Research Article

Effect of Effective Microorganisms on intestinal morphology and morphometry in Japanese quails

Michał GESEK[1],* , **Rajmund SOKÓŁ**[2] , **Barry D. LAMBERT**[3] , **Iwona OTROCKA-DOMAGAŁA**[1]

[1]Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland
[2]Department of Parasitology and Invasive Diseases, Faculty of Veterinary Medicine,
University of Warmia and Mazury in Olsztyn, Poland,
[3]Sustainability and Ecosystem Sciences, Tarleton State University, Stephenville, TX, USA

Abstract: The aim of this study was to determine the effect of supplementation with Effective Microorganisms (EM) on the morphology and the morphometry of the intestine, and on proliferation activity of crypt cells in Japanese quails. The EM group (16 birds) received EM for 30 days in water (3 L/1000 L of water) and in feed (5 kg/1000 kg of feed) mixed with standard diet. The second group (16 birds) was established as the control group (no EM supplementation). Histopathological analysis revealed infiltration of lymphoid cells mainly in the duodenum and jejunum similarly in the EM and the control groups. The EM group showed irregular villus surfaces when compared to the control group. Morphometric analysis revealed significant differences in the EM group for villus width in the duodenum (186.0 μm to 170.7 μm; $P \leq 0.05$), in crypt depth (129.2 μm to 110.7 μm; $P \leq 0.05$), muscular layer thickness in the jejunum (70.88 μm to 57.44 μm) ($P \leq 0.05$), and muscular layer thickness in the ileum (76.88 μm to 64.76 μm; $P \leq 0.05$). Immunohistochemical analysis of proliferating cell nuclear antigen activity was similar for the EM and the control groups: in the duodenum 90.82% in the control group and 91.89% in the EM group, in the jejunum 93.32% in the control group and 94.53% in the EM group, and in the ileum 92.53% in the control group and 93.92% in the EM group. Supplementation with EM resulted in improved structure and functions of the villi and changed their surface, height, width, and crypt depth.

Key words: Effective Microorganisms, histopathology, Japanese quail, morphometry

1. Introduction

It is known that morphology of the alimentary system, especially the structure of the villi in different parts of the gut, influences digestion and absorption of nutrients. Increased height and width of the villi increases the digestion and absorption surface area, thereby increasing utilization of available nutrients. Dietary supplements such as seeds of plants, proteins, various sources of fiber, vitamins, amino acids, and microelements influence the morphology of the gut. Previous research concerning various protein levels in broiler chickens' diet on histological features of intestinal mucosa showed that a medium-protein diet (20.5% crude protein) caused lengthening of villi, which supported digestive enzyme action, and resulted in more effective transport of nutrients at the villus surface (1). Quail diets high in fiber resulted in longer villi due to stimulation of crypt cells, and resulted in longer overall small intestine in quails (2). Murakami et al. (3) found better development of the mucosa in broilers (with supplementation of glutamine and vitamin E), especially morphometrically

(villus height and crypt depth) during the entire fattening period. Moreover, genetic progress pointed to higher final body mass, improved feed conversion due to improved gut morphology, increased villous surface area, greater crypt depth, and higher enterocyte migration rate, which is 40% higher in fast-growing broilers (4).

There are studies demonstrating the effects of probiotics on growth, feed efficiency, and intestinal morphology. Timmerman et al. (5) studied the effects of chicken-specific probiotics, consisting of 7 *Lactobacillus* species, on broiler chickens and observed reduced mortality and increased productivity in birds with probiotic supplementation compared to a control group. Kalavathy et al. (6) analyzed the effects of 12 strains of *Lactobacillus* on broiler chickens. They found improved weight gain and feed conversion, and lower total cholesterol, low-density lipoprotein cholesterol, and triglycerides in the treatment group. Probiotic supplementation also changed the mitotic index in the crypts, and proliferation activity of the crypt cells was higher in treated groups (7). Probiotic and prebiotic

* Correspondence: michal.gesek@uwm.edu.pl

addition in broiler chickens was evaluated with positive effects concerning intestinal villus height, crypt depth, and villus surface (8). Administration of synbiotics has been shown to increase final body weight (9) and had positive effects on intestinal morphology and morphometry (10). Yeast products as growth promoters have also been of interest to poultry researchers. Yeast products resulted in increased vitamin absorption as well as synthesis of enzymes and protein metabolism. Gao et al. (11) reported improved growth performance in broiler chickens, due to increased villus morphometry, and Santin et al. (12) also found improved body weight and feed conversion in broilers receiving *Saccharomyces cerevisiae* cell wall.

