Influence of dietary organic acid blend supplementation and interaction with delayed feed access after hatch on broiler growth performance and intestinal health

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ABSTRACT: A trial was conducted to investigate the effects of a dietary organic acid blend for a period of 35 days on the growth performance, intestinal histomorphology and microflora of male broiler chicks with delayed access to feed. One hundred and ninety two one day old broiler chicks (ROSS 308) were randomly distributed into 4 groups housed in four replicate pens with 12 birds in each. A 2 × 2 factorial design was implemented. Four experimental groups were formed by two levels of dietary organic acid blend supplementation (Control and Fysal Dry®) and two periods of delayed feed access (0 and 36 h). At 36 h after hatching body weight and body weight change of chicks were significantly ($P < 0.001$) lower than groups fed immediately after hatching. Delayed feed access had an adverse impact ($P < 0.001$) on the body weight and feed consumption of broiler chickens on days 14 and 28. Between the days 28 and 35 of the feeding period, these differences disappeared. The relative weight of gizzard ($P < 0.05$), pancreas ($P < 0.01$) on day 6 and intestine ($P < 0.05$) on day 10, and gizzard ($P < 0.01$) on day 10 were reduced in birds subjected to delayed feed access. Dietary organic acid blend inclusion increased villus length ($P < 0.001$), whereas delayed feed access decreased villus length ($P < 0.05$) and increased the incidence of epithelial degeneration and basal membrane separation of the propria mucosa of villus in the jejenum. A significant decrease in Enterobacteriaceae count ($P < 0.01$) was noted in organic acid blend supplemented groups on day 25. Pectoral muscle malondialdehyde levels were decreased ($P < 0.01$) with dietary organic acid blend supplementation at day 10. Delayed feed access significantly increased ($P < 0.05$) the heterophil:lymphocyte ratio at day 6. Overall, dietary organic acid blend supplementation helped broiler chicks to develop a healthier intestinal microflora and this may, in turn, inhibit the delayed feed access-induced increase in malondialdehyde in the early growing period. However, the inclusion of organic acid blend to broiler diets may not be a protective management practice in preventing delayed feed access-related growth depression of broiler chickens.

Keywords: broiler; organic acid; delayed feed access; growth performance; intestinal health

In recent years, there has been an increase in the use of acidifiers as substitutes for antibiotic growth promoters because of the fears of antibiotic resistance and the implications for human health (Radcliffe 2000). Organic acids have been used in poultry diets for decades and seem to elicit a positive response in growth performance (Vogt et al. 1982; Patten and Waldroup 1988; Skinner et al. 1991). An Important objective of dietary acidification is the inhibition of intestinal bacteria competing with the host for available nutrients, and a reduction of possible toxic bacterial metabolites. In this regard, a number of studies have suggested that organic acids affect the concentration of bacteria in the ceca and small intestine (Vogt et al. 1982), and that they are bactericidal for salmonellae in the crop (Hinton and Linton 1988; Thompson and Hinton 1997).

The composition of the gut microflora plays an important role in digestion, with beneficial, negative or neutral effects (Garrido et al. 2004). Modifications to the gastrointestinal microflora which reduce pathogen attachment may have a profound effect on the structure of the intestinal wall.

For example, Uni et al. (2003) reported that stress (delayed feed access after hatch, bacteria etc.), in the digestive tract was associated with changes in intestinal morphology, such as shorter villi and deeper crypts.

In practice, hatching and transportation procedures delay the feeding of chicks by 10 to 60 hours (Noy and Sklan 1999). Delayed feeding in the first few days of life span reduces final BW (Noy and Sklan 1999), and probably affects immunological capacities (Dibner et al. 1998). Indeed, the immune system of the hatchling, particularly the mucosal immune system, requires oral feed intake for its full and rapid development. In addition, the immediate post-hatch period seems to be critical for intestinal development in chicks although fasting often occurs in practice (Uni et al. 2003).

Previous reports (Noy and Sklan 1999; Noy et al. 2001; Bigot et al. 2003; Gonzales et al. 2003; Halevy et al. 2003; Uni et al. 2003) have shown that delayed access to feed and water decreased broiler post hatch performance. However, no studies have been carried out to investigate the response of fasted broiler chickens to the inclusion of organic acid blend in corn soybean diets. It is thus necessary to understand precisely the consequences of delayed feeding and organic acid supplementation on growth performance. The other aim of the present study was to test the influence of dietary organic acid addition on microbial and morphological characteristics of the intestine as a possible intervention to inhibit the detrimental effects of fasting after hatch. In this sense, in addition to the growth performance parameters, the determination of gut characteristics (intestinal microflora composition and jejenum histomorphology) and immune responses (plasma and muscle malondialdehyde levels, heterophil:lymphocyte ratio) were the main objectives of this study.

MATERIAL AND METHODS

Birds, housing, experimental design and diets

One hundred and ninety two, one day old male ROSS 308 (Egetav Tavukçuluk San. ve Tic. A.S., Izmir, Turkey) broiler chicks were obtained from a commercial hatchery 0–4 h posthatch and transported within one hour to an experimental unit. Broiler chicks were held in transportation boxes at a temperature (32 ± 1 °C) and humidity (65–70%) controlled room to prevent dehydration before feed access. The birds were initially weighed individually so that the pens had similar initial weight distribution and were randomly assigned to four experimental groups, with four replicates of 12 chicks each. Chicks were housed in floor pens with fresh wood shavings-based litter at 8 cm deep. These four treatment groups were formed by supplementation or not of a dietary organic acid blend (OAB) (Control and Fysal Dry®) and feeding male broiler chicks at the time of arrival to the experimental unit and after 36 hours holding time at optimal environmental conditions prior to feeding (2 × 2 factorial arrangement). Broilers were fed with a corn and soybean meal-based diet (Table 1) that contained the critical nutrients recommended

Table 1. Composition and calculated analysis of experimental diets

Ingredients	Diets		
	starter (0–14 days)	grower (15–28 days)	finisher (29–35 days)
Corn, ground	53.70	56.80	58.95
Soy bean meal	39.10	36.00	33.50
Vegetable fat	3.00	3.90	4.50
Calcium carbonate	1.20	1.00	1.00
Dicalcium phosphate	1.60	1.30	1.25
Salt	0.35	0.35	0.30
DL-methionine	0.35	0.15	0.10
L-Lysine	0.10	–	–
Vitamin and mineral premix*	0.30	0.30	0.30
Organic acid blend (Fysal Dry®)**	0.30	0.20	0.10
Calculated analysis			
Metabolisable energy (kcal/kg)	3050	3150	3200
Crude protein (%)	23.5	22	21
Calcium (%)	0.96	0.80	0.78
Available phosphorus (%)	0.40	0.35	0.32

