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RESEARCH ARTICLE

Different Dietary Levels of Lysine have Beneficial Effects on Intestinal Morphology in Japanese Quail (Coturnix Coturnix Japonica)

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A B S T R A C T

This study was designed to check the dietary effect of different levels of lysine on intestinal morphology, cecal tonsils and goblet cell count in Japanese Quails. For this Purpose, a total of 100, day old chicks were randomly divided in four groups. These groups were further sub-divided into five replicas per group, each replica having five birds. NRC has recommended 13g/kg of lysine in basal diet in 1994. The birds of control group (first) were fed with basal diet containing 13 g/kg of lysine, second experimental groups fed contained lysine at the rate of 11.7g/kg of lysine, third group 13.6 g/kg of lysine and fourth group 14.9 g/kg, respectively for 35 days. The results showed a significant increase ($P<0.05$) in villus height (VH), villus width (VW), villus surface area (VSA) and muscular mucosa thickness (MMT) in all parts of the small intestine of 13.65 g/kg supplemented birds. In jejunum, VH, VW, VSA, lamina propria thickness (LPT) and MMT was increased at levels 13.65 g/kg and 14.95 g/kg. Crypt depth (CD) and muscularis externa thickness (MET) did not vary in both duodenum and jejunum, in ileum CD and MET was increased ($P<0.005$) in treated groups 13.65 g/kg and 14.95 g/kg. LPT was increased ($P<0.005$) in duodenum and jejunum, while in ileum it was insignificant. Total goblet cells count was increased in all duodenum, jejunum and ileum. In duodenum acidic mucin and total GC count was significant ($P<0.005$) in lysine treated groups at 13.65 g/kg and 11.7 g/kg level while mixed mucin GC count was non-significant. In jejunum and ileum portion acidic mucin, mixed and total GC count was higher ($P<0.005$) in 13.65 g/kg group. In cecal tonsils length, width, area and number of lymphatic nodule and was significant ($P<0.005$) in 13.65 g/kg. Lysine supplementation of 13.65 g/kg is beneficial feed additive for Japanese Quails on intestinal morphology.

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INTRODUCTION

In 1960, poultry production had started in Pakistan and now it is providing a significant portion of daily proteins to the people. Approximately 1.5 million of population in Pakistan directly or indirectly earn from the poultry sector. Poultry meat is significantly maintaining health status; it is 26.7% of total meat production in Pakistan. The demand for meat is increasing due to increase in the population of Pakistan. So, the use of growth promoters like antibiotics is increasing to maintain the higher meat production (Hussain *et al.,* 2015). In recent years, researchers have directed their attention to find the safe alternatives of these antibiotic growth promoters (Hruby and Cowieson, 2006).

In poultry sector throughout the world, antibiotics are being used to enhance growth performance and intestinal morphology. Although, excessive increase in unwise use of antibiotics may increase resistant pathogens to antibiotics, which result as residues in gastrointestinal

tract, disturbance of normal bacterial count, and decrease in the number of in useful organisms in poultry digestive tract (Anwar, 2013). As a result, many countries have banned antibiotics in livestock sector. However, researchers are looking to find out such supplementations, which replace antibiotics to maintain health and performance in commercial zone of poultry (Ghadban, 2002).

Lysine (Lys) is considered to be the 2nd limiting amino acid (AA) for diets in poultry, due to the reason that it is only used for protein formation, under the ideal protein concept the percentage of lysine is selected to express all other essential amino acids (Chen *et al.,* 2015). Lysine is an important amino acid for boosting the breast yield as it can increase body to weight gain and feed conversion ratio equally (Li *et al.,* 2018). Lysine supplementation has been effective in intestinal mucosal morphology (Vaezi *et al*., 2011). A stimulatory effect of dietary supplementation of lysine can increase crypt depth, cell hyper-genesis, resulting in higher crypt depth and villus height (Shazali *et al.,* 2019). As such, this study was designed to check the effect of lysine on intestinal morphology and it can be used in poultry as growth promotor.

Protein diets are thought to be the main part of muscle tissues, which forms carcass (Suida 2001). It is a known fact that dietary needs of birds mainly contain amino acids, instead of only raw protein, so it is very important to accurately make a diet for birds, which contains a balance amount of amino acids, and it will be helpful in genetic potential expression (Li *et al.,* 2018). A balance diet of amino acids is important to prevent the detrimental effects on locomotion, feathering, immunity and growth performance (Kidd *et al*., 2001). Amino acids supply is related with protein formation and deposition like lysine has direct effect on carcass yield (Trindade Neto *et al.,* 2010).