Effective Microorganisms (EM) is a combination of lactic acid bacteria, photosynthetic bacteria, yeasts, actinomycetes, and fermenting fungi (*Lactobacillus hilgardii, L. zeae, L. casei, L. plantarum, L. perolens, L. diolivorans, Bacillus subtilis, B. thuringiensis, B. amyloliquefaciens, B. pumilis, B. megaterium, Acetobacter trophicans, A. lovaniensis, A. syzygii, Saccharomyces cerevisae, Moraxella osloensis, Rhodopseudomonas palustris, Dermacoccus nishinomiyaensis, Brachybacterium paraconglomeratum, Brevibacillus brevis, Devosia chinhatensis, Candida ethanolica, Issatchenkia terricola, I. orientalis, Methylobacterium mesophilicum, Kluyveromyces marxianus, Lophodermium agathidis, Brettanomyces custersianus, Novosphingobium* spp., *Mesorhizobium* spp., *Promicromonospora* spp.). EM is reported to enhance the quality of soil and the growth of plants, to suppress odor, prevent infestation of insects, and limit proliferation of pathogenic bacteria (13–16). There are many studies concerning the effects of EM on living animals. Simeamelak et al. (17) studied body weight, egg production, egg quality, and feed conversion ratio in laying hens during EM supplementation. Gnanasesigan et al. (18) conducted similar studies with layers, and focused on egg production, mortality, and egg composition. In broiler chickens, Jwher et al. (19) reported positive effects on final body weight and morphometry in the jejunum in the EM group. Wondmeneh et al. (20) compared mortality level, weight gain, and FCR between the control and the EM groups of native chickens, and found no differences, while other studies reported positive results of EM supplementation in broilers chickens (21). Studies of the effect of EM on blood parameters in layers have also yielded in various results (22).

There is no available literature concerning histopathology and morphometry during EM supplementation. Thus, the aim of the present study was to determine the effect of EM supplementation on the morphology of the alimentary system, morphometry of the intestine (height and width of the villi, depth of the crypt, and muscular layer thickness of the gut in the duodenum,

jejunum, and ileum), and proliferation activity of the cells in the crypts in Japanese quails.

2. Materials and methods

The study was conducted on thirty-two 14-day-old female Japanese quails (*Coturnix coturnix japonica*). The birds were obtained from a commercial farm and were kept in cages in two separate groups of 16 birds each. No vaccination program was performed. One group of 16 birds (the EM group) received two commercial preparations of EM for 30 days mixed in water and in feed. In water, "Multikraft" product was used in dose 3 L/1000 L of water, produced from the basic EM concentrate (Multikraft, Pichl bei Wels, Austria), and according to the manufacturer's specification contained 1.3×10^7 colony forming units (cfu) of lactic acid bacteria mL^{-1}, 3.3×10^4 cfu of photosynthetic bacteria mL^{-1}, and 1.3×10^4 cfu yeasts mL^{-1}. "MultiPROFIT" product was used in feed in dose 5 kg/1000 kg mixed in standard diet, produced from the basic EM concentrate, and according to the manufacturer's specification contained 1.3×10^7 cfu of lactic acid bacteria mL^{-1}, 3.3×10^4 cfu of photosynthetic bacteria mL^{-1}, and 1.3×10^4 cfu yeasts mL^{-1} (MultiPROFIT, Agro Concept, Poland). Both products were mixed with water and feed every day before supplementation. The animals were fed ad libitum and mixed product in feed and water was available all day. The second group (16 birds) was established as the control group and received standard diet (ad libitum) and fresh drinking water. Table 1 presents the standard feed parameters (commercial feed for laying quails production).

After 30 days, all the birds (44-day-old layers) were slaughtered (decapitation) following the approved protocols established by the Local Ethics Committee in Olsztyn.