Vitamin and mineral premix include per kilogram of diet: Supplied per kilogram of diet: 11 025 IU of vitamin A, 3 528 IU of vitamin D3, 33 IU of vitamin E, 0.91 mg of vitamin K, 2 mg of thiamine, 8 mg of riboflavin, 55 mg of niacin, 18 mg of Ca pantothenate, 5 mg of vitamin B6, 0.221 mg of biotin, 1 mg of folic acid, 478 mg of choline, 28 µg of vitamin B12, 75 mg of zinc, 40 mg of iron, 64 mg of manganese, 10 mg of copper, 2 mg of iodine, and 0.3 mg of selenium

**Fysal® DRY contains: sorbic acid (0.3%), formic acid (12%), acetic acid (6.7%), lactic acid (0.5%), propionic acid (11%), ammonium formate (17%), L-ascorbic acid (< 0.1%), citric acid (0.4%), 1,2-propanediol (0.2%), silicon dioxide (31.9%)

by the ROSS 308 broiler manual up to 35 days. From 1 to 10 days of age, they received a starter diet (23.5% crude protein; 3050 kcal/kg ME, 3 kg/t OAB) from 11 to 28 days of age a grower diet (22% crude protein; 3150 kcal/kg ME, 2 kg/t OAB) and from 29 to 35 days of age a finisher diet (21% crude protein; 3200 kcal/kg ME, 1 kg/ton OAB). Fysal Dry® used in the experiment contains sorbic acid, formic acid, acetic acid, lactic acid, propionic acid, L-ascorbic acid, citric acid, ammonium formate and 1,2 propendiol. Each pen (1.3 m^2) was supplied with hanging feeders and nipple drinkers to provide *ad libitum* access to feed and water, whereas lighting was provided on a 24 h light schedule. The experiment was conducted under appropriate animal care regulations.

Sampling and measurements

Broiler chicks were weighed before and after the holding period and their body weight (BW) loss was recorded. Feed consumption (FC) and pen BWs were recorded on days 14, 28 and 35. Mortality was recorded daily. FC, BW gain, and feed efficiency (FE) were adjusted for mortality and calculated for the following growth periods: 0 to day 14, day 15 to day 28 and day 28 to day 35. The probable cause of death or reason for removal was documented.

On days 6, 10 and 25, one bird from each pen (16 birds per treatment, 48 birds) was randomly selected and euthanised by cervical dislocation to determine organ weights, intestinal histology and microflora. Intestine, breast muscle, gizzard, liver, pancreas and some immune organs such as spleen and bursa of Fabricius were sampled. The weights of these organs were expressed as grams of slaughter weight; the entire length of the intestine was measured in cm.

Following the necropsy examinations, tissue samples taken from the jejunum were fixed in 10% neutral buffered formalin, processed routinely, sectioned at 5 µm, and stained with haematoxylin and eosin (H&E). In addition, in order to calculate goblet cell number per villus (at 20× microscope objective) in the jejunum sections, periodic acid-Schiff reaction (PAS) was also used (Culling et al. 1985). For each histopathological parameter (epithelial degeneration and separation in propria mucosa of villus and hyperplasia in crypts), 10 replicate measurements were taken per bird and the average of these values was used for statistical analysis. Villus length and width were measured in at least 10 well-oriented villi at 10× microscope objective. In addition, using 40× magnifications, the crypt depths of at least five well-oriented villi were also measured and recorded.

In addition, intestinal content and tissue were collected from euthanised birds and analysed for pH, microflora and histomorphology. The carcasses were subsequently opened and the entire gastrointestinal tract was removed aseptically. The gastrointestinal tract was then divided into sections (i.e., ileum, ceca, and colon) that were ligated with light twine before separating the content from the ileum. For bacterial enumeration in digesta per bird, appropriately stored samples, frozen at –80 °C, were thawed and removed from storage bags. Intestinal contents (ileum) were then aseptically emptied into a new sterile bag and were immediately diluted 10-fold (i.e., 10% wt/vol) with sterile ice-cold anoxic PBS (0.1M; pH 7.0) and subsequently homogenized for 3 min in a stomacher (Bagmixer 100 Minimix, Interscience, Arpents, France). Each digesta homogenate was serially diluted from 10^{-1} to 10^{-7}. Dilutions were subsequently plated on duplicate selective agar media for enumeration of target bacterial groups. In particular, total aerobes, total anaerobe bacteria, *Enterobactericeae, coliforms, Lactobacillus spp*. and *Salmonella* were enumerated using nutrient agar, violet red bile glucose agar, violet red bile lactose agar, Rogosa agar and Brillant green agar according to Hartemink and Rombouts (1999). Plates were then incubated at 37 °C for 24 to 72 h aerobically and colonies were counted. Anaerobic incubation was achieved using appropriate catalysts (Anaerocult A, Merck, Darmstadt, Germany) in sealed anaerobic jars (Oxoid, Basingstoke, UK). Results were expressed as $\log_{10}$ colony-forming units per gram of digesta (Hartemink and Rombouts 1999).

Intestinal contents from ileum were collected manually for pH measurement. Intestinal pH was measured after the content was mixed (as 1/10) and homogenised with deionized water.

At days 6, 10 and 25, blood samples from each euthanised bird from every treatment were taken from the jugular vein and transferred to heparinised sterile tubes immediately. Collected blood was centrifuged (Nüve-Bench Top Centrifuge, NF 800R, Turkey) at 3000 rpm/min, for 10 min and plasma was separated. The dissected breast muscle (*m. pectoralis major*) was immediately rinsed in ice-cold phosphate-buffered saline. Tissues were

homogenised (2000 rpm/min for 1 min, 1/10 w/v) using a stirrer (IKA Overhead Stirrer; Germany) in 10% 150mM phosphate buffer (pH 7.4) in ice bath. The homogenate was centrifuged (Nuve-Bench Top Centrifuge, NF 800R, Turkey) at 6000 g for 10 min at 4 °C. The plasma and supernatants were frozen at –80 °C (Glacier Ultralow Temperature Freezer, Japan) in aliquots until used. In supernatants, total protein levels were determined by a spectrophotometer (Shimadzu UV-1601, Japan) using a commercially available kit (Archem diagnostic Ind. Co., Turkey). The results are expressed as nmol/mg protein. The plasma and tissue homogenates were used for lipid peroxidation estimation, which was done by measuring the formation of thiobarbutric acid reactive substances (TBARS) according to the method of Yoshioka et al. (1979). Absorbance was measured using a spectrophotometer at 532 nm. The concentration of malondialdehyde (MDA) was calculated by the absorbance coefficient of the MDA-TBA complex (absorbance coefficient ε = 1.56 × 105/M/cm) and is expressed as nmol/ml plasma and nmol/mg tissue protein.