Currently, Japanese Quail (Coturnix Coturnix Japonica) has attained an importance in poultry sector. Japanese Quail is being used for genetics and biological studies because it is smaller in size, minimum disease risk, easy to handle, and a limited space is required to grow a large number of birds. They rapidly become sexual mature and have a short generational period. In Japanese Quail egg production is high, a number of off-springs are available from a certain number of parents (Nkukwana *et al.,* 2014).

It is an oviparous bird, so it is also beneficial for embryological studies (Ayasan and Okan*,* 2001). Japanese Quail (Coturnix Coturnix Japonica) is one of the most produced varieties in poultry, kept for the commercial eggs and meat production. It has some unique characteristics of rapid growth, early sexual maturity, high egg production rate, short generation period and shorter incubation time-period that makes it capable for diversified animal agriculture. They do not need to be vaccinated, as they are very well resistant to diseases. Because they have less weight and volume, so less dietary feed and space for rearing is required, a less capital investment is required for commercial quail farming as compared to other poultry animals with almost the same profit margin (Kaur and Mandal 2015).

MATERIALS AND METHODS

Birds and Dietary treatments: A total of 100-day-old chicks (Coturnix Coturnix japonica) were randomly divided into four groups with five replicates per group, each having five birds. The birds were maintained under standard conditions for 8-10 days. The temperature and relative humidity were maintained at 34±1.5°C and 65±5%, respectively from first day. 37°C temperature was maintained on first day then decreased 3°C per week until it was 26°C on 21 day and remained same until the end of experiment. Birds were reared at Avian Research and Training Centre (ART) Lahore, Pakistan. Post-trial processing of samples was carried out in Department of Anatomy and Histology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The experimental shed was cleaned and sterilized. Birds were provided with free access to the water and corn-based diet mixed with different levels of lysine based on four groups for 35 days. In control (first) group, birds were only given basal diet (BD) (Table 1) along with 13 g/kg of lysine as per NRC recommendations. Second experimental was given BD supplemented with 11.7g/kg of lysine. Third experimental group was treated with BD supplemented along with 13.65 g/kg of lysine, fourth experimental group was given BD supplemented with 14.95 g per kg of lysine.

Histo-morphometric studies: At the end of experiment on 35th day, two birds were selected randomly from each replicate and killed by cervical dislocation. Then 5cm of small intestinal segments duodenum, jejunum and ileum along with cecal tonsils were removed, from pylorus to distal portion of duodenal loop, duodenum was fragmented, jejunum was excised from the distal portion of duodenal loop to the Meckel's diverticulum and ileum from the Meckel's diverticulum to the anterior portion of ileal-ceacal junction then were washed by normal saline and then fixed in 10% formaline. Then tissues were dehydrated and after that tissues were cut of 4-5µm by microtome. Then tissues were mounted on slides and after that staining was done by H & E. For goblet cells, Alcian blue-periodic acid-Schiff (AB-PAS) was used (Sayrafi *et al.,*2011). And at the end, histomorphometry was done by commercial programmed (Prog Res®2.1.1Capture Prog Camera Control Software). Five villi were selected for measurements with intact lamina propria. The intestinal parameters were villus height, width, crypt depth, lamina propria thickness, muscular mucosa thickness and muscular externa thickness (Ashraf *et al.,* 2013). The villus height (µm) was measured from the top of villus to the villus-crypt junction, crypt depth (µm) was measured from depth of invagination between adjacent villi. Villus width was measured at three points that were close to the bottom then at the midpoint and close to the tip of the villus then average was done. Then surface area of villus (µm²) was measured by formula that is $(2\pi) \times (VW/2) \times (VL)$ (khan *et al.,* 2017).

Statistical analysis: Statistical analysis was carried out by SPSS (V.13.3, Chicago IL, USA). The data was analyzed by using one way-ANOVA and presented as Standard error of the mean (SEM). Differences between the groups were compared by Duncan's Multiple Range Test and were considered significant at $P<0.05$.

