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The effects of in ovo pollen extract injection on growth parameters, ileal histomorphology and caecal microflora in fasted broiler chicks

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ABSTRACT

This study was carried out to determine the effects of in ovo pollen extract injection on growth parameters, ileal histomorphology, and caecal microflora of fasted broiler chicks. In this experiment, 2×2 factorial experimental design was used. One d old, 120 healthy broiler chicks were allocated to 4 treatment groups and 6 replicates (5 mixed sex chicks allocated each replicates). Treatment groups were: A) Pollen extract injection and 24 h fasting (P24); B) Pollen extract injection and 48 h fasting (P48), C) Control, no injection 24 h fasting (C24) and D) Control, no injection 48 h fasting (C48). The experiment lasted 21 days. Live weight, feed consumption, feed conversion ratio were recorded weekly. Ileal histomorphology, caecal microbiota, organ weight were recorded at 21th days of experiment. In ovo pollen extract injection did not affect hatchability rate. At the end of 21 days, in ovo pollen extract injection did not affect feed intake, live weight gain, feed conversion ratio, inner organ development and ileal villi width irrespective to fasting 24 h and 48 h. In ovo pollen extract injection increased ileal villi length, caecal lactic acid bacteria and *Saccharomyces Cerevisiae* count, decreased caecal *Enterobactericaea* count. In conclusion, in ovo pollen extract injection can be applied for broiler eggs to improve weight gain, better digestion and gut health.

Key words: Broiler, In ovo injection, Performance, Pollen extract.

INTRODUCTION

Time is very important factor for commercial hatchery. After hatching, when transportation of chicks for commercial production is delayed more than 48 hours, performance of chicks reduces. It has shown that chicks lose about 25% of their live weight (Bigot *et al*., 2003), with reducing growth performance, inner organ development, immune system and digestive enzyme activity (Shira *et al*., 2005) when transportation is postponed more than 48 hours. Noy and Sklan, (1998), Panda *et al*., (2006) and Kornasio *et al*., (2011) have reported that early feeding necessary after hatch to get rid of these negative effects. However, early feeding of all chicks may have not always been possible due to handling applications (drying, vaccination) after incubation, transportation and other factors. Because of these applications, most of the chicks may have exposed to fasting more than 48 hours (Kornasio *et al*., 2011). Ferket, (2006) have reported that chicks are developed in eggs by in ovo nutrient injection. Different in ovo nutrients were used to increase embryonic development such as sucrose, maltose, dextrin, β-hidroksy-β- methylbutyrate, arginine, albumin, zinc-metiyonin, L-carnitine (Uni and Ferket, 2004; Uni *et al*., 2005; Tako *et al*., 2004; Foye *et al*., 2005a, 2005b; Foye *et al*., 2003a, 2003b, 2006; Tako *et al*., 2005; Keralapurath *et al*., 2010). Other alternative nutrients must be studied to get better embryonic development. Pollen extract should be a candidate for these nutrients. Erdogan and Dodologlu, (2005) reported that pollen include all essential and non-essential amino acids as being unique source of nutrients for bees. Also, Moreira *et al*., (2008) stated that pollen contains proteins (25–30%), carbohydrates (30–55%), lipids, fatty acids and sterols (1–20%), vitamins and minerals and pollen is rich in carotenoids, flavonoids, phytosterols, polyphenols and other beneficial compounds. On the literature, it has been determined that the effects of pollen extracts on in ovo feeding are not sufficient. As well as studies about the pollen and pollen extracts addition to broiler feed on broiler performance is not sufficient. Wang *et al*., (2007) have reported that supplementation of pollen to broiler feed increased thickness and length of gastro intestinal tract and increased length of the villi in duodenum, jejunum and ileum, respectively. Wang *et al*., (2007) demonstrated that pollen promoted early development of digestive tract of broilers and suggesting that pollen may be used as feed additive for

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broilers. Attia *et al*., (2011) reported that inclusion 200 mg pollen increased New Zealand White Bucks growth performance. Also, abouda *et al*., (2011); Kačániová *et al*., (2013) and Tekeli *et al*., (2010) reported that pollen have an antimicrobial effects on pathogenic microorganisms in the gut of broilers chicks. In literature, there are studies about pollen on performance, gut histology and gut microbiota, but there is no study about in ovo injection of pollen extract on performance, gut histology and gut microbiota of broiler chicks. The aim of this study was to determine the effect of in ovo pollen extract injection to fertile broiler eggs and fasting time on growth performance, inner organ development, ileal histomorphological parameters and caecal microorganisms count of broiler chicks.