Whole blood samples containing EDTA were obtained via subcutaneous venipuncture from each chicken into appropriate collection tubes for estimating the heterophil:lymphocyte ratio. Whole blood samples were smeared onto glass slides and stained with May-Grunwald-Giemsa. Briefly, the blood films were thoroughly air-dried in a staining rack and, then, were fixed in 100% methanol for 5 min. After fixation, smears were washed in tap water for 1 min and were stained with Giemsa (4% diluted deionized water) for 20 min. At the end of the staining procedure, smears were washed in slowly running tap water and were dried in upright position at room temperature. Two-hundred heterophil and lymphocyte cells were manually counted on each slide, using a light microscope at 1000× magnification. The heterophil to lymphocyte ratio was determined by dividing the number of heterophil cells by the number of lymphocyte cells.

Statistical analyses

Data were analyzed by means of the GLM procedure using Duncan's multiple range test with SAS statistical software (SAS 2003). Relative organ weight data were subjected to arc sine transformation, which showed a similar statistical trend. Differences were considered significant at $P < 0.05$ (Steel et al. 1997).

RESULTS AND DISCUSSION

Growth performance

The results for live performance are presented in Tables 2 and 3. The present trial indicated that there was a significant ($P < 0.001$) effect of DFA on BW prior to feeding. Between 0 and 36 h posthatch, chicks with DFA reduced BW by approximately 9%. Moreover, DFA and OAB interaction in BW was not significant for the first 36 h. Mean BW (day 14: 422 vs. 471g; day 28: 1485 vs. 1585), BW gain (day 14: 373 vs. 422g; day 28: 1436 vs. 1536) was significantly ($P < 0.001$) lower in birds denied feed for 36 h than in those with access to immediate feed (Table 3). Beside this, feed efficiency of chicks with delayed feed access was significantly ($P < 0.05$) increased (1.4 vs. 1.3) in the first 14 day period. Extended posthatch holding (in the hatcher) has been reported to dehydrate chicks, reduce growth performance, and depress immune response (Casteel et al. 1994). Similarly, Bigot et al. (2003) found a significant BW loss (7%) in chicks delayed access to feed for two days post-hatching. Moreover, Saki (2005) reported that BW was de-

Table 2. Body weight (g) and body weight change (%) at 0 and 36 hours of male broilers subjected to delayed feed access and dietary organic acid blend inclusion

Treatments		BW (g)		BW change (%)
DFA	OAB	0 h	36 h	0–36 h
0	0	49.04	63.07	28.65
36	0	49.13	44.55	–9.32
0	1	49.30	61.25	24.20
36	1	49.07	44.45	–9.42
SEM[1]		0.55	1.11	1.90
DFA				
0		49.17	62.16	26.42
36		49.10	44.50	–9.37
OAB				
0		49..08	53.81	9.67
1		49.19	52.85	7.39
ANOVA			*P*	
DFA		NS	***	***
OAB		NS	NS	NS
Interaction		NS	NS	NS

NS = not significant at $P > 0.05$, ***$P < 0.001$
[1]SEM = pooled standard error of the mean

Table 3. Body weight, body weight gain, feed consumption and feed efficiency of male broilers subjected to delayed feed access and dietary organic acid blend inclusion

Treatments		Body weight (g)			Body weight gain (g)			Feed consumption (g)			Feed efficiency (g:g)[1]		
		day											
DFA	OAB	14	28	35	0–14	0–28	0–35	0–14	0–28	0–35	0–14	0–28	0–35
0	0	467	1581	2196	418	1532	2147	490	2340	3524	1.4	1.5	1.6
36	0	430	1480	2045	380	1431	1996	593	2161	3435	1.3	1.5	1.7
0	1	476	1590	2145	426	1541	2096	505	2368	3434	1.4	1.5	1.6
36	1	415	1490	2134	366	1441	2084	579	2175	3374	1.3	1.5	1.6
SEM[2]		6.6	21.1	42.9	6.6	21.2	43.1	7.6	32.2	116.3	0.1	0.1	0.1
DFA													
0		471	1585	2171	422	1536	2122	587	2354	3479	1.4	1.5	1.7
36		422	1485	2089	373	1436	2040	497	2168	3405	1.3	1.5	1.6
OAB													
0		448	1531	2121	399	1481	2072	542	2250	3480	1.4	1.5	1.7
1		445	1540	2139	396	1490	2090	542	2272	3404	1.4	1.5	1.6
ANOVA		*P*											
DFA		***	***	NS	***	***	NS	***	***	NS	*	NS	NS
OAB		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Interaction		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS = not significant at $P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$
[1]Feed efficiency was calculated by dividing feed consumption (g) by BW gain (g) per pen basis
[2]SEM = pooled standard error of the mean

creased in those chicks which were denied feed compared with groups fed with a starter diet immediately after hatching. These findings are consistent with the several reports which demonstrate that delay in feed intake after hatch adversely affects the posthatch performance of chicks (Pinchasov and Noy 1993; Bigot et al. 2003; Gonzales et al. 2003). The present results for initial BW loss following hatch is mainly due to metabolism and possibly some dehydration occurring during the holding time in the incubator and post-incubator as has been previously reported by Noy et al. (2001). In previous reports (Noy and Sklan 1999), and in the current study, BW began to increase 24 to 48 h after access to feed. This finding confirms that DFA clearly causes a negative energy balance and chicks invariably lose weight.

In this trial, the BW and FC of birds was depressed due to DFA for the first 28 days but this negative difference was equalized by the end of the experiment (Table 3). However, DFA improved (1.4 vs. 1.3; $P < 0.05$) feed efficiency from 0 to 14 days of age presumably due to reduced FC. Our findings associated with overall growth performance are in agreement with the study of Gaglo-Disse et al. (2010) which showed that the BW in the late growing period was similar for groups with and without DFA. In addition, Hooshmand (2006) did not observe any difference for overall FC and FE in chicks with DFA. It is also known that BW gain in young stressed animals may elicit positive performance results (compensatory growth) after elimination of stress factors over the flock. The duration and the severity of stressors become more relevant in these conditions. Our results suggest that chicks held for 36 h were affected earlier/more by stressors and these birds had better FE than birds with access feed immediately after hatch. Overall, mortality rates (data not shown) in the present experiment were found to be similar to some previous studies (Hooshmand 2006; Kidd et al. 2007). Moreover, bird losses due to mortality and culling were at low levels, and were not affected by the feeding program or feed supplement.