Samples of the duodenum (medial portion), jejunum (medial portion posterior to the bile ducts and anterior to Meckel's diverticulum), ileum (medial portion posterior to Meckel's diverticulum and anterior to the ileocecal junction), and cecum (medial portion) were fixed in 10% neutralized formalin and embedded in paraffin blocks for microscopic evaluation. The paraffin sections (4 μm) were stained with hematoxylin and eosin. The morphometric indices for villus height were measured from the tip of the villi to the top of the crypt, crypt depth was measured from the base of the villi to the submucosa, and villus width were measured in the middle of the villi with an average of 15–20 measures per tissue. Each section was imaged using a Panoramic Scanner MIDI (3DHISTECH, Hungary). The measurement data of the alimentary system were prepared using Panoramic Viewer software (3DHISTECH, Hungary).

The endogenous peroxidase activity was quenched by incubating the specimens for 5 min with Peroxidase Block (EnVision, Dako, Denmark). The slides were then

Table 1. Chemical composition of the diets.

Item	Day 0 to 7[1] (Starter)	Day 8 to 28[1] (Grower I)	Day 29 to 42[1] (Grower II)	From day 42[1] (for laying birds)
Chemical composition %				
Crude protein	26.0	23.5	20.0	21.0
Gross fiber	3.0	3.1	3.4	3.8
Lysine	1.50	1.25	1.00	1.15
Methionine	0.69	0.53	0.44	0.45
Methionine + Cysteine	1.10	0.93	0.80	0.81
Ca	1.00	0.88	0.95	3.2
Na	0.17	0.16	0.15	0.16
Cl	0.16	0.15	0.14	0.15
Total P	0.50	0.40	0.40	0.55
ME (kcal/kg)	2975	2900	2800	2800
The vitamin and mineral content				
Vitamin A IU/kg	13,200			
Vitamin D3 IU/kg	3120			
Vitamin E (IU/kg)	68			
Vitamin K3 (mg/kg)	4.80			
Vitamin B1 (mg/kg)	2.2			
Vitamin B2 (mg/kg)	7.2			
Vitamin B6 (mg/kg)	5.0			
Vitamin B12 (mg/kg)	0.01			
Biotin (mg/kg)	0.03			
Cu (mg/kg)	8			
Fe (mg/kg)	116			
Mn (mg/kg)	80			
Zn (mg/kg)	100			
I (mg/kg)	0.80			
Se (mg/kg)	0.20			

[1]Ingredients: Corn (24.0%), wheat (38.0%), soybean meal (30.0%), calcium carbonate (5.0%), monocalcium phosphate (1.7%), NaCl (0.3%), premix (1.0%).

incubated with antiproliferating cell nuclear antigen (PCNA) monoclonal mouse antibody clone PC10 (Dako) diluted 1:200 in Dako Antibody Diluent at room temperature for 30 min in a humid chamber. For the negative control, this primary antibody was replaced with Dako Mouse IgG2a (1:200). The primary antibody was detected using a system based on a horseradish peroxidase-labeled polymer conjugated with secondary antibodies (EnVision, Dako). The incubation time was 30 min in a humid chamber. PCNA-positive cells were then visualized using 3,3-diaminobenzidine (DAB) substrate-chromogen (EnVision, Dako), which resulted in a brown precipitate at the antigen site. The specimens were counterstained with hematoxylin (3 min) and were dehydrated and mounted Rusing Canadian balm (Merck, Darmstadt, Germany). A commercial human tonsil specimen was used as a positive control (Dako). The brown-stained cells were considered PCNA positive. Immunohistochemical reactions for PCNA in the nucleus were measured with Nuclear Quant software (3DDHISTECH, Hungary).

Data for villus height and width, crypt depth, and muscular layer thickness were analyzed using ANOVA (GraphPad Prism 6.07, La Jolla, CA, USA). The model contained the effects of treatment. When significant effects were detected in the model ($P \leq 0.05$), differences between means were estimated using Fisher's LSD test.

