

## Effects of organic acid supplementation on antioxidant capacity and immune responses of broilers challenged orally with *Salmonella enterica* subsp. *enterica* Typhimurium

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(Received 22 June 2014; Accepted 18 September 2014; First published online 13 November 2014)

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### Abstract

Four commercial organic acids and a reference antibiotic, Neoxyval, were administered to commercial broilers to evaluate the efficacy of these products during pre- and post-challenge with *Salmonella enterica* subsp. *enterica* Typhimurium (*S. Typhimurium*) on selected indicators of their antioxidant status and immune responses in serum. Two hundred and eight broilers received one of the following seven treatments: positive control group (CTL+); negative control (CTL-); antibiotic Neoxyval, (NEOX); Gallimix (GALI); FormaXOL (FORMA); Fysal (FYS); and Selko-pH in drinking water (SELK). The chicks in treatments T2 to T7 were challenged with ( $3 \times 10^9$  cfu/mL) of *S. Typhimurium* on day 16. Each treatment consisted of 10 cage replicates with four birds per cage for a starter (0 - 14 days of age) and a finisher (15 - 42 days of age) period. The results revealed that the total antioxidant capacity (TAC) value was highest when birds were supplemented with organic acids, with the highest value for FORMA (0.70 mM), which was not different from those of the FYS (0.67 mM), SELK (0.66 mM) and GALI (0.64 mM) treatments. Birds receiving treatment, CTL-, produced the highest H<sub>2</sub>O<sub>2</sub> value (75.9 µM), while, FYS, FORMA and SELK lowered H<sub>2</sub>O<sub>2</sub> concentration significantly (51.1, 53.8 and 55.0 µM, respectively). Anti-*Salmonella* titre was significantly improved by dietary organic acid supplementation. The highest *Salmonella* titre was recorded for birds that received FORMA (1.54) as compared with all other treatments. Week of sampling showed a significant effect on *Salmonella* and Newcastle disease titres. In summary, the tested organic acids, especially FORMA, had a positive influence on TAC and *Salmonella* titres and reduced the H<sub>2</sub>O<sub>2</sub> concentration in serum.

**Keywords:** Broilers, immune responses, organic acids, oxidative stress, total antioxidant capacity

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### Introduction

Organic acids are among the alternative growth promoters that have been used in practice for decades and have been shown to stimulate growth performance in poultry (Dibner & Winter, 2002; Dibner, 2004). Several studies have suggested that organic acid supplementation to poultry diets acts as a bactericidal for *Salmonella* in the crop (Thompson & Hinton 1997), ceca and small intestine (Vogt *et al.*, 1982) and ileum (Cengiz *et al.*, 2012), thus providing better intestinal health for the bird to obtain maximum nutrient absorption. Organic acid supplementation to poultry diets has been shown to suppress the growth of certain species of bacteria, mainly acid-intolerant species such as *Salmonella*, *E. coli*, *Clostridium perfringens*, *Listeria monocytogenes* and *Campylobacter* (Van Immerseel *et al.*, 2002). Several other reports have shown that organic acids might be helpful in improving immunity and gut health in broilers (García *et al.*, 2007; Abdel-Fattah *et al.*, 2008; Khodambashi *et al.*, 2013).

Organic acids have been shown to lower the pH in the animal intestines and as a result, disturbed bacterial growth. The non-ionized (undissociated) organic acids can infiltrate the lipid membrane of the bacterial cell wall and interrupt the normal physiology of certain types of bacteria by disrupting DNA and protein synthesis in the bacterium (Nursey, 1997; Ricke, 2003).

Limited studies have been conducted to determine the effect of organic acids on antioxidant status and immune responses of broilers during pre- and post-challenge with *Salmonella enterica* subsp. *enterica* Typhimurium (*S. Typhimurium*). Therefore, in the present study, the objective was to evaluate the effect of

four commercial organic acids on antioxidant status and immune responses of broiler chickens that were subjected to a *S. Typhimurium* challenge.

### Materials and Methods

Two hundred and eighty Cobb 500 broiler chicks were randomly assigned to seven treatments. Each treatment included 10 replicates, with four chicks per replicate. The birds were allocated randomly to the seven treatments: T1 = control group, unmedicated diet, unchallenged birds (CTL+); T2 = unmedicated diet + *S. Typhimurium* (CTL-); T3 = medicated diet with Neoxyval (0.05 g/kg diet) + *S. Typhimurium* (NEOX); T4 = Gallimix (1 g/kg diet) + *S. Typhimurium* (GALI); T5 = FormaXOL (1 g/kg diet) + *S. Typhimurium* (FORMA); T6 = Fysal (2.5 g/kg feed) + *S. Typhimurium* (FYS); and T7 = Selko-pH (1 mL/kg drinking water) + *S. Typhimurium* (SELK).