Either no effects or negative effects of dietary OAB on chick growth performance were observed in the study. There were no significant interactions of DFA and OAB for growth performance.

Similarly, previous researchers (Izat et al. 1990; Waldroup et al. 1995) also reported no effect on BW of broiler chicken with the use of formate and propionic acid in the broiler diet. However, Vogt et al. (1982) and Skinner et al. (1991) found a positive influence on chick growth performance after the dietary inclusion of several organic acids at levels up to 2%. There are a number of possible causes for the differing results among these trials. One potential reason is the organic acid dose used in the diets. The results of this study indicate that there was no significant effect on growth performance with the suggested doses of OAB in the diet. Another possible reason is variations in the specific acids used, which may have different impacts on the performance of the birds. Citric acid, ascorbic acid, acetic acid, lactic acid, formic acid, propionic acid, and sorbic acid are commonly used acids in the poultry diets. Variations in feedstuffs and nutrient levels may also influence results. The presence of microbial challenges in various trials (Hinton and Linton 1988; Thompson and Hinton 1997, Fernandez-Rubio et al. 2009) may also alter results. Because organic acids have antimicrobial activity, beneficial effects may have resulted from their use when these challenges were present. Data from this study suggest that the adverse effects of DFA on growth performance cannot be alleviated by dietary OAB supplementation.

Relative weights of intestines and other organs

Changes in the weight and length of the intestine are presented in Table 4. The effects of restriction time and dietary organic acid supplementation on the length of intestines was not significant whereas the relative weight of the intestine was reduced (13.06 vs 12.29; $P < 0.05$) by holding time at day 10. The reduction in intestinal weight had disappeared at day 25. The proportions of several organs as a fraction of BW are shown in Table 5. The results indicate that holding chicks for 36 h prior to feeding reduced the relative weight (g/100 g BW) of the gizzard (9.79 vs. 9.03; $P < 0.05$) and pancreas (0.55 vs. 0.37; $P < 0.01$) at day 6, gizzard (7.94 vs. 6.99; $P < 0.01$) at day 10, whereas the weight of the bursa

Table 4. Length (cm) of the intestine, relative weight (g/100g body weight) of the intestine and pH level of intestinal contents (ileum) of male broiler chickens subjected to delayed feed access and dietary organic acid blend inclusion

Treatments		Length of intestine (cm)			Relative weight of intestine			pH	
					day				
DFA	OAB	6	10	25	6	10	25	6	10
0	0	92.25	126.80	173.23	14.73	13.38	7.51	6.83	6.32
36	0	94.73	127.20	173.95	13.47	12.14	7.68	6.62	6.22
0	1	106.20	135.45	183.05	15.67	12.74	8.18	6.90	6.53
36	1	91.48	120.38	188.80	17.52	12.43	7.84	6.49	6.41
SEM[1]		4.96	5.13	6.66	1.24	0.30	0.52	0.29	0.34
DFA									
0		99.30	131.13	178.14	15.20	13.06	7.85	6.87	6.42
36		93.10	123.79	181.38	15.49	12.29	7.76	6.55	6.31
OAB									
0		93.49	127.00	173.59	14.09	12.76	7.60	6.72	6.27
1		98.84	127.91	185.92	16.59	12.58	8.01	6.70	6.47
ANOVA					*P*				
DFA		NS	NS	NS	NS	*	NS	NS	NS
OAB		NS	NS	NS	NS	NS	NS	NS	NS
Interaction		NS	NS	NS	NS	NS	NS	NS	NS

NS = not significant at $P > 0.05$, *$P < 0.05$
[1]SEM = pooled standard error of the mean

Table 5. Relative organ weights (g/100g body weight) of male broiler chickens subjected to delayed feed access and dietary organic acid blend inclusion

Treatments		Day 6						Day 10						Day 25					
DFA	OAB	pectoral muscle	gizzard	liver	pancreas	spleen	bursa of fabricius	pectoral muscle	gizzard	liver	pancreas	spleen	bursa of fabricius	pectoral muscle	gizzard	liver	pancreas	spleen	bursa of fabricius
0	0	11.17	9.47	3.65	0.52	0.08	0.14	14.78	7.91	3.69	0.51	0.09	0.22	20.71	4.53	2.17	0.23	0.08	0.23
36	0	10.03	8.87	3.35	0.36	0.09	0.22	14.50	7.08	3.43	0.50	0.08	0.26	23.66	4.46	2.33	0.30	0.08	0.17
0	1	10.82	10.12	3.80	0.58	0.11	0.19	14.17	7.96	3.98	0.47	0.09	0.19	19.18	4.26	2.31	0.30	0.08	0.19
36	1	9.67	9.18	3.71	0.38	0.09	0.20	15.01	6.89	3.68	0.46	0.07	0.24	20.47	4.14	2.44	0.32	0.10	0.19
SEM[1]		0.53	0.35	0.18	0.05	0.01	0.03	0.48	0.26	0.16	0.04	0.01	0.02	1.87	0.005	0.18	0.03	0.02	0.03
DFA																			
0		11.00	9.79	3.72	0.55	0.09	0.16	14.47	7.94	3.83	0.49	0.09	0.21	19.94	4.39	2.24	0.30	0.08	0.21
36		9.85	9.03	3.53	0.37	0.09	0.21	14.75	6.99	3.56	0.48	0.08	0.25	22.07	4.30	2.39	0.27	0.09	0.18
OAB																			
0		10.60	9.17	3.50	0.44	0.09	0.18	14.64	7.49	3.56	0.50	0.08	0.24	22.19	4.49	2.25	0.26	0.08	0.20
1		10.25	9.65	3.75	0.48	0.10	0.20	14.59	7.43	3.83	0.46	0.08	0.22	19.82	4.20	2.38	0.31	0.90	0.19
ANOVA											*P*								
DFA		NS	*	NS	**	NS	NS	NS	**	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
OAB		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Interaction		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS = not significant at $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$
[1]SEM = pooled standard error of the mean

