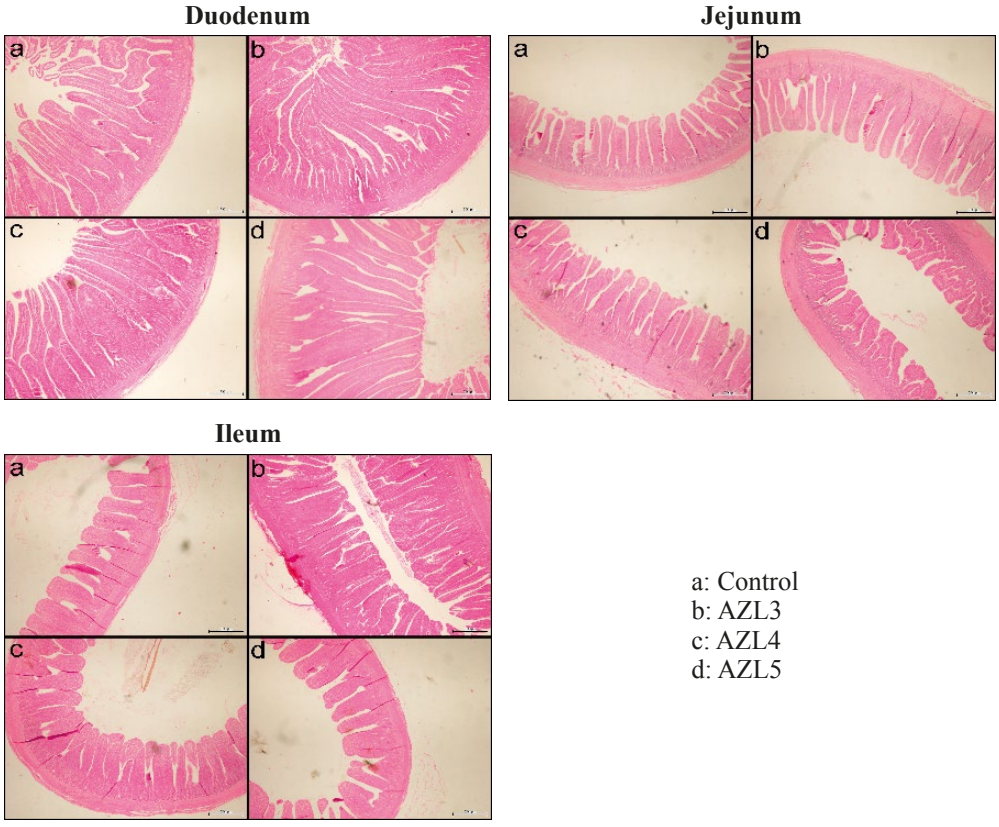

Fig. 2. Representative photomicrographs of transverse section of the duodenum, jejunum and ileum (Stained with hematoxylin and eosin, Bar: 500 µm)

than the jejunum and ileum regions. In the duodenum and jejunum sections of the small intestine, VH was significantly increased in AZL3 group compared to the other groups ($P < 0.05$). On the other hand, although there was no significant difference in terms of VH between AZL3 and AZL4 groups, the lowest VH was found in AZL5 group ($P < 0.05$). When compared to the control, jejunal VH was significantly higher ($P < 0.05$) only in AZL3 group than the control group, but the differences between the other experimental groups and the control group were statistically insignificant. It is well known that villi and crypts in the absorptive epithelium of the small intestine play a key role in the digestion and assimilation of nutrients (43). In this study, when compared with the control and other experimental groups, the highest VH was detected in the AZL3 group and this difference was statistically significant for duodenum and jejunum ($P < 0.05$). In ileum, the AZL3 group had significantly higher VH than only the control and AZL5 group ($P < 0.05$).