3. Results

Histopathological analysis revealed several lesions as presented in Table 2. Infiltration of lymphoid cells was the main lesion observed in both the duodenum and the jejunum (9 cases in the control group and 8 cases in the EM group, and 10 cases in the control group and 8 cases in the EM group, respectively). An interesting observation was the irregular surface of the villi in 6 cases in the EM group and none in the control group in the jejunum, 4 cases in the EM group with one in the control group in the ileum, and 2 cases in both groups in the duodenum. One case of fibromuscular dysplasia was diagnosed in the artery in the cecum in the control group. Other lesions were accidental findings and did not change intestinal morphology. The histopathological examination revealed no significant differences.

Morphometric analysis of diagnosed segments of the alimentary system showed no differences for villus height after EM supplementation in any segment of the small intestine (Table 3). When the width of the villi was examined, duodenal villi were wider in the EM-treated group. Crypt depth was greater in the jejunum and muscle layer thickness was greater in the jejunum and ileum of the EM-supplemented birds.

Immunohistochemical analysis of PCNA activity showed similar observations in the EM and control groups,

regardless of the analyzed segment. In the duodenum, PCNA activity was 90.82% in the control group and 91.89% in the EM group, in the jejunum 93.32% in the control group and 94.53% in the EM group, and in the ileum 92.53% in the control group and 93.92% in the EM group.

4. Discussion

Histopathological analysis of the alimentary system showed only minimal changes. Mostly, infiltration of lymphoid cells with similar intensity in the control and EM groups similarly within the duodenal and jejunal mucosa was diagnosed. Infiltration of the inflammatory cells is part of the immune system and creates protection from dietary pathogens. Similar observations in the EM and the control groups suggest no influence of EM supply on gut immunostimulation. Interestingly, surface irregularities in the intestine were observed. In 6 cases in the jejunum, 4 cases in the ileum, and 2 cases in the duodenum in the EM group, irregular surfaces of the villi were found. We observed wavy surfaces of villi, which resulted in a larger number of enterocytes on the villi, and therefore increased digestion and absorption surface area is presumed. This observation was diagnosed more often in EM-supplemented quails, suggesting a causative effect of EM stimulation and proliferation of the cells. This is the

Table 2. The number of morphological changes in alimentary system in the EM and control groups in Japanese quails.

Part of alimentary system	Morphological changes	Control group n = 16	EM group n = 16
Duodenum	Congestion of the mucosa	0	0
	Infiltration of lymphoid cells in the mucosa	9	8
	Infiltration of myeloid cells in the mucosa	1	0
	Fusion of the villi	0	1
	Irregular surface of the villi	2	2
	Focal necrosis of the enterocytes	1	0
Jejunum	Congestion of the mucosa	3	0
	Infiltration of lymphoid cells in the mucosa	10	8
	Dilatation of the crypts	1	2
	Focal necrosis of the enterocytes	1	0
	Edema of the villi	1	0
	Fusion of the villi	4	2
	Irregular surface of the villi	0	6
Ileum	Congestion of the mucosa	1	3
	Infiltration of lymphoid cells in the mucosa	2	0
	Fusion of the villi	2	1
	Irregular surface of the villi	1	4
Cecum	Infiltration of lymphoid cells	2	2
	Fibromuscular dysplasia – artery	1	0

Table 3. Effect of EM supplementation on intestinal morphometry in Japanese quails[1].

	Duodenum		Jejunum		Ileum		Pooled SEM	P-value
	Control	EM	Control	EM	Control	EM		
Villus height, μm	1225[A]	1186[A]	581.5[B]	629.8[B]	375.2[C]	425.4[C]	25.1	<0.0001
Villus width, μm	170.7[B]	186.0[A]	123.1[C]	139.5[C]	116.7[C]	126.1[C]	7.6	<0.0001
Crypt depth, μm	154.0[A]	161.7[A]	110.7[C]	129.2[B]	82.40[D]	92.64[D]	9.2	<0.0001
Muscular layer thickness, μm	73.44[A]	74.90[A]	57.44[B]	70.88[A]	64.76[B]	76.28[A]	6.7	0.0144

[A–D]Different letters within the same row indicate a difference (P ≤ 0.05).
[1]Each value represents the mean of 16 birds per group.

first such observation of irregular surfaces, although the observations made by Khambualai et al. (7) with use of a scanning electron microscope after probiotic supply are similar. They revealed protuberant, rough surfaces of the villi, which in our opinion make the villi irregular, as is the case in our study. Furthermore, the authors hypothesized that the rough surface might be due to hypertrophy of the cells, but our hypothesis is that the surface of the villi is pleated/wrinkled equally like the EM villi in our study and, as a result, increased digestion and absorption surface area.