**Table 1** Dietary ingredients and chemical composition of starter diets

Ingredients (%)	T1 <sup>§</sup> *	T3 <sup>‡</sup>	T4 <sup>‡</sup>	T5 <sup>‡</sup>	T6 <sup>‡</sup>
Maize	63.01	63.01	62.91	62.91	62.76
Soybean meal	31.15	31.15	31.15	31.15	31.15
Palm oil	1.72	1.72	1.72	1.72	1.72
Dicalcium phosphate	1.96	1.96	1.96	1.96	1.96
Limestone	0.73	0.73	0.73	0.73	0.73
Salt	0.25	0.25	0.25	0.25	0.25
VM Mix <sup>1</sup>	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.25	0.25	0.25	0.25	0.25
Lysine-HCL	0.18	0.18	0.18	0.18	0.18
Threonine	0.07	0.07	0.07	0.07	0.07
Sodium bicarbonate	0.12	0.12	0.12	0.12	0.12
Choline chloride	0.05	0.05	0.05	0.05	0.05
Neoxyval		0.005			
Gallimix			0.1		
FormaXOL				0.1	
Fysal					0.25
Total	100	100	100	100	100
<b>Chemical composition</b>					
ME, MJ/kg	12.6				
Crude protein, g/kg	205				
Methionine, %	0.55				
Lysine, %	1.2				
Sulphur amino acids, %	0.89				
Threonine, %	0.85				
Calcium, %	0.95				
Phosphorus, %	0.41				

<sup>1</sup>Vitamin-mineral premix contains in the following per kg: 2400000 IU vitamin A; 1000000 IU vitamin D; 16000 IU vitamin E; 800 mg vitamin K; 600 mg vitamin B<sub>1</sub>; 1600 mg vitamin B<sub>2</sub>; 1000 mg vitamin B<sub>6</sub>; 6 mg vitamin B<sub>12</sub>; 8000 mg niacin; 400 mg folic acid; 3000 mg pantothenic acid; 40 mg biotin; 3000 mg antioxidant; 80 mg cobalt; 2000 mg copper; 400 mg iodine; 1200 mg iron; 18000 mg manganese; 60 mg selenium, 14000 mg zinc.

<sup>§</sup> Same diet was fed to T2 and T7.

<sup>\*</sup> T1 = control group, unmedicated diet, unchallenged birds, (+CONT); T2 = unmedicated diet + bacterial challenge (CTL-); T3 = medicated diet with Neoxyval (0.05 g/kg diet) + bacterial challenge (NEOX); T4 = Gallimix (1 g/kg diet) + bacterial challenge (GALI); T5 = FormaXOL (1 g/kg diet) + bacterial challenge (FORMA); T6 = Fysal (2.5 g/kg feed) + bacterial challenge (FYS); T7 = Selko-pH (1 mL/kg drinking water) + bacterial challenge (SELK).

The experiment was conducted on broilers from the age of 1 to 42 days. Feed and water were provided *ad libitum*, and the birds were maintained in a 24-hour light schedule. The chicks had been vaccinated for Marek's disease, Newcastle disease and Infectious bronchitis. A two-phase regimen, starter and finisher diets, were formulated in mashed form, which met or exceeded the recommendations in commercial practice in Saudi Arabia. The composition of the diets is presented in Tables 1 and 2.

**Table 2** Dietary ingredients and chemical composition of finisher diets

Ingredients (%)	T1 <sup>§</sup>	T3	T4	T5	T6
Maize	70.09	70.09	69.99	69.99	69.84
Soybean meal	23.08	23.08	23.08	23.08	23.08
Palm oil	2.98	2.98	2.98	2.98	2.98
Dicalcium phosphate	1.87	1.87	1.87	1.87	1.78
Limestone	0.59	0.59	0.59	0.59	0.59
Salt	0.25	0.25	0.25	0.25	0.25
VM Mix <sup>1</sup>	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.25	0.25	0.25	0.25	0.25
Lysine-HCL	0.17	0.17	0.17	0.17	0.17
Threonine	0.07	0.07	0.07	0.07	0.07
Sodium bicarbonate	0.11	0.11	0.11	0.11	0.11
Choline chloride	0.04	0.04	0.04	0.04	0.04
Neoxyval		0.005			
Gallimix			0.1		
FormaXOL				0.1	
Fysal					0.25
Total	100	100	100	100	100
<b>Chemical composition</b>					
ME, MJ/kg	13.2				
Crude protein, g/kg	173				
Methionine, %	0.51				
Lysine, %	0.98				
Sulphur amino acids, %	0.80				
Threonine, %	0.73				
Calcium, %	0.85				
Phosphorus, %	0.38				

<sup>§</sup> Same diet was fed to T2 and T7; <sup>1</sup> Same as in starter diet (Table 1).