RESULTS

Histomorphometric measurements of small intestinal mucosa, ceacal tonsil and histochemisty of goblet cells: The data regarding the histomorphometric measurements of duodenum, jejunum and ileum is presented in Table 2. Supplementation of lysine in basal diet of Japanese Quails at 13.65 g per kg increased (P<0.05) villus height, villus width, villus surface area and muscular mucosa thickness in all segmental area in small intestine. In jejunum segment lysine levels 13.65 g/kg and 14.95 g/kg increased villus height, villus width, villus surface area, lamina propria thickness and muscular mucosa thickness (P<0.005). Parameters like crypt depth and muscularis externa thickess did not vary in both duodenum and jejunum segments, while in ileum crypt depth and muscularis externa thickness was increased in treated groups 13.65 g per kg and 14.95 g/kg (P<0.005). Lamina propria thickness was increased in duodenum and jejunum, while in ileum it remainsed unchanged. Goblet cell count data was presented in Table 3. In duodenum acidic and total goblet cell count was significant (P<0.005) in lysine treated groups at 13.65 g per kg and 11.7 g per kg level while mixed goblet cell count was non-significant. In jejunum acidic, mixed and total goblet cell count was high (P<0.005) in 13.65 g per kg group. Similarly, in ileum acidic, mixed and total goblet cell count was high (P<0.005) in 13.65 g/kg group. Morphometric measurements of cecal tonsils was presented in Table 4. In cecal tonsils the parameters like length, width and area of lymphatic nodule and their total number were increased significantly (P<0.005) in group 13.65 g/kg as compared to all other groups. Length, width, area and total number lymphatic nodules did not significantly increase in 11.7 g/kg and 14.95 g /kg but their values were significantly higher as compared to control group.

Table 1: Composition of Japanese Quail diet.

INGREDIENTS	CP 23%
Maize	35.13
Rice polish	0.00
Wheat Brant	1.00
Canola Meal	15.00
Rapeseed Meal	4.00
Soybean Meal	15.65
Corn Gluten Meal	1.20
Poultry Byproduct meal	0.00
Fish Meal	2.00
Marble Chips	0.80
DCP	0.80
Lysine*	0.57
DL Methionine	0.09
Threonine	0.06
Molasses	0.63
Premix	0.24
Salt	0.18
Phyzyme	0.05
Rice Broken	22.60
%	100.00

*Commercially available Lysine was used as a basal diet (BD): *Provided per kg of diet: vitamin A, 8,000 IU; cholecalciferol, 2,000 ICU; vitamin E, 30 mg; manadione, 2 mg; riboflavin, 5.5 mg; pantothenic acid, 13 mg; niacin, 36 mg; choline, 500 mg; vitamin B12, 0.02 mg; folic acid, 0.5 mg; thiamin, 1 mg; pyridoxine, 2.2 mg; biotin, 0.05 mg; ethoxiquin, 125 mg; Mn, 65 mg; Fe, 55 mg; Cu, 6 mg; Zn; 55 mg.

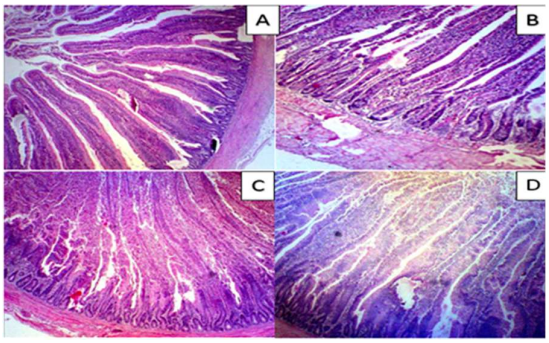

Fig. 1: Histomicrograph showing villus height and width in duodenal portion of small intestine A) Control group (first group) containing 13g/kg of lysine B) second group contains 11.7 g/kg of lysine, C) third group contains 13.65 g/kg group, D) Fourth group contains 14.95 g/kg group (40X) H&E staining.