One case of fibromuscular dysplasia (FMD) was found in the artery in the cecum. FMD is an idiopathic, noninflammatory, and nonatherosclerotic disease of the vessels, where the lumen is obstructed by a plug originating from the media of the vessel or reduced by cells proliferating from different layers of the vessel wall, leading to lumen stenosis (23). We diagnosed the case as the medial fibromuscular dysplasia subtype of FMD, similar to in Braga et al. (24) in intramuscular arteries and Gesek et al. (25) in the cecum artery in Japanese quails and in arteries and in a vein in broiler chickens with no any ischemic or necrotic changes in the tissues (23).

Morphometric analysis of the gut segments revealed significant differences (P ≤ 0.05) in the duodenum (villus width), jejunum (crypt depth, muscular layer thickness), and ileum (muscular layer thickness). Although the remainder of the measurements did not show significant differences, the data are worthy of discussion, and in our opinion EM supplementation changed the architecture of the gut segments. In the duodenum, we found no differences in villus height, but the villus width showed significant differences between the groups. The crypt depth showed no significant difference but increased depth was noted in the EM group. Hassanpour et al. (9) after 42 days of synbiotic administration found higher duodenal villi, but on day 32 there was no difference between the treated and the control groups, which is similar to our findings after 30 days of supplementation. Awad et al. (10)

found no difference in duodenal villus height and width after synbiotic supply. The same result of no difference after probiotic supply within duodenal villus height and crypt depth was reported by Tsirtsikos et al. (26). Pelicano et al. (8) proved higher crypt depth in the duodenum after *Bacillus subtilis* addition compared to the control group but there was no difference in villus height. Yeast addition in the study by Santin et al. (12) significantly improved villus height in the duodenum but only in the first 7 days and all the benefits had disappeared by the end of the experiment. Gao et al. (11) also studied yeast culture effects and reported varying results depending on gut segment and yeast addition. They revealed the highest villi in duodenum with 2.5 g/kg addition on day 21 but with 7.5 g/kg on day 42. The crypt depth was significantly different compared with the control group on day 42, with 7.5 g/kg addition. Although our findings in the duodenum did not reveal higher villi, the greater villus width in the EM group obviously increased digestive and absorption area. This observation confirms findings from the study by Smith et al. (4), who reported that increased digestive and absorption area influence weight gain.

In the present study, crypt depth and muscular layer thickness were greater in the jejunum, although the villus height and width were not increased in the EM group compared to the control group. Hassanpour et al. (9) in a synbiotic group in the jejunum (after 42 days) found decreased width of the villi, which differs from our study. After *Bacillus subtilis* supplementation, significant higher villus height and crypt depth were noted in the jejunum in Pelicano et al.'s (8) research. Yeast addition in Santin et al.'s (12) studies showed positive effects in the jejunum only in the first 7 days, but at the end of the experiment villus height in the treated and control groups did not differ. Contrary to our findings, some showed significant decreased crypt depth during yeast supply, where in our studies significant crypt depth was noted in the EM group, similar to the study by Gao et al. (11), where the authors found significant differences in the 7.5 g/kg addition

group. Gao et al. (11) did not find positive effects of yeast supplementation on the jejunal villus height, similar to the current study. Jwher et al. (19), using EM, reported higher jejunal villi, similar to our study, but they also showed significant differences between the EM and control groups. Equally, our EM groups had significant differences within crypt depth, which confirms the positive effect of EM supplementation on cell turnover in the crypt in the jejunum.