of Fabricius was significantly increased (0.21 vs. 0.25; $P < 0.05$) at day 10. Delayed feeding after hatch also tended to diminish (11.0 vs. 9.85, not significant at $P < 0.05$) the relative weight of the pectoralis muscle at six days of age (Table 5). As it is known, the first days of life of broiler chickens are a critical stage of development with regard to feeding factors. Several research papers focus on the effect of DFA in broiler production because of its significant effects on muscle and organ development (Noy and Sklan 1999; Noy et al. 2001; Uni et al. 2003). Moreover, some of the important metabolic pathways prior to hatch are also described in a recent review (De Oliveira et al. 2008) which emphasizes the liver, pectoral muscle, hatching muscle and intestine, as most affected by changes toward hatch. In the present study, the relative growth rates differed in the different organs; the relative growth of the gizzard and pancreas depressed more rapidly and dramatically than the other organs. It is clear from the present data that compensatory growth was observed during the last two weeks of trial to compensate for the retardation in body, intestine and organ weight gains caused by the fasting period. Results for the relative weight of intestine (day 10) and pectoral muscle (day 6) were relatively similar with the previous studies of Bigot et al. (2003) and Moore et al. (2005) who reported that posthatching starvation impaired intestinal growth, retarded pectoral muscle weight gain and that weight increase occurred only after chicks had access to feed.

Neither the relative weight of organs (gizzard, liver, pancreas, spleen, bursa of Fabricius) nor the pectoralis muscle evaluated in this study was affected by the addition of OAB to broiler diets. There are a limited number of studies evaluating the effects of organic acids on relative organ weights. Wang et al. (2010) also did not observe any significant effects on relative weights of gizzard, liver, spleen, bursa of Fabricius and breast muscle by dietary organic acid supplementation.

Intestinal histomorphometry

Addition of OAB to the diet affected the histological properties of the jejunum at days 6 and 10

Table 6. Villus length (µm-10×), villus width (µm-10×), crypt depth (µm-40×), crypt count (40×) and goblet cell count (40×) measured in the jejenum of broiler chickens subjected to delayed feed access and organic acid blend inclusion

Treatments		Day 6					Day 10					Day 25				
DFA	OAB	villus length	villus width	crypt depth	crypt count	goblet cell count	villus length	villus width	crypt depth	crypt count	goblet cell count	villus length	villus width	crypt depth	crypt count	goblet cell count
0	0	676.35	102.28	91.08	4.00	59.50	835.43	110.20	82.83	2.75	60.75	883.68	103.08	81.15	4.0	73.75
36	0	699.78	115.70	105.25	3.00	55.00	835.85	90.05	86.48	3.50	54.25	907.08	152.90	96.15	3.5	69.00
0	1	776.98	104.35	82.55	2.50	74.00	1073.15	98.10	70.43	3.25	69.75	1026.90	130.28	121.20	3.5	65.00
36	1	735.33	105.28	82.73	2.50	53.50	926.68	109.65	84.38	3.00	59.50	893.73	110.68	111.08	3.5	74.25
[1]SEM		77.59	8.21	13.41	0.29	4.11	30.44	8.13	2.27	0.31	0.88	89.17	11.58	16.03	0.25	3.47
DFA																
0		726.66	103.31	93.99	3.25	66.75	954.29	104.15	85.43	3.00	65.25	955.29	116.68	101.18	3.75	71.63
36		717.55	110.49	86.81	2.75	54.25	881.26	99.85	76.63	3.25	56.88	900.40	131.79	103.61	3.50	69.38
OAB																
0		688.06	108.99	98.16	3.50	57.25	835.64	100.13	77.40	3.13	57.50	895.38	127.99	88.65	3.75	69.63
1		756.15	104.81	82.64	2.50	63.75	999.91	103.88	84.65	3.13	64.63	960.31	120.48	116.14	3.50	71.38
ANOVA									*P*							
DFA		NS	NS	NS	NS	*	*	NS	**	NS	***	NS	NS	NS	NS	NS
OAB		NS	NS	NS	**	NS	***	NS	**	NS	***	NS	NS	NS	NS	NS
Interaction		NS	NS	NS	NS	NS	*	NS	*	NS	NS	NS	*	NS	NS	NS

NS = not significant at $P > 0.05$, *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
[1]SEM = pooled standard error of the mean

(Table 6). The villus length (1000 vs. 836 µm-10×; $P < 0.001$) was significantly higher in birds on the OAB diet compared to those in birds fed on the control diet whereas the crypt depth (85 vs. 77 µm-40×; $P < 0.01$) on day 10 and number of crypts (3.5 vs. 2.5; $P < 0.01$) on day 6 were reduced. Moreover, the interaction between delayed feed access and organic acid blend treatments for villus length ($P < 0.05$) and crypt depth ($P < 0.05$) was significant at day 10 (Figures 1A and 1B). In addition, OAB inclusion to broiler diets significantly ($P < 0.01$) reduced (65 vs. 58%) the number of goblet cells per 100 villus cells at day 10 (Figure 2A). The reductions in the number of goblet cells in the jejenum villi also occurred in birds with delayed feed access (Table 6). DFA decreased the number of goblet cells per area on day 6 (67 vs. 54; $P < 0.05$) and day 10 (65 vs. 57; $P < 0.01$) significantly. In addition to this, villus length ($P < 0.05$) and crypt depth were significantly ($P < 0.01$) decreased in birds exposed to DFA on day 10. Nor DFA neither OAB supplementation changed the histological characteristics of the jejunum at day 25, but the interaction of delayed feed access and organic acid blend treatments was significant for villus width on day 25 (Figure 1C). Moreover, histopathological observations indicate that birds with delayed feed access had an increased incidence of epithelial degeneration and basal membrane separation (Figure 2B) on day 6, an increased incidence of hyperplasia in crypts and epithelial degeneration on day 10 (Figure 2C), and increased incidence of basal membrane separation on d 25. Beside this, OAB supplementation also had significant effects on jejunum mucosa. The incidence of epithelial degeneration on days 6, 10 and 25, and basal membrane separation on day 10 and 25 was increased significantly by dietary OAB supplementation (data not shown).