Duodenal VW was highest in the AZL3 group ($P < 0.05$), jejunal VW was highest in the AZL3 and AZL4 groups ($P < 0.05$) and ileal VW was highest in the AZL3 group ($P < 0.05$). Duodenal VSA was highest in AZL3 group ($P < 0.05$) and there was no significant difference between AZL4 and AZL5 groups. However, VSA detected in the experimental groups was significantly higher than the control group ($P < 0.05$). Jejunal and ileal VSA values were highest in AZL3 and AZL4 groups ($P < 0.05$), but there was no significant difference between AZL5 and control groups. Duodenal, jejunal and ileal CD values were the lowest in the AZL3 group ($P < 0.05$). However, in terms of VH/CD ratio, the AZL3 group had a higher ratio than the control and other experimental groups and this difference was statistically significant ($P < 0.05$). Since shorter villi offer less surface area for the absorption of nutrients, the simultaneous observation of short villi and deep crypts is considered an indicator of adverse intestinal development (14). In this study, the highest VH and lowest CD for the duodenum, jejunum and ileum were observed simultaneously in the AZL3 group and therefore the highest VH/CD ratio was detected in this group, which indicated that the addition of 3% Azolla to diet was associated with greater improvement in intestinal development compared to the control and other experimental groups. Moreover, a smaller CD is desirable. Indeed, larger crypts may be associated with an increase in cell turn-over, which may lead to an increase in energy requirements for gut maitenance and hence the use of nutrients for digestive functioning rather than growth (44). In addition, considering that rapid growth in chickens is directly related to the morphological and functional integrity of the digestive system (43), the higher final BW and feed efficiency (i.e., lower FCR) in the AZL3 group in this study may probably be a result of the fact that this group had higher values of VH, VW, VSA and VH/CD and lower values of CD than the control and other experimental groups. Also, Qaisrani et al. (32) observed that lower VHs and greater CDs could lead to poor digestion and less absorption of nutrients and consequently poor performance in animals. Except for the AZL3 group, there was no significant difference in duodenal, jejunal and ileal MsT among the groups, and the highest MsT in all three sections of the small intestine was found in AZL3 group ($P < 0.05$). Similarly, the highest McT in all three sections of the small intestine was detected in AZL3 group ($P < 0.05$). There was no significant difference among the control, AZL4 and AZL5 groups in the duodenum and ileum, except for the jejunum section of the small intestine in terms of McT. In the jejunum, the lowest McT was detected in the AZL5 group ($P < 0.05$), and there was no significant difference between the control and AZL4 group. It was also thought that increased thickness of the *Tunica muscularis* and the *Tunica mucosa* contributed to growth performance by improving intestinal strength and movement, lubrication, passage and absorption of nutrients in the intestine.

Based on the results of this study, the addition of Azolla to the diet did not cause a significant difference in feed intake during the experimental period, but the best results in terms of final body weight and FCR were obtained with the addition of 3% Azolla to the diet. In the other experimental groups supplemented by more than 3% Azolla, a decrease in growth performance parameters was observed. The absence of any mortality during the experiment was an indication that the addition of Azolla to the diet did not cause deleterious effects on broilers. In the intestinal histomorphometric measurements performed at the end of the experiment, the best results on villus height, villus width, crypt depth, villus surface area, ratio of villus height to crypt depth, thickness of *Tunica muscularis* and *Tunica mucosa* were obtained with 3% Azolla addition to the diet. In conclusion, this study showed that the addition of Azolla exceeding 3% to broiler diets had no significant advantage on both performance and intestinal histomorphometric parameters, which are indicative of normal intestinal development. In other words, this study indicates that the optimal inclusion level of Azolla in broiler diets should be limited to 3%, as exceeding this threshold offers no measurable improvements in growth performance or intestinal development parameters. Although there have been studies in the past years with different results on the effects of Azolla on growth performance in broilers, there are very few studies investigating the effects of this non-conventional feed additive on intestinal development and histology. In this context, there is a need for comprehensive studies on the effects of Azolla on intestinal development and histomorphology in poultry.