MATERIALS AND METHODS

This study was conducted at the Poultry Research Unit of Ahi Evran University, Kirsehir, Turkey. The practices and procedures for this experiment were reviewed and approved by the Ahi Evran University, Animal Ethics Committee (02.12.2014/1/9). Fertile 180 eggs were provided from a breeder flock at 48 wk of age (Ross 308). These eggs were incubated under optimal conditions. After unfertilized or with dead embryos were discarded by illumination at 12 d of incubation, fertile eggs weighed and divided into 2 equal weight eggs groups (control no injection and in ovo injection of pollen extract). At 18 d of incubation, the blunt side of the egg was sterilized with 70% ethanol. In ovo administration of pollen extract at 0.2 ml per egg was applied through air sac of the blunt side of the eggs by using a 21-gauge needle. Then, after hatching, each treatment group was divided into 2 fasting groups (24 h fasting and 48 h fasting).

After hatching, 120 day old healthy chicks were housed according to belonging treatment groups with 6 replicates and 5 chicks per replicate for 21 days. After hatch, mixed sexs 120 Ross 308 broilers chicks were transferred to three-tier cages. Battery cages were equipped with wire mesh, dropping trays, nipple drinkers and trough feeders. The battery cages were placed in an environmentally controlled room with windows. The experiment was designed in a 2 X 2 factorial design. The factors were in ovo injection (no injection as control and pollen injection) and the 24 or 48 h fasting. Treatment groups were: A) Pollen extract injection and 24 h fasting (P24), B) Pollen extract injection and 48 h fasting (P48), C) Control 24 h fasting (C24) and D) Control 48 h fasting (C48). The experiment lasted for 21 days. Chicks were fed in mash form feed and watered ad libitum with 23 h continuous illumination by fluorescence lamp per day. Birds were weighed weekly and feed intake, feed conversion ratio and weight gain was calculated. Two birds (1 female and 1 male) randomly from each replication (12 birds per treatment) were slaughtered at day 21 to determine proventriculus, gizzard, liver, gastro intestinal weight and length. Weights of edible inner organs (gizzard, heart and liver) were recorded as g/100 g body weight.

Experimental diet includes 3.080 Mcal metabolizable energy (ME) kg^{-1} and 22.39 g crude protein (CP) kg^{-1} (Table 1) and provided from commercial feed company at Kayseri in Turkey.

In the study, pollen extract provided from Ordu Apiculture Research Institute in Turkey. Pollen collected from Ordu province at May 2015, pollens is dried in dark room condition and collected in glass bottle at -18 degree in deepfreeze. Pollen structure is formed by season flowers pollens. The extraction method which was applied by Aytug *et al.* (1991) was used for preparing the extracts of collected pollen, as an extractive Coca solution and for sterilization sterile filtration technique was used. 500 mg Pollen and 4.5 ml coca solution was added in falcon tube and mixed 24 hours +4 ÚC degree in magnetic stirrer. Pollen extract centrifuged (2750 rpm) and filtered 8 times for avoid of solid residues. Coca solution is formed from NaCl (9 gr), NaHCO3 (3 gr), C6H5OH (5 gr) and distilled water (983 ml). Coca solution pH was balanced to 8.2 with a few drops of 10% NaOH (Aytug *et al.,* 1991).

Ileum samples were taken and cut into 1.5 cm pieces and placed into 10% formalin for further processing. Tissues sections were placed into tissue cassettes for dehydration

Table 1. Composition of experimental diet (%).

Ingredients	%
Maize	44.00
Soybean meal (44)	41.15
Meat and bone meal	4.00
Soybean oil	6.50
Dicalciumphosphate	2.50
L-lysine HCl	0.70
DL-methionine	0.35
Sodiumchloride	0.30
Vitamin Premix*	0.25
Mineral Piremix#	0.25
Analyzed nutrient composition	
ME [kcal/kg]	3080
Crude protein	22.39
Crude fibre	2.80
Ether extract	8.4
Met + Cys	1.20
Methionine	0.7
Lysine	1.5
Calcium	1.0
Non-phytate P	0.5

Premix provided per kg of diet: * Vitamin A, 12.000 IU; Vitamin D3, 2.400 IU; Vitamin E, 30 mg; Vitamin K3, 4 mg; Vitamin B1, 3 mg; Vitamin B2, 7 mg; Vitamin B6, 5 mg; Vitamin B12, 15 µg; niacin, 25 mg; # Fe, 80 mg; folic acid, 1 mg; pantothenic acid, 10 mg; biotin, 45 mg; Choline, 125000 mg; Cu, 5 mg; Mn, 80 mg; Zn, 60 mg; Se, 150 µg.

process and were embedded in paraffin blocks, and subsequently cut 5-µ thickness and placed on a slide. Each ileal histomorphological tissue sample was prepared and stained with hematoxylin and eosin solution by using standard paraffin-embedding methods (XU *et al.,* 2003). After embedding process villus length and villus width were photographed and evaluated by using an image processing and analysis system (ZEN 2012 SP2).