It is hypothesised that the increase in beneficial microbial activity resulting from dietary OAB supplementation may influence gut morphology and consequently affect gut maturation. In the present study, inclusion of OAB in the diet markedly affected the arrangement of the villi and increased

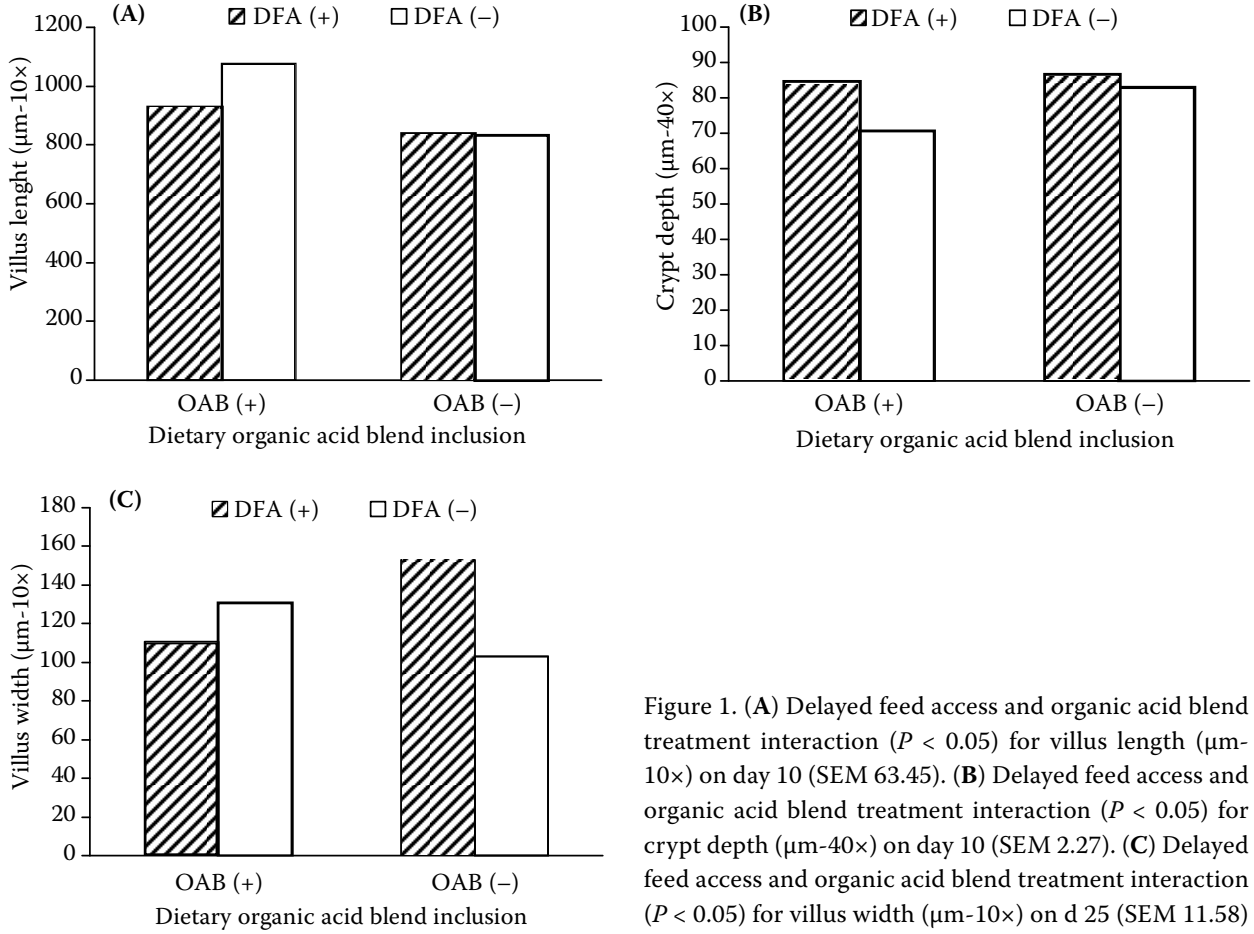

Figure 1. (**A**) Delayed feed access and organic acid blend treatment interaction ($P < 0.05$) for villus length (µm-10×) on day 10 (SEM 63.45). (**B**) Delayed feed access and organic acid blend treatment interaction ($P < 0.05$) for crypt depth (µm-40×) on day 10 (SEM 2.27). (**C**) Delayed feed access and organic acid blend treatment interaction ($P < 0.05$) for villus width (µm-10×) on d 25 (SEM 11.58)

their length, thereby enhancing the absorptive area in the early growing period. These observations are in broad agreement with several reports which suggest that dietary organic acid supplementation increases villus length in chicks (Sun et al. 2005; Gunal et al. 2006; Samik et al. 2007). Previously, Pelicano et al. (2005) reported that supplementation of dietary probiotics in the gut augmented growth and stability of specific bacteria which produce organic acids and this, in turn, might increase the height of the villi. The latter conclusion corresponds with the findings of the present study as exposure to DFA decreased the number of goblet cells per 100 villus cells. Goblet cells are responsible for the secretion of mucin that is used for the mucinous lining of the intestinal epithelium (Schneeman 1982). Thus, a decreased number of goblet cells may result in a reduction in the secretion of mucin. Changes in mucin content or in the composition of the mucosal surface may also decrease nutrient absorption and increase the energy requirement for gut maintenance (Uni et al. 2003). Delayed access to feed after hatch has also been reported to cause a reduction in crypt size and villus length, particularly in the jejunum as has been previously reported (Geyra et al. 2001). This finding was accompanied by a decrease in the number of goblet cells in the jejunum villi. Intestinal growth correlated with the number of cells in the crypts, the number of cells along the villus and the segment surface area (Geyra et al. 2001). In previous reports, rats, pigs, mouse, and chickens (Langhout et al. 1999; Sharma et al. 1997) have shown alterations in the number of goblet cells due to changes in diets or because of malnutrition. Similar results were obtained by Dunsford et al. (1991) who showed, in pigs, that early weaning altered the numbers of goblet cells as examined by AB and PAS staining. The development of the goblet cells in the broiler occurs in the late embryonic and immediate posthatch period (Uni et al. 2003). Goblet cells exert protective and transport functions through the formation of the mucus layer, and their number is influenced by the time of access to feed. Moreover, a decrease in the number of goblet cells may inhibit immune functions resulting in decreased resistance to stressors.