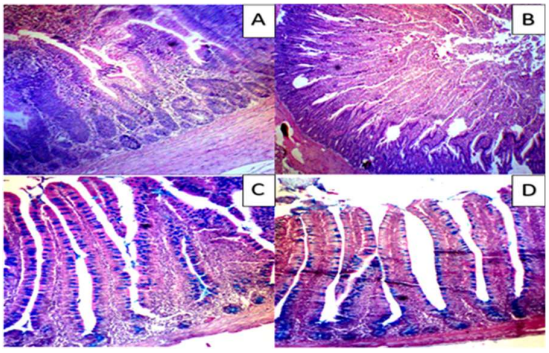

Fig. 2: Histomicrograph of Jejunum villus height and width A) first group, containing 13 g/kg of lysine B) Second group 11.7 g/kg group, C) Third group containing 13.65 g/kg of lysine, D) fourth group containing 14.95 g/kg (40X) H&E staining.

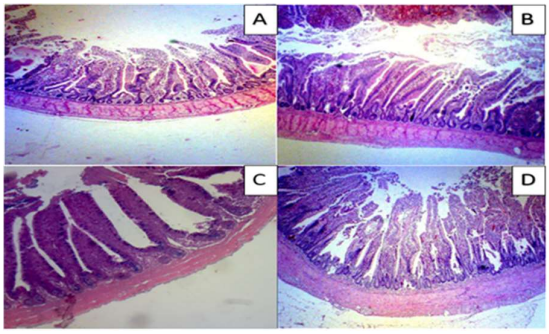

Fig. 3: Histomicrograph of Ileum villus height and width A) First control group containing 13 g/kg of lysine B) Second group 11.7 g/kg of lysine, C) Third 13.65 g/kg of lysine, D) Fourth 14.95 g/kg of lysine. (40X) H&E staining.

DISCUSSION

Mucosa of intestine is primarily responsible for the digestion and absorption of nutrients which regulates the growth in animals (Chen *et al.,* 2015). Histological parameters of intestinal in this study were villus height, width, surface area, thickness of lamina propria, muscular mucosa thickness, muscularis externa thickness, crypt depth, goblet cell count and cecal tonsils. In present study on quail chicks revealed that different dietary levels of lysine like 13.65 g/kg and 14.95 g/kg showed more encouraging results then control group 13.0 g/kg as they

increased villus width and surface area (Neu *et al.,* 2005). Increase in villus height is because it increased the epithelial cells turnover. Longer villus length depicts that there is an increase in the surface area, which directly increases the absorption rate and is good indicator of improved intestinal health (Xu *et al.,* 2003). Our results are in accordance with a previous study which shows that with the incorporation of enzymes and amino acids i.e. lysine at a level of 13.6 g/kg, digestibility of nutrients has also been maximized as the surface area of villus enlarged with an increase in absorption rate of bile acids (Jiang *et al.,* 2020). Increase in villus height when observed together with an increase in villus height:crypt depth ratio has a direct correlation with enhanced epithelial cell turnover and activated cell mitosis (Awad *et al.,* 2009; Ashraf *et al.,* 2013).

Moreover, increase in crypt depth of villi improved the cell yield of intestine by which villus regeneration occurred (Awad *et al.,* 2009). Thickness of lamina propria is an indication that the gut health is improved. It contains dendritic cells, which activate the active immune response which provides the defense against infections, this immune response increases the gut mortality by adjusting the mucin production (Macpherson and Harris, 2004). In this experiment, the thickness of lamina propria was significant in treated group 13.65 g/kg. Lysine supplementation is effective and concluded that it can positively affect crypt depth, cell number, resulting in higher crypt depth and villus height in supplemented groups 13 and 14.95 g/kg (Vaezi *et al.,* 2011). Increase in crypt depth may be due the normal sloughing and increasing demand of rapid turnover for renewal of villus tissues (Awad *et al.,* 2009).

Intestinal epithelium consists of goblet cells which has glycoprotein filling in the form of mucin. Goblet cells are important components of innate gut immune system. They produce a mucus layer composed of mucin glycoproteins, which be responsible for protection, lubrication and transport between epithelial lining and luminal contents of intestine (Pelaseyed *et al.,* 2014;

Table 2: Effect of Lysine supplementation on morphometric parameters of small intestine in Japanese Quails