Samples of the caecal contents were collected into sterile glass tubes in which they were kept on ice until subsequent inoculation into agars. MRS agar (MERCK, Darmstadt, Germany, 1.10660) was used for enumeration of lactic acid bacteria (LAB) at 37 °C for a 3-d incubation period and malt extract agar (MERCK, Darmstadt, Germany, 1.05398) was used for enumeration of yeast at 25 °C for a 3-d incubation period. VRB (Violet Red Bile) (MERCK, Darmstadt, Germany, 1.01406) agar was used for enumeration of *Enterobacteriaceae* at 37°C for a 18 – 20 h incubation period.

Bacterial colonies were counted with determining the average number of live bacteria for per gram caecal contents. LAB, *Saccharomyces Cerevisiae* and *Entero bacteriaceae* counts of the samples were converted into logarithmic colony forming units (cfu g^{-1}).

The data were analyzed using the general linear models procedure of SPSS software (SPSS 15). Differences between groups' means were separated by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Hatchability rate after in ovo injection are given in Table 2. In ovo pollen extract injection into air sac of fertile eggs did not affect hatchability rates. Hatchability is the most crucial factor for commercial poultry production to profitability. If the hatchability rate decreases after in ovo injection, there is no commercial importance of scientific results. In our preliminary study, we applied 0.2, 0.3, 0.4 and 0.5 ml in ovo pollen extract injection into air sac and hatchability decreased about 86.67, 83.33, 76.67 and 53.33% respectively (unpublished data). In this study, in ovo pollen injection carried out 0.2 ml into air sac of fertile eggs to not reduce hatchability and to see the effect of low dose pollen extract on chicks growth, ileal histomorphology and gut microbiota. Earlier studies of in ovo feeding showed that injection volume decreases hatchability and injection volume (Zhai *et al.*, 2011), injection site (Ohta *et al.*, 2001) has important effect on hatchability rate. Ebrahimnezhad *et al.*, (2011) stated that 0.5 ml in ovo solution injection into amniotic fluid caused allergic cavity, stop breathing and embryonic death due to 0.5 ml in ovo solution increase osmotic pressure in eggs (Pedroso *et al.*, 2006).

Table 2. The effects of in ovo pollen extract injection into fertile eggs on hatchability.

	Pollen injection	Control	P value
H	%88.00	%86.67	0.77
(n)	(90)	(90)	

H: hatchability

The effect of in ovo pollen extract injection and fasting of hatching hicks for 24 and 48 h on growth parameters are given in (Table 3). At the end of the study, feed intake, live weight gain and feed conversion ratio were not affected. Although in ovo pollen injection did not affect live weight gain in 24 and 48 h fasted broiler, chicken growth at 21 days tent to increase in pollen extract injected groups compared to non-injected groups. Kornasio *et al.*, (2011) reported that in ovo nutrient injection increased broiler growth performance at 36 h fasting time after 0.6 ml in ovo solution applied into amnion. In the study, we applied 0.2 ml in ovo injection solution and chicken growth did not affect according to fasting time. This is the first record of in ovo pollen extract injection on growth performance of chickens. Similarly, number of studies about pollen supplementation to broiler feed for growth performance is not sufficient. In this studies, It has been reported that pollen supplementation to diet increase broiler growth performance (Fazayeli *et al.*, 2015; Attia *et al.*, 2014).

The effects of in ovo pollen extract injection on ileal histomorphological parameters in 24 and 48 h fasted chicks are given in (Table 5). It was determined that in ovo pollen injection increased ileal villi length in fasted 24 h broiler chickens. Villi width was not affected by in ovo pollen extract injection or fasting. Fazayeli *et al.*, (2015) have reported that dietary supplementation of pollen increased ileum villi length. Similarly Wang *et al.*, (2007) have reported that dietary pollen supplementation to broiler feed increased villi length in duodenum, jejunum and ileum respectively. Our results about ileal villi length confirmed the results of Fazayeli *et al.*, (2015) and Wang *et al.*, (2007) studies. The effects of in ovo pollen extract injection on inner organ developments are given in Table 5. Inner organ development was not affected by treatments.

Table 3. The effects of in ovo pollen extract injection on fasted 24 h and 48 h chicken's 21 d growth parameters.