References

1. *Abdelatty A. M., Mandouh M. I., Mohamed S. A., Busato S., Badr O. A. M., Bionaz M., Elolimi A. A., Moustafa M. M. A., Farid O. A. A., Al-Mokaddem A. K.*: Azolla leaf meal at 5% of the diet improves growth performance, intestinal morphology and p70S6K1 activation, and affects cecal microbiota in broiler chicken. Animal 2021, 15, 100362, doi: 10.1016/j.animal.2021.100362.
2. *Adzman N., Goh S. J., Johari A., Alam M. Z., Kamaruddin M. J.*: Preliminary study on Azolla cultivation and characterization for sustainable biomass source. J. Phys.: Conf. Ser. 2022, 2259, 012018. IOP Publishing, doi: 10.1088/1742-6596/2259/1/012018.
3. *Alagbe J. O., Soares D. M.*: Effects of feeding different levels of Azolla pinnata, Polyalthia longifolia, Tithonia diversifolia, Moringa olifera, Azadiracta indica leaf meal infusion as an organic supplement on the performance and nutrient retention of growing grass cutters. Greener J. Agric. Sci. 2018, 8, 01-11, doi: 10.15580/GJAS.2018.1.010818002.
4. *Alalade O. A., Iyayi E. A.*: Chemical composition and the feeding value of Azolla (Azolla pinnata) meal for egg-type chicks. Int. J. Poult. Sci. 2006, 5, 137-141.
5. AOAC: Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists, Arlington, VA, USA 2007.
6. *Aptekmann K. P., Artoni S. M. B., Stefanini M. A., Orsi M. A.*: Morphometric analysis of the intestine of domestic quails (Coturnix coturnix japonica) treated with different levels of dietary calcium. Anat. Histol. Embryol. 2001, 30, 277-280.
7. *Ara S., Adil S., Banday M. T., Khan M. A.*: Feeding potential of aquatic fern-azolla in broiler chicken ration. J. Poult. Sci. Technol. 2015, 3, 15-19.
8. *Ara S., Adil S., Wani M. A., Banday M. T., Kamil S. A.*: Effect of Azolla cristata with or without enzyme supplementation on blood biochemistry and intestinal histomorphology of broiler chicken. Indian J. Anim. Sci. 2022, 92, 1133-1136, doi: 10.56093/ijans.v92i9.120118.
9. *Arram H. M., Aal M. H. A., Iraqi M. M., El-Sayed A. I. M., Radwan A. A.*: Effect of Azolla and probiotic feeding on broilers performance, and blood parameter traits. Egypt. Poult. Sci. 2023, 43, 333-347, doi: 10.21608/epsj.2023.305149.
10. *Bancroft J. D., Gamble M.*: Theory and practice of histological techniques (6th ed.). Churchill Livingstone/Elsevier, New York 2008, p. 744.
11. *Becerra M., Preston T. R., Ogle B.*: Effect of replacing whole boiled soya beans with azolla in the diets of growing ducks. Livest. Res. Rural Dev. 1995, 7, 1-11.
12. *Bhattacharyya A., Shukla P. K., Roy D., Shukla M.*: Effect of Azolla supplementation on growth, immunocompetence and carcass characteristics of commercial broilers. J. Anim. Res. 2016, 6, 941-945, doi: 10.5958/2277-940X.2016.00122.4.
13. *Buckingham K. W., Ela S. W., Morris J. G., Goldman C. R.*: Nutritive value of the nitrogen fixing aquatic fern Azolla filliculoides. J. Agric. Food Chem. 1978, 26, 1230-1234.
14. *Caspary W. F.*: Physiology and pathophysiology of intestinal absorption. Am. J. Clin. Nutr. 1992, 55, 299-308, doi: 10.1093/ajcn/55.1.299s.
15. FAO: Feedipedia: An online encyclopedia of animal feed, retrieved from www.feedipedia.org on 27.05.2015.
16. *Gupta S. K., Chandra R., Dey D., Mondal G., Shinde K. P.*: Study of chemical composition and mineral content of sun dried Azolla pinnata. J. Pharmacogn. Phytochem. 2018, 7, 1214-1216.