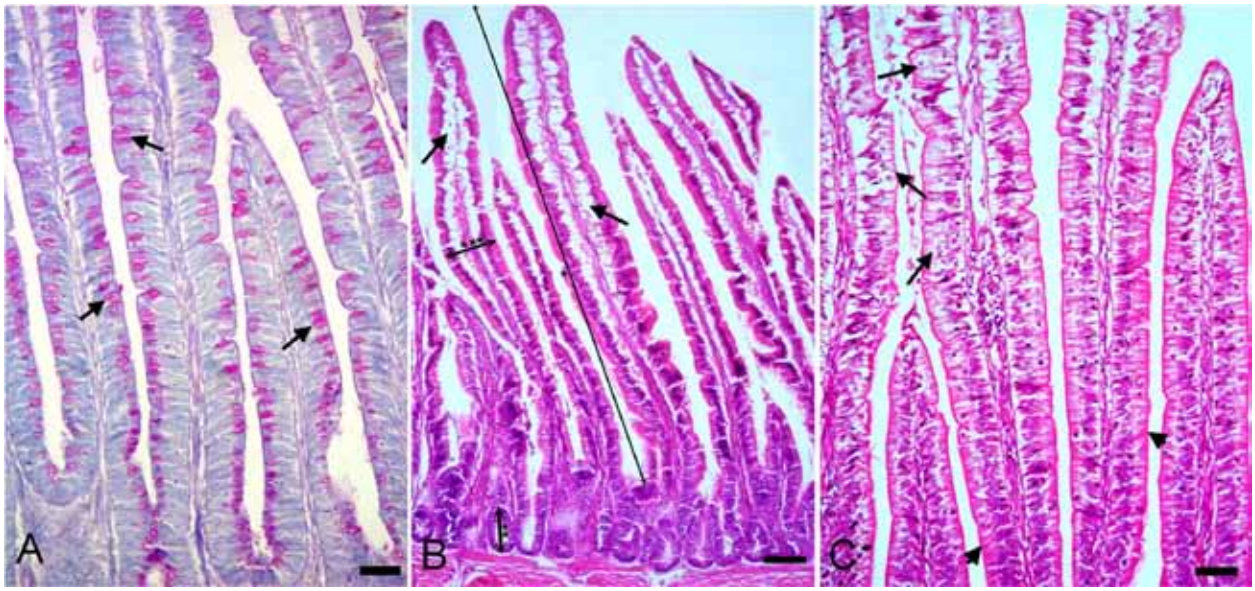

Figure 2. (**A**) Appearance of the goblet cells (arrows) in the villus on day 10; PAS, bar 50 µm. (**B**) Separations in the propria mucosa on day 6 (arrows, *villus lenght, **crypt depth, ***villus width); HE, bar 100 µm. (**C**) Degeneration in the epithelium of the villus (arrows) and appearance of the normal epithelium (arrowheads) on day 10; HE, bar 50 µm

Histological findings suggest that dietary OAB supplementation to broiler diets may help to prevent the detrimental effects of DFA on absorptive surface area in the small intestine, but this improvement did not affect the overall performance of the broiler chickens in the present study.

Intestinal microbial population

The population of total bacteria, total anaerobic bacteria, coliform bacteria, *Enterobacteriaceae* and lactobacilli in the digesta content of the small intestine is presented in Table 7. Dietary supplementation with OAB reduced (4.30 vs. 3.40 $\log_{10}$ cfu/g; $P < 0.01$) the *Enterobacteriaceae* count at 25 days of age. Moreover, the interaction between delayed feed access and organic acid blend was significant with respect to the *Enterobacteriaceae* count ($P < 0.05$) at day 25 (Figure 3). The feed sample analysis showed that dietary OAB supplementation did not influence total bacteria, total anaerobic bacteria, coliform bacteria, *Enterobacteriaceae* or lactobacilli count compared with the diet had no organic acid supplementation (data not shown). Salmonella was absent from the feed and intestinal content samples. There were no significant effects of post-hatch holding time on microbial populations of broiler chickens in the current study. The inhibitory effect of dietary organic acid on microbial flora colonisation was reported previously. Alp et al. (1999) reported a reduced *Enterobacteriaceae* count in the ileum of broilers in response to the separate or combined inclusion of an organic acid blend containing lactic, fumaric, propionic, citric, and formic acid.

Of note, lactobacilli are capable of growing at a relatively lower pH and thus are more resistant to changes in gut milieu induced by dietary acidification (Russell and Diez-Gonzalez 1998). It is in-

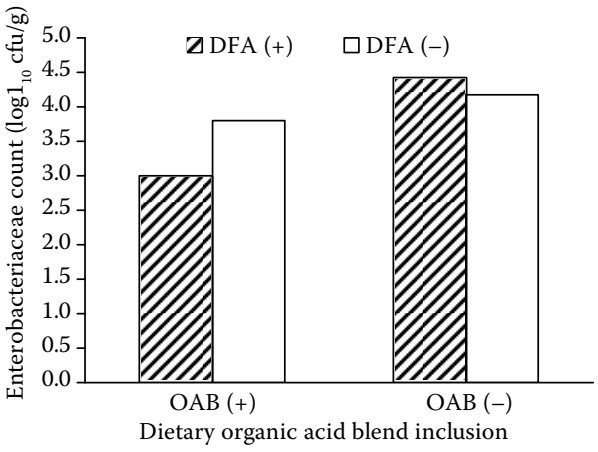

Figure 3. Delayed feed access and organic acid blend treatment interaction ($P < 0.01$) for plasma malondialdehyde level (nmol/ml) on day 10 (SEM 1.13)

Table 7. Microbiological analysis of intestinal contents (ileum) of male broiler chickens subjected to delayed feed access and dietary organic acid blend inclusion at days 6, 10 and 25

Treatments		Total bacteria			Total anaerobe bacteria			Coliform bacteria			Enterobacteriaceae			Lactobacilli		
								$\log_{10}$ cfu/g								
DFA	OAB	6	10	25	6	10	25	6	10	25	6	10	25	6	10	25
								day								
0	0	8.00	8.44	7.47	7.65	7.64	6.57	5.34	4.30	5.63	6.26	4.87	4.18	5.19	5.08	5.05
36	0	7.95	8.35	6.65	7.43	7.76	7.24	5.51	4.15	5.16	5.65	4.93	4.43	4.94	4.95	5.19
0	1	8.21	7.15	7.80	7.71	7.65	7.71	5.44	4.00	5.43	5.95	4.30	3.80	5.40	5.58	6.07
36	1	7.81	8.22	7.23	7.62	7.83	6.84	4.79	4.00	4.92	5.66	4.75	3.00	5.71	5.63	5.39
SEM[1]		0.37	0.54	0.35	0.25	0.64	0.43	0.44	0.21	0.21	0.55	0.92	0.22	0.65	0.65	0.50
DFA																
0		8.10	7.79	7.63	7.68	7.65	7.14	5.39	4.15	5.53	6.11	4.59	3.99	5.30	5.33	5.56
36		7.88	8.28	6.94	7.52	7.79	7.04	5.15	4.08	5.04	5.65	4.84	3.72	5.32	5.29	5.29
OAB																
0		7.97	8.39	7.06	7.54	7.70	6.91	5.42	4.23	5.40	5.96	4.90	4.30	5.06	5.02	5.12
1		8.01	7.68	7.51	7.66	7.74	7.28	5.12	4.00	5.18	5.80	4.52	3.40	5.56	5.60	5.73
ANOVA									*P*							
DFA		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
OAB		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS
Interaction		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS

NS = not significant at $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$
[1]SEM = pooled standard error of the mean

triguing to that in the present trial the Lactobacilli count in digesta collected from OAB-treated birds was numerically higher than that of the un-treated birds, although the difference was not significant. It has been reported that the antimicrobial activity of organic acids is restricted to the crop and gizzard and any drastic changes in pH in the distal part of the small intestine due to supplementation of organic acid are unlikely (Thompson and Hinton 1997). Supplementation with OAB did not alter the pH of the ileal content significantly which corroborated the above hypothesis (Table 4). This is probably because of the strong buffering action of the poultry gastrointestinal tract. This result supports the previous findings reported by Izat et al. 1990 and Hernandez et al. 2006, indicative of an antimicrobial action of the OAB in the upper alimentary tract. Therefore, the antimicrobial action of OAB in the gizzard might have reduced the number of the *Enterobacteriaceae* and increased the numbers of *Lactobacillus* in the small intestine as a compensatory mechanism. Moreover, the magnitude of the antimicrobial effects of organic acids varies from one acid to another and is also dependent on concentration and pH (Chaveerach et al. 2002). These properties may explain the inconsistent results reported from studies in which chicks are exposed to organic acids.

Biochemical and haematological variables

With regard to plasma biochemical and haematological variables, it was observed that muscle MDA levels decreased (19.04 vs. 11.11 nmol/mg protein; $P < 0.01$) at day 10 when broiler chickens were supplemented with dietary OAB during the growing period (Table 8). Moreover, delay to feed access significantly increased (1.32 vs. 0.43; $P < 0.05$) the heterophil to lymphocyte ratio on day 10, whereas muscle MDA concentration tended to increase at 6 days of age ($P > 0.05$). Dietary OAB supplementation did not have any effect on muscle and plasma MDA levels, or the heterophil to lymphocyte ratio. Moreover, OAB numerically reduced the plasma heterophil counts, causing a

Table 8. Heterophil/lymphocyte ratio, malondialdehyde levels in muscle (nmol/mg protein) and plasma (nmol/ml) of male broilers subjected to delayed feed access and dietary organic acid blend inclusion

Treatments		Heterophil/lymphocyte		Malondialdehyde (muscle)			Malondialdehyde (plasma)		
		day							
DFA	OAB	6	10	6	10	25	6	10	25
0	0	0.33	0.38	13.33	21.22	56.94	15.13	14.94	8.39
36	0	1.92	0.55	12.76	16.85	46.24	14.63	11.37	11.15
0	1	0.53	0.40	12.52	10.93	39.19	12.75	11.51	8.46
36	1	0.72	0.45	19.56	11.30	60.87	11.86	15.29	10.02
SEM[1]		0.32	0.12	5.15	2.43	11.41	1.78	1.13	1.83
DFA									
0		0.43	0.39	12.92	16.08	48.06	13.94	13.22	8.42
36		1.32	0.50	16.16	14.07	53.56	13.24	13.33	10.59
OAB									
0		1.12	0.46	13.04	19.04	51.59	14.88	13.15	9.77
1		0.63	0.42	16.04	11.11	50.03	12.30	13.40	9.24
ANOVA					*P*				
DFA		*	NS	NS	NS	NS	NS	NS	NS
OAB		NS	NS	NS	**	NS	NS	NS	NS
Interaction		0.048	NS	NS	NS	NS	NS	**	NS

NS = not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$
[1]SEM = pooled standard error of the mean

decrease in the heterophil:lymphocyte ratio at day 6 (Table 8). Besides this, the delayed feed access and organic acid blend inclusion interaction with respect to plasma MDA levels (nmol/ml) was significant ($P < 0.01$) at day 10 (Figure 4). An elevated heterophil to lymphocyte ratio is considered as an indicator of stress (Gross and Siegel, 1983); therefore, the results reflect that the chicks experienced stress, which was also evident from their early BW and organ growth retardation that differed from birds with access to feed and water immediately after hatch. Our results also reveal that OAB supplementation in the diets of young chicks may help to alleviate the inhibitory effects of DFA on immune functions. However, the mechanism by which organic acids affect the immune responses is not well elucidated. Therefore, further research is needed to investigate the effects of different organic acids on intestinal microflora, intestinal morphology and the immunity of broilers exposed to stress factors.

Based on the results of the present study, it can be stated that organic acids added to the diets of young chicks alter the microbial population of the small intestine and inhibit the DFA-induced increase in MDA levels in the early growing period. However, the addition of OAB to broiler diets may not be a protective feeding practice in preventing DFA-related growth depression of broiler chickens.

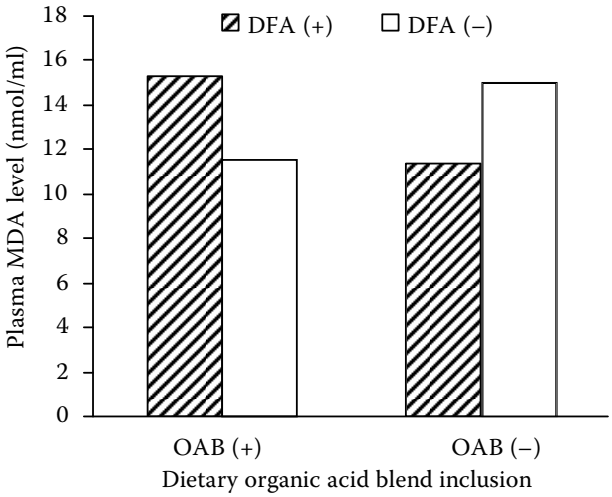

Figure 4. Delayed feed access and organic acid blend treatment interaction ($P < 0.05$) for Enterobacteriaceae count ($\log_{10}$ cfu/g) on day 25 (SEM 0.22)

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