						P Value		
	C24	P24	C48	P48	SEM	F	PI	F X PI
LWG (gr)	529.33	555.08	519.44	532.25	8.258	0.171	0.113	0.572
FI (gr)	872.92	868.83	861.06	859.31	5.56	0.406	0.818	0.926
FCR	1.65	1.57	1.66	1.62	0.014	0.493	0.128	0.629

FI: Feed intake. LWG: Live weight gain. FCR: Feed conversion ratio. F: Fasting. PI: Pollen injection. SEM: Standard error of means.

Table 4. The effects of in ovo pollen extract injection on fasted 24 h and 48 h chicken's inner organ development.

	C24	P24	C48	P48	SEM	P Value F	P Value PI	P Value F X PI
EIO[1]	6.26	5.96	5.99	5.77	0.12	0.343	0.385	0.855
Proventriculus[1]	0.66	0.65	0.63	0.62	0.01	0.643	0.300	0.959
Pankreas[1]	0.48	0.49	0.44	0.47	0.02	0.690	0.378	0.824
GITL[2]	21.83	21.11	23.33	21.03	0.75	0.359	0.662	0.625
GITW[1]	5.97	5.68	5.59	5.63	0.13	0.650	0.438	0.559
Bursa Fabricius	0.26	0.32	0.23	0.35	0.02	0.066	0.985	0.482

SEM: Standard error of means, EIO: Edible inner organs (Heart, Liver And Gizzard), GITL: GIT length, GITW: GIT weight, 1)- g/100g live weight, 2)- cm/100 gr live weight, F: fasting, PI: pollen injection.

Table 5: The effects of in ovo pollen extract injection on fasted 24 h and 48 h chicken's ileal histomorphological parameters caecal microbiota.

	C24	P24	C48	P48	SEM	P Value F	P Value PI	P Value F X PI
Villi Length (μ)	758.29b	900.60a	746.13b	800.46b	16.15	0.001	0.056	0.132
Villi Width (μ)	155.12	139.95	148.48	138.23	3.65	0.092	0.575	0.741
Enterobacter (10^{-6})	5.09a	4.50b	5.19a	4.34b	0.061	0.629	0.001	0.024
Yeast (10^{-6})	5.18a	6.02c	5.41b	6.04c	0.069	0.072	0.001	0.145
LAB (10^{-6})	6.64a	6.98b	6.51a	7.08b	0.054	0.890	0.001	0.187

[a-b] Means for the same treatment and effect or interaction effects with no common superscript differ significantly respectively ($P<0.05$).
LAB: Lactic acid bacteria, SEM: Standard error of means, VL: Villi length and VW: Villi width, F: fasting, PI: pollen injection.

The effects of in ovo pollen extract injection on caecal *Enterobacteriaceae*, *Saccharomyces Cerevisiae* and *Lactobacillus Spp*. count are given in Table 4. In ovo pollen injection increased *Saccharomyces Cerevisiae* and *Lactobacillus Spp*. and decreased *Enterobacteriaceae* count in 21 d chicken's caecum. We have determined that in ovo pollen extract injection into fertile eggs works like a symbiotic in gut. Because, in earlier study (Coskun *et al*., 2015), we injected symbiotic to fertile eggs and we found same effect on *Saccharomyces Cerevisiae* and *Lactobacillus Spp*. colonization in gut. Also the current results are similar to those of Abouda *et al*., (2011); Kačániová *et al*., (2013) and Tekeli *et al*., (2010). Abouda *et al*., (2011) reported that bee pollen inhibited the proliferation of some microorganisms. Kačániová *et al*., (2013) demonstrated that *Enterobacteriaceae* genera number decrease in gastro intestinal tract, *Lactobacillus spp*. and *Enterococcus spp*. counts increase when pollen increases in broiler diet. Also Tekeli *et al*., (2010) have reported that inclusion of pollen into the diet increase *lactobacillus spp*. in gastro intestinal tract. Pereira *et al*., (2007) and Estevinho *et al*., (2008) reported that antimicrobial activity pollen extracts is associated with total phenolic compounds of pollens. Also Mercan *et al*., (2007) have reported that pollens have antimicrobial effects on *E Coli* and other pathogen microorganisms and they have stated that pollens and bee honey more effective on bacteria than antibiotics.

To conclude 0.2 ml in ovo pollen extract injection into the air sac of fertile eggs at 18 d of incubation can be used to improve villi morphology, suppress pathogenic microorganisms (*Enterobactericaea*) and increase the *Saccharomyces Cerevisiae* and *Lactobacillus Spp*. in caeca of broilers for better digestion reflecting to better growth performance.

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