17. *Hassen W., Tafese W., Amza N., Gudeta S., Beyene A., Muleta E.*: Effect of partial substitution of soybean meal (Glycine max) by mosquito fern (Azolla pinnata) on growth performance and carcass characteristic of Cobb 500 broiler chickens. Ethiop. J. Appl. Sci. Technol. 2019, 10, 22-28.
18. *Iji P. A., Saki A., Tivey D. R.*: Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. Br. Poult. Sci. 2001, 42, 505-513, doi: 10.1080/00071660120073151.
19. *Islam M. A.*: Effect of Azolla (Azolla pinnata) on growth and lipid profiles of broiler chickens. Ann. Bangladesh. Agric. 2017, 21, 73-78.
20. *Joysowal M., Aziz A., Mondal A., Singh S. M., Boda S. S., Chirwatkar B., Chhaba B.*: Effect of Azolla (Azolla pinnata) feed on the growth of broiler chicken. J. Entomol. Zool. Stud. 2018, 6, 391-393.
21. *Kamel E. R., Hamed E.*: Effect of dried azolla on growth performance, hematological, biochemical, antioxidant parameters, and economic efficiency of broiler chickens. Adv. Anim. Vet. Sci. 2021, 9, 1886-1894, doi: 10.17582/journal.aavs/2021/9.11.1886.1894.
22. *Khare A., Chatterjee A., Mondal M., Karunakaran M., Ghosh M. K., Dutta T. K.*: Effect of supplementing Azolla microphylla on feed intake and blood parameters in growing crossbred female calves. Indian J. Anim. Nutr. 2016, 33, 224-227, doi: 10.5958/2231-6744.2016.00039.6.
23. *Khursheed I., Masud S., Khan A., Khan N., Kour S., Dua S., Khursheed I.*: Proximate evaluation of Azolla pinnata as sustainable feed supplement for poultry. J. Pharmacogn. Phytochem. 2019, 8, 3157-3160.
24. *Kumar M., Dhuria R. K., Jain D., Sharma T., Nehra R.*: Performance of broilers different phases fed on different levels of Azolla meal. J. Entomol. Zool. Stud. 2018, 6, 792-795.
25. *Kumar M., Dhuria R. K., Jain D., Sharma T., Nehra R., Prajapat U. K.*: Effect of feeding Azolla pinnata on the growth and performance of broiler chicks. Int. J. Chem. Stud. 2018, 6, 3284-3290.
26. *Kumari R., Dhuria R. K., Patil N. V., Sawal R. K., Singh S.*: Chemical composition and pellet quality of Azolla pinnata grown in semi-arid zone of India. Int. J. Chem. Stud. 2018, 6, 2031-2033.
27. *Kunjiappan S., Chowdhury R., Bhattacharjee C.*: A green chemistry approach for the synthesis and characterization of bioactive gold nanoparticles using Azolla microphylla methanol extract. Front. Mater. Sci. 2014, 8, 123-135, doi: 10.1007/s11706-014-0246-8.
28. *Lakshmi R. K. S., Seshiah C. V., Reddy P. R., Nagaraja K., Kumar I.*: Influence of incorporation of Azolla meal on performance of laying Japanese quails. Indian J. Anim. Nutr. 2019, 36, 47-50, doi: 10.5958/2231-6744.2019.00008.2.
29. *Mishra S., Khune V. N., Bara S., Banjara S.*: Nutritional evaluation of Azolla pinnata. Pharma Innovation 2020, 9, 16-17.
30. *Munnarwar S. R., Chavan S. D., Shegokar S. R., Kahate P. A., Nage S. P., Shelke R. R., Bidwe K. U., Rathod K. D.*: Seasonal effect of azolla powder supplementation on cost of production of Giriraja poultry birds. Pharma Innovation 2022, SP-11, 697-701.