The analysis of the ileum showed significant differences in muscular layer thickness, but, similar to preceding segments, in the EM group increased values were noted compared to the control group. These results look promising when data from the study by Hassanpour et al. (9) are taken into consideration. After 32 days, Hassanpour et al. (9) found no difference in the height or width of the villi in the ileum, but, after 42 days of synbiotic supply, a significant decrease was observed in the height of villi and surprisingly increased width of villi (0.1% synbiotic supply). Awad et al. (10) in a study with synbiotic addition stressed that ileal villus height was significantly increased compared to the control group, but crypt depth was decreased. Significantly increased height of villi and crypt depth, opposite to our findings, were found by Pelicano et al. (8) after *Bacillus subtilis* addition in broiler chickens. Santin et al. (12) revealed significant differences in ileal villus height after yeast administration but only after 7 days, and at the end of the experiment there was no difference. Various results were shown by Gao et al. (11) in villus height with yeast addition. On day 21 they reported higher villi in the ileum in the treated group (2.5 g/kg), but on day 42 all villi in the treated group were smaller compared to those in the control group. Crypt depth in the treated groups was also reduced compared to the control groups. Samli et al. (27) found significant differences in ileal villus height after probiotic

(*Enterococcus faecium*) supplementation. They showed no difference in villus width and crypt depth in their treated and control groups, similar to our observation but we found significant differences within muscular layer thickness, contrary to the findings of those authors. Tsirtsikos et al. (26) after probiotic addition, consisting of *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pedicoccus acidilactici*, and *Lactobacillus salivarius* similar to findings in the duodenum did not reveal any differences in villus height or crypt depth in the ileum. Thus, our morphometrical observation in the ileum did not show significant differences within the mucosa, but increased values in the treated group look promising.

Analysis of the immunohistochemical reaction showed similar activity in different segments. The highest PCNA reaction was observed in the jejunum with the lowest in the duodenum, but values were consistently above 90%. Similar observations were reported by Khambualai et al. (7) in broiler chickens after commercial probiotic product supplementation with higher cell mitosis in crypts in the duodenum and jejunum in probiotic groups compared with the control group. A PCNA-positive reaction is expressed during DNA synthesis of the cell cycle. In the alimentary system within crypt cells, this is the standard finding and suggests high turnover of the enterocytes.

In conclusion, EM supplementation improved the structure and function of the alimentary system as a result of changed villus surface, villus height and width, and crypt depth, and, consequently, increased immune function, and digestive and absorption surface area. In our opinion, EM protects enterocytes from pathogens, and stimulates turnover of the cells in the crypts similar to other probiotics and synbiotics, and are a potential alternative to antibiotics used as growth promoters and comprise a future of natural production systems.

References

1. Laudadio V, Passantino L, Perillo A, Lopresti G, Passantino A, Khan RU, Tufarelli V. Productive performance and histological features of intestinal mucosa of broiler chickens fed different dietary protein levels. Poult Sci 2012; 91: 265-270.

2. Starck JM, Rahmaan GHA. Phenotypic flexibility of structure and function of the digestive system of Japanese quails. J Exp Biol 2003; 206: 1887-1897.

3. Murakami AE, Sakamoto MI, Natali MRM, Souza LMG, Franco JRG. Supplementation of glutamine and vitamin E on the morphometry of the intestinal mucosa in broiler chickens. Poult Sci 2007; 86: 488-495.

4. Smith MW, Mitchell MA, Peacock MA. Effects of genetic selection on growth rate and intestinal structure in domestic fowl (*Gallus domesticus*). Comp Biochem Physiol A Mol Integr Physiol 1990; 97: 57-63.

5. Timmerman HM, Veldman A, van den Elsen E, Rombouts FM, Beynen AC. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. Poult Sci 2006; 85: 1383-1388.

6. Kalavathy R, Abdullah N, Jalaludin S, Ho YW. Effects of *Lactobacillus* cultures on growth performance, abdominal fat deposition, serum lipids and weight of organs of broiler chickens. Br Poult Sci 2003; 44: 139-144.

7. Khambualai O, Yamauchi K, Ruttanavut J, Incharoen T, Kashimura J. Effect of sugar cane extract, commercial probiotic and their mixture on growth performance and intestinal histology in broiler chickens. Am J Anim Vet Sci 2010; 5: 132-138.

8. Pelicano ERL, Souza PA, Souza HBA, Figueiredo DF, Boiago MM, Carvalho SR, Bordon VF. Intestinal mucosa development in broiler chickens fed natural growth promoters. Braz J Poultry Sci 2005; 7: 221-229.

9. Hassanpour H, Zamani Moghaddam AK, Khosravi M, Mayahi M. Effects of synbiotic on the intestinal morphology and humoral immune response in broiler chickens. Livest Sci 2013; 153: 116-122.