Neoxyval® (Sogeval Laboratories, France) was used as the reference antibiotic and was compared to four organic acids (Gallimix®, FormaXOL™, Fysal® and Selko®-pH). Gallimix® (Intermedicavet) is an association of protected organic acids and essential oils. FormaXOL™ (Kemin Industries, Iowa, USA) is an antimicrobial compound composed of organic acids and essential oils. Fysal® (Selko® Feed Additives) is a blend of organic acids and their ammonium salts. Selko®-pH is a blend of free organic acids, which was included in the drinking water during the trial period.

At 16 days of age, chicks in treatments T2 to T5 were challenged with *S. Typhimurium* ( $3 \times 10^9$  CFU/mL). The strain used in this experiment was *S. Typhimurium* ATCC13311. Chicks were gavaged with 1 mL of a cocktail containing  $3 \times 10^9$  CFU/mL *S. Typhimurium*, which was obtained commercially (Microbiologics, Cloud, Minn., USA), according to the procedure described by Abudabos *et al.* (2014).

Serum was used to estimate total antioxidant capacity (TAC), superoxide dismutase (SOD, EC 1.15.1.1) activity and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentration, using analytical kits (Cell Biolabs, Inc., San

Diego, Calif., USA). For this purpose, blood samples were collected on day 42 from seven selected chicks from each treatment via brachial venipuncture into plain tubes. The blood was centrifuged at 5 °C and 3000 rpm for 10 min. Serum was harvested and then transferred into Eppendorf tubes and stored at -80 °C until the time of analysis.

The immune responses to *Salmonella* antibody were determined by the enzyme-linked immunosorbent assays (ELISA) (BioChek, London, UK). Blood samples were collected from seven selected chicks from each treatment on days 7 (before bacterial challenge), 21, 28 and 35 (after the challenge), and serum was harvested. For the chicken *S. Typhimurium* antibody test, microtitre plates were used that had been pre-coated with inactivated *S. Typhimurium* antigen. Serum samples were diluted (1 : 100) with a diluent reagent and were added to the microtitre wells where any anti-*S. Typhimurium* antibodies present will bind and form an antigen-antibody complex. Anti-chicken IgG labelled with the enzyme, alkaline phosphatase, was added to the wells. Then the substrate was added in the form of pNPP chromogen, and absorbance was measured at 405 nm with a microtitre plate reader.

The Newcastle disease virus antibody test kit (IDEXX Laboratories, Inc., Westbrook, Maine, USA) was used to detect antibodies to Newcastle disease. Serum samples were diluted (1 : 500) with a diluent reagent and 100 µL of samples were added to the microtitre wells. Wells were incubated for 30 minutes at 18 - 26 °C, and then washed with 350 µL distilled water. Then 100 µL of conjugate were dispensed into each well, after incubation for 30 minutes at 18 - 26 °C, 100 µL of TMB substrate solution were dispensed into each well and 100 µL of a stop solution were dispensed to stop the reaction. Absorbance was measured at 650 nm.

At days 41, 42 and 43, three birds were selected per treatment/day, totalling nine birds per treatment, for measurement of organ weight. After euthanasia, feathers, head and shanks were removed and the remaining carcass was weighed. The liver, bursa of Fabricius, spleen and thymus were collected and weighed. The weight of each part was calculated as a percentage of dressed carcass weight.

Data were analysed using the general linear model procedure of SAS (2002-2003). Seven treatments were arranged in 10 replications in a randomized complete block design. Means for measurements showing significant differences in the analysis of variance were tested using the PDIFF option. The overall level for statistical significance was set at  $P < 0.05$ . All values were expressed as statistical means  $\pm$  standard error of the mean (SEM).