Parameters	Control	11.7 g/kg	13.65 g/kg	14.95 g/kg	P-Value
duodenum					
VH (um)	797.8±10.14^{c}	682.33±10.60^{d}	880.83±36.34^{a}	869.43±15.59^{b}	.000
VW (um)	96.21±3.817^{c}	104.62±3.052^{b}	133.2±3.821^{a}	93.24±0.833^{d}	.000
VSA (um^2)	78.65±2965^{b}	62.06±3150^{c}	99.41±0512^{a}	80.40±1392^{b}	.000
LPT (um)	62.10±2.22^{c}	76.39±1.90^{b}	109.0±3.47^{a}	63.13±2.17^{c}	.000
CD (um)	81.40±2.02^{c}	107.8±15.71^{a}	104.3±7.51^{b}	105.4±2.46^{a}	.104
MMT (um)	18.23±1.18^{a}	13.47±0.65^{c}	18.74±0.83^{a}	14.49±0.78^{b}	.001
MET (um)	55.22±5.10^{c}	45.80±1.48^{d}	66.67±4.67^{a}	60.28±5.39^{b}	.181
Jejunum					
VH (um)	787.89±10.14^{c}	672.33±10.60^{d}	850.83±36.34^{b}	859.43±15.59^{a}	.000
VW (um)	96.21±3.81^{c}	114.6±3.05^{b}	133.2±3.82^{a}	93.24±0.833^{c}	.000
VSA (um^2)	68.65±2965^{b}	62.06±3150^{c}	79.41±0512^{a}	66.40±1392^{b}	.000
LPT (um)	62.1±2.22^{c}	76.3±1.90^{b}	109.0±3.47^{a}	63.1±2.17^{c}	.001
CD (um)	85.4±2.02^{d}	108.8±15.7^{a}	105.3±7.51^{b}	102.4±2.46^{c}	.100
MMT (um)	14.2±0.83^{b}	13.4±0.65^{c}	18.7±1.18^{a}	14.4±0.78^{b}	.005
MET (um)	55.2±5.10^{d}	45.8±1.48^{c}	56.67±15.51^{b}	60.28±5.39^{a}	.180
Ilium					
VH (um)	150.11±5.56^{c}	272.2±14.39^{b}	317.56±6.01^{a}	276.47±7.57^{b}	.000
VW (um)	57.49±0.78^{d}	61.11±5.98^{c}	99.84±12.4^{a}	70.83±6.15^{b}	.000
VSA (um^2)	68.65±2965^{b}	62.06±3150^{c}	79.41±0512^{a}	66.40±1392^{b}	.000
LPT (um)	40.87±3.34^{d}	81.83±2.27^{a}	44.49±5.34^{b}	42.37±4.15^{c}	.201
CD (um)	52.85±1.58^{c}	58.03±3.41^{b}	74.69±2.39^{a}	46.41±6.41^{d}	.000
MMT (um)	10.68±0.57^{c}	15.29±0.72^{a}	17.22±1.75^{a}	12.43±0.12^{b}	.000
MET (um)	28.79±5.10^{d}	56.95±4.79^{b}	69.06±5.10^{a}	32.34±1.9^{c}	.000

*a-d within the same row, means with different superscripts are significantly different (P<0.05); Values represent the Mean± SEM of four groups. VH = Villus height, VW= Villus width, VSA = villus surface Area, CD = Crypt Depth, LPT= Lamina propria thickness, MMT = Muscularis mucosa thickness, MET = Muscularis externa thickness.

Table 3: Effect of Lysine supplementation on goblet cells count of duodenum, jejunum and ileum in Japanese Quails

Intestinal segment	Goblet Cells	Control	11.7 g/kg	13.65 g/kg	14.95 g/kg	P-Value
Duodenum	AGC	16.4±0.50^{c}	33.2±26.6^{a}	33.6±1.86^{a}	22.0±5.26^{b}	.000
	MGC	28.0±2.30^{c}	43.0±36.0^{b}	47.2±2.70^{a}	44.2±7.34^{b}	.021
	TGC	34.4±2.24^{d}	74.2±3.67^{b}	80.8±2.05^{a}	66.2±4.95^{c}	.005
Jejunum	AGC	14.2±1.56^{c}	24.0±2.46^{b}	27.8±1.74^{a}	25.6±5.72^{b}	.000
	MGC	32.0±1.94^{d}	37.2±3.39^{c}	49.0±2.88^{a}	41.8±1.82^{b}	.000
	TGC	46.2±2.41^{d}	61.2±3.69^{c}	76.8±4.48^{a}	67.4±1.77^{b}	.004
Ileum	AGC	20.8±1.77^{b}	16.0±2.07^{c}	33.4±3.00^{a}	17.8±1.62^{c}	.000
	MGC	23.0±0.89^{c}	30.8±1.31^{b}	41.0±1.09^{a}	31.9±1.58^{b}	.000
	TGC	61.8±2.08^{b}	39.0±2.54^{d}	66.4±4.22^{a}	46.6±3.32^{c}	.000

*a-d within the same row, means with different superscripts are significantly different (*P*<0.05). Values represent the Mean± SEM of four groups. AGC = Acidic goblet cells, MGC = Mixed goblet cells, TGC = Total goblet cells.