10. Awad W, Ghareeb K, Bohn J. Intestinal structure and function of broiler chickens on diets supplemented with a synbiotic containing *Enterococcus faecium* and oligosaccharides. Int J Mol Sci 2008; 9: 2205-2216.

11. Gao J, Zhang HJ, Yu SH, Wu SG, Yoon I, Quigley J, Gao YP, Qi GH. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. Poult Sci 2008; 87: 1377-1384.

12. Santin E, Maiorka A, Macari M, Grecco M, Sanchez JC, Okada TM, Myasaka AM. Performance and intestinal mucosa development of broiler chickens fed diets containing *Saccharomyces cerevisiae* cell wall. J Appl Poult Res 2001; 10: 236-244.

13. Zhao Q. Effect of EM on peanut production and soil fertility in the red soil region of China. In: Proceedings of the Conference on Effective Microorganisms for a Sustainable Agriculture and Environment. France; 1995. pp. 99-102.

14. Xu HL. Effects of a microbial inoculant and organic fertilizers on the growth, photosynthesis and yield of sweet corn. J Crop Prod 2001; 3: 183-214.

15. Yamada K, Xu HL. Properties and applications of an organic fertilizer inoculated with Effective Microorganisms. J Crop Prod 2001; 3: 255-268.

16. Yan PS, Xu HL. Influence of EM Bokashi on nodulation, physiological characters and yield of peanut in nature farming fields. J Sustain Agr 2002; 19: 105-112.

17. Simeamelak M, Solomon D, Taye T. The effect of effective microorganisms on production and quality performance of Rhode Island Red layers. International Journal of Livestock Production 2013; 4: 22-29.

18. Gnanadesigan M, Sandhanasamy I, Ponnusamy S, Lakshmanan R, Natarajan M, Rajagopal R. Quality evaluation of egg composition and productivity of layers in EM (Effective Microorganisms) treatments: a field report. Egyptian Journal of Basic and Applied Sciences 2014; 1: 161-166.

19. Jwher DMT, Abd SK, Mohammad AG. The study of using effective microorganisms (EM) on health and performance of broiler chicks. Iraqi Journal of Veterinary Sciences 2013; 27: 73-78.

20. Wondmeneh E, Getachew T, Dessie T. Effect of effective microorganisms (EM®) on the growth parameters of Fayoumi and Horro chicken. International Journal of Poultry Science 2011; 10: 185-188.

21. Wondmeneh E, Melesse A, Dessie T. Effect of effective microorganisms on growth parameters and serum cholesterol levels in broilers. Afr J Agric Res 2011; 6: 3841-3846.

22. Sokół R, Michalczyk M, Spodniewska A, Barski D. The influence of administering "effective microorganisms" to pullets on chosen haematological and biochemical blood indexes. Pol J Vet Sci 2009; 12: 519-522.

23. Gesek M, Paździor K, Otrocka-Domagała I, Rotkiewicz T, Szarek J. Fibromuscular dysplasia in arteries and in a vein in broiler chickens. Pol J Vet Sci 2013; 16: 93-99.

24. Braga III IS, Tanaka S, Itakura C, Mizutani M. Fibromuscular dysplasia in intramuscular arteries of Japanese Quail (*Coturnix coturnix japonica*). J Comp Pathol 1996; 114: 123-130.

25. Gesek M, Sokół R, Welenc J, Tylicka Z, Korzeniowska P, Kozłowska A, Wiśniewska AM, Otrocka-Domagała I. Histopathological observations of the internal organs during toltrazuril (Baycox®) treatment against naturally occurring coccidiosis in Japanese Quail. Pak Vet J 2015; 35: 479-483.

26. Tsirtsikos P, Fegeros K, Balaskas C, Kominakis A, Mountzouris KC. Dietary probiotic inclusion level modulates intestinal mucin composition and mucosal morphology in broilers. Poult Sci 2012; 91: 1860-1868.

27. Samli HE, Senkoylu N, Koc F, Kanter M, Agma A. Effects of *Enterococcus faecium* and dried whey on broiler performance, gut histomorphology and intestinal microbiota. Arch Anim Nutr 2007; 61: 42-49.