31. NRC: Nutrient Requirements of Poultry. 9th Rev Ed. National Academy Press, Washington, D.C. 1994.
32. *Qaisrani S. N., Moquet P. C., Krimpen M. M., van Kwakkel R. P., Verstegen M. W. A., Hendriks W. H.*: Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 2014, 93, 3053-3064, doi: 10.3382/ps.2014-04091.
33. *Rengma D. J., Savino N., Vidyarthi V. K.*: Effect of dietary inclusion of Azolla powder on performance of broiler chicken. Livest. Res. Int. 2019, 7, 144-150.
34. *Riaz A., Khan M. S., Saeed M., Kamboh A. A., Khan R. U., Farooq Z., Imran S., Farid M. U.*: Importance of Azolla plant in poultry production. Worlds Poult. Sci. J. 2022, 78, 789-802, doi: 10.1080/00439339.2022.2054752.
35. *Sakamoto K., Hirose H., Onizuka A., Hayashi M., Futamura N., Kawamura Y., Ezaki T.*: Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats. J. Surg. Res. 2000, 94, 99-106, doi: 10.1006/jsre.2000.5937.
36. *Samad F. A., Idris L. H., Abu Hassim H., Goh Y. M., Loh T. C.*: Effects of Azolla spp. as feed ingredient on the growth performance and nutrient digestibility of broiler chicken. J. Anim. Physiol. Anim. Nutr. 2000, 104, 1704-1711, doi: 10.1111/jpn.13345.
37. *Shambhvi, Katoch S., Chauhan P., Mane B. G.*: Effect of feeding Azolla pinnata in combination with direct-fed microbial on broiler performance. Trop. Anim. Health Prod. 2021, 53, 1-9, doi: 10.1007/s11250-020-02437-w.
38. *Sharma R. K., Pathak A. K., Sharma R. K., Sharma N.*: Azolla cultivation to produce sustainable feed ingredient: Chemical composition and its impact on performance of broiler chickens. J. Anim. Res. 2020, 10, 1067-1075, doi: 10.30954/2277-940X.06.2020.29.
39. *Soest P. J. van, Robertson J. B., Lewis B. A.*: Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 1991, 74, 3583-3597, doi: 10.3168/jds.S0022-0302(91)78551-2.
40. *Uni Z., Ganot S., Sklan D.*: Posthatch development of mucosal function in the broiler small intestine. Poult. Sci. 1998, 77, 75-82, doi: 10.1093/ps/77.1.75.
41. *Vahedi V., Hedayat-Evrigh N., Holman B. W. B., Ponnampalam E. N.*: Supplementation of macro algae (Azolla pinnata) in a finishing ration alters feed efficiency, blood parameters, carcass traits and meat sensory properties in lambs. Small. Rum. Res. 2021, 203, 106498, doi: 10.1016/j.smallrumres.2021.106498.
42. *Varadharajan A., Gnanasekar R., Kothandaraman S.*: Studies on feeding value of azolla in quails in relationship to its carcass traits. Pharma Innovation 2019, 8, 1143-1145.
43. *Wang J. X., Peng K. M.*: Developmental morphology of the small intestine of African ostrich chicks. Poult. Sci. 2008, 87, 2629-35, doi: 10.3382/ps.2008-00163.
44. *Williams J., Mallet S., Leconte M., Lessire M., Gabriel I.*: The effects of fructooligosaccharides or whole wheat on the performance and digestive tract of broiler chickens. Brit. Poult. Sci. 2008, 49, 329-339, doi: 10.1080/00071660802123351.
45. *Yadav S. K., Mishra A. K., Gupta N. M., Singh A. K., Goyal G., Gaur S.*: Effect of Azolla supplementation on production performance of Narmadanidhi birds. J. Anim. Res. 2022, 12, 131-134, doi: 10.30954/2277-940X.01.2022.20.

Corresponding author: F. Yigit, Department of Histology and Embryology, Faculty of Veterinary Medicine, Istanbul Univesity-Cerrahpaşa, Istanbul, Türkiye; e-mail: fyigit@iuc.edu.tr