Table 4: Effect of Lysine supplementations on cecal tonsil morphometric parameters in Japanese Quails

Cecal tonsils	Control	11.7 g/kg	13.65 g/kg	14.95 g/kg	P-Value
LLN (mm)	0.16± 0.037^{c}	0.19± 0.017^{b}	0.29± 0.033^{a}	0.27± 3.041^{a}	.002
WLN (mm)	0.09±0.018^{c}	0.14±0.013^{b}	0.19±0.024^{a}	0.14±0.034^{b}	.040
ALN (mm2)	0.010±0.12^{d}	0.020±0.063^{c}	0.037±0.018^{a}	0.023±1.033^{b}	.016
LN (Number)	4.30±1.78^{c}	5.15±1.43^{b}	9.14±1.97^{a}	7.40±1.59^{b}	.003

*a-c within the same row, means with different superscripts are significantly different (*P*<0.05). Values represent the Mean± SEM of four groups. LLN = Length of lymphatic nodule, WLN = width of lymphatic nodule, ALN = Area of lymphatic nodule, LN = Lymphatic nodule numbers.

Zaneb *et al.,* 2016). Goblet cells are divided into three types i.e. acidic, mixed and total. Acidic mucin plays protective role against pathogen while mixed neutral mucin facilitates feed movement due to less viscosity (Duritis and Mugurevics, 2015). Goblet cells are speckled amongst intestinal columnar cells which are mainly accountable for defense of intestinal mucosa by emission of mucilage which control the immune function of intestine (Chen *et al.,* 2015). In this study, goblet cells (mixed, acidic and total) count was increased in all treated groups like 13.65 g per kg and 14.95 g per kg above the control amount 13.0 g per kg clearly indicates that the villus height and width will surely be increased and making the digestibility more efficient because goblet cells are responsible for mucilage and improved the digestibility function of intestine.

Gut associated lymphoid tissue (GALT) generate protective immunity against systematic and local pathogens for this it attracted much response (Revolledo *et al.,* 2006). About 45.7 percent of lymphatic nodules are accumulated in the cecal tonsils of adult Japanese Quails, in avian species it is the largest gut associated lymphoid tissue (Odedeyi *et al.,* 2014). Cecal tonsils provide immunity in avian species and in poultry against some infectious diseases like ND and AI (Hamedi *et al.,* 2016). In this experiment lysine supplementation in-group 13.65 g/kg and 14.95 g/kg increased length, width, area and total number of lymphatic nodules in ceccal tonsils. This may show the immune-stimulatory effect of lysine supplementation on GALT. These increased morphometric measurements of lymphatic nodules in lysine treated group 13.65 g/kg are also helpful in increasing contact between immune cells and microorganisms. This thing may increase antigen presentation and higher antibody production. To confirm this observation, more complex methodologies of cytokine profiling and studying changes in the subpopulations of T/B lymphocytes are needed. The results obtained from this study implicate that supplementation of Lysine (13.65g/kg) in diet improved intestinal health through ameliorated gut microbial microarchitecture and increased cellular count, with a prominent immunomodulatory effect in Japanese Quails.

Conclusions: In this study, the dietary effect of different levels of lysine on intestinal morphology, cecal tonsils and goblet cell count in Japanese Quails was checked. It was examined that Lysine supplementation at dose rate of 13.65 g/kg is beneficial as feed additive for Japanese Quails based on its intestinal and cecal tonsils microbial architecture. As such, the use dietary use of Lysine might be useful for Quails raised for commercial purpose.

Authors contribution: JN and SM conceived and designed the experiments; JN and SQ, IR performed the experiments; FK, AD, SAD, JAG, GML, and AM contributed reagents, materials, and analysis tools; JN wrote the manuscript.

Competing interests: The authors declare no competing interests.

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