## Results and Discussion

*Salmonella* Typhimurium is an enteric bacterium that can colonize chickens, and is a serovar that is known to cause salmonellosis in broiler chickens (Ribeiro *et al.*, 2007; Van Immerseel *et al.*, 2009). Growth performance parameters in broilers may be regarded as indication of the severity of *S. Typhimurium* infection. Body weight gain was found to decrease from 14% to 33%, and feed conversion ratio to increase by 5% to 20% (Chalghoumi *et al.*, 2009; Abudabos *et al.*, 2014).

Oxidative stress is a physiological condition in which there is an imbalance between concentrations of reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> and antioxidants. Excessive accumulation of ROS will lead to cellular injury, such as damaged DNA, proteins and lipid membranes. It is accepted that changes in various antioxidant enzyme activities can be used to estimate the level of oxidative stress and total antioxidant status. In this trial, serum from birds was used to estimate TAC, SOD activity and H<sub>2</sub>O<sub>2</sub> concentrations with ELISA kits and the results are presented in Table 3. Under normal physiological conditions, cellular ROS generation is counterbalanced by the action of cellular antioxidant enzymes and redox molecules. The TAC value was highest ( $P < 0.001$ ) for birds that received FORMA (0.7 mM) and this value was not significantly different from birds that received FYS, SELK or GALI (0.67, 0.66 and 0.64 mM, respectively). The lowest value was recorded in birds that received treatment CTL+ (0.54 mM), which was not different to that of NEOX (0.61 mM). According to Erel (2004), TAC reflects precisely the antioxidative status of the organism. On the other hand, SOD activity was not affected by treatment ( $P > 0.05$ ). Superoxide dismutase catalyzes the dismutation of the superoxide anion into H<sub>2</sub>O<sub>2</sub> and molecular oxygen is considered one of the most antioxidative enzymes.

The H<sub>2</sub>O<sub>2</sub> concentration was highest ( $P < 0.001$ ) for birds that received treatment CTL- (75.9 µM) and this value was not significantly different from that of birds that had received treatments CTL+, NEOX or GALI, (70.5, 68.4 and 65.4 µM, respectively). The lowest value was obtained from birds that received FYS, FORMA and SELK (51.1, 53.8 and 55.0 µM, respectively). Hydrogen peroxide is one of the best-documented ROS produced under oxidative stress conditions, which is a toxic product of normal aerobic metabolism and pathogenic ROS production involving oxidase and SOD reactions.

The results for antibody responses against *Salmonella* are presented in Table 4. There was a significant treatment x week interaction ( $P < 0.001$ ). The *Salmonella* titre continued to increase from week 1 through to week 5 for birds that received treatments CTL-, NEOX, FYS and SELK. For treatment CTL+, the

**Table 3** Antioxidant capacity and oxidative stress in serum of broiler chickens fed the experimental diets

Treatments	TAC	SOD	H <sub>2</sub> O <sub>2</sub>
	mM	μL	μM
Control	0.54 <sup>c</sup>	0.16	70.5 <sup>a</sup>
Negative control	0.63 <sup>b</sup>	0.16	75.9 <sup>a</sup>
Neoxyval	0.61 <sup>bc</sup>	0.31	68.4 <sup>ab</sup>
Gallimix	0.64 <sup>ab</sup>	0.21	65.4 <sup>abc</sup>
FormaXOL	0.70 <sup>a</sup>	0.40	53.8 <sup>cd</sup>
Fysal	0.67 <sup>ab</sup>	0.20	51.1 <sup>d</sup>
Selko-pH	0.66 <sup>ab</sup>	0.21	55.0 <sup>bcd</sup>
SEM	± 0.026	0.05	5.12
<i>P</i> value	**	ns	**

Each mean is based on seven birds.

<sup>abcd</sup> Means in the column with different superscripts differ significantly;

\*\**P* < 0.01.

TAC: total antioxidant capacity; SOD: super oxide dismutase; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide.

**Table 4** *Salmonella* titre in serum of broiler chickens fed the experimental diets

Treatment	<i>Salmonella</i> titre	
Control	1.10 <sup>b</sup>	
Negative control	1.10 <sup>b</sup>	
Neoxyval	1.17 <sup>b</sup>	
Gallimix	0.91 <sup>b</sup>	
FormaXOL	1.54 <sup>a</sup>	
Fysal	1.18 <sup>b</sup>	
Selko-pH	1.00 <sup>b</sup>	
SEM	0.11	
<i>P</i> value	**	
<b>Week</b>	<b>Challenge</b>	
1	Before	
		0.435 <sup>d</sup>
3	After	0.863 <sup>c</sup>
4	After	1.227 <sup>b</sup>
5	After	2.080 <sup>a</sup>
SEM		0.08
<i>P</i> value		***

For treatment, each mean is based on 28 birds. For week, each treatment is based on 49 birds.

<sup>abcd</sup> Means in the column with different superscripts differ significantly;

\*\**P* < 0.01; \*\*\**P* < 0.001.

titre increased from week 1 to week 3, then decreased in week 4. For GALLI, the titre increased until week 4, then decreased in week 5. For FORMA, the titre decreased from week 3 compared with week 1, then it

increased in weeks 4 and 5. Treatment, on the other hand, had a significant effect on *Salmonella* titre: The highest titre was obtained from birds that received the organic acid, FORMA ( $P < 0.001$ ). Beneficial effects of organic acids supplementation on broilers immunity have been reported (Abdel-Fattah *et al.*, 2008; Khodambashi *et al.*, 2013). It was suggested that improvement in bird immunity could be related to the inhibitory effects of organic acids on gut system pathogens (Khodambashi *et al.*, 2013). On the other hand, week showed a significant effect on *Salmonella* titre: Week 5 had the highest value (2.08) and week 1 had the lowest value (0.435) ( $P < 0.001$ ).

The Newcastle disease titre results are shown in Table 5. Treatment showed no effect on Newcastle disease titre. However, there was a strong effect for the week ( $P < 0.001$ ): The titre was high in the first week before the challenge, then dropped significantly after the challenge in week 3 (2.78 vs. 1.90). In weeks 4 and 5, it increased to a level higher than week 3, but less than in week 1. The Newcastle disease titre values were 2.78, 1.90, 2.44, and 2.40 for weeks 1, 3, 4 and 5, respectively. Lessard *et al.* (1995) found that *S. Typhimurium*-infected birds had higher antibody titres in response to Newcastle disease vaccination compared with uninfected birds. Houshmand *et al.* (2012) concluded that the dietary addition of organic acids significantly increased the antibody titres against Newcastle disease compared with the control group.

**Table 5** Newcastle titre in serum of broiler chickens fed experimental diets

Treatment		Newcastle titre
Control		2.40
Negative control		2.28
Neoxyval		2.29
Gallimix		2.54
FormaXOL		2.42
Fysal		2.32
Selko-pH		2.43
SEM		0.11
<i>P</i> value		ns
Week	Challenge	
1	Before	2.78 <sup>a</sup>
3	After	1.90 <sup>c</sup>
4	After	2.44 <sup>b</sup>
5	After	2.40 <sup>b</sup>
SEM		
<i>P</i> value		***

For treatment, each mean is based on 28 birds. For week, each treatment is based on 49 birds.

<sup>abc</sup> Means in the column with different superscripts differ significantly;

\*\*\* $P < 0.001$ .

The mean weight of organs as a percentage of carcass weight is documented in Table 6. Treatment had no effect ( $P > 0.05$ ) on weight of the liver, bursa of Fabricius, spleen or thymus relative to dressed carcass weight. Conversely, Abdel-Fattah *et al.* (2008) concluded that organic acid supplementation to broilers resulted in heavier immune organs (bursa of Fabricius and thymus).

## Conclusions

In summary, organic acids used in this trial (especially FormaXOL) increased the total antioxidant capacity, improved the immunological responses of broiler chicks, especially for *Salmonella*, and reduced oxidative stress conditions in broilers by lowering the concentration of  $H_2O_2$  in the serum. *S. Typhimurium*-challenged birds (CTL) had the highest oxidative stress. Organic acids were able to eliminate the negative effects of the *S. Typhimurium* challenge and improve the immune system.

**Table 6** Effect of treatments on organ weight relative to dressed carcass weight

Treatment	Liver	Bursa*	Spleen	Thymus
	%	%	%	%
Control	2.91	0.22	0.132	0.537
Negative control	2.62	0.19	0.129	0.587
Neoxyval	2.91	0.18	0.131	0.541
Gallimix	3.00	0.20	0.172	0.646
FormaXOL	2.79	0.21	0.136	0.663
Fysal	2.81	0.20	0.135	0.594
Selko-pH	2.84	0.19	0.146	0.487
SEM	±	0.155	0.019	0.056
<i>P</i> value	ns	ns	ns	ns

ns: not significant.

\* Bursa: bursa of Fabricius.

### Acknowledgements

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for funding this research through Research Group Project No. RGP-VPP-273.

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