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RESEARCH ARTICLE

Efficacy of Organic Acids and Kanamycin as Feed Additives in Improving the Immune Status of Broiler chickens Infected with *Salmonella* Typhimurium

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Abstract

This investigation was performed to demonstrate the effect of continuous feeding of organic acids and antibiotic supplemented diets on some immunological and biochemical parameters in healthy and *Salmonella* Typhimurium experimentally infected broiler Chickens. One hundred and twenty-one day old Ross 308 chicks were divided into six equal groups. Group 1 negative control. Group 2: infected broilers with 0.5 mL of $1x10^8$ CFU/mL *S.* Typhimurium per os as a positive control. Group 3: infected chickens and treated intramuscularly with 0.1 mL / kg body weight kanamycin for 5 successive days. Group 4 administered with propionic acid by a dose of 2 kg/ ton diet then infected with *S.* Typhimurium. Group 5: treated with formic acid by 1 mL/ L drinking water then infected with *S.* Typhimurium. Group 6: administered with propionic acid by a dose of 2 kg/ ton diet + 1 mL formic acid / L drinking water then infected with *S.* Typhimurium. The results revealed that group 2 infected by *Salmonella* showed loss of appetite, depression, and diarrhea, with mortality rate up to 20%. Group 3 showed mild clinical signs with 15% mortality rate. Groups treated with organic acid showed mild clinical signs with 10% mortality rate. *S.* Typhimurium infected chickens (G2) revealed leukocytosis, heterophilia, monocytosis, significant increase in uric acid, creatinine, liver enzymes activities, IgA and IgG and significant decrease in albumin and total antioxidant capacity (TAOC). While treatment of *Salmonella* infection with kanamycin, organic acids alone or combination of propionic acid and formic acid resulted in a positive effect in treatment, a significant improvement in some immunological and biochemical parameters and ameliorating the severity of infection. In conclusion, the combination of organic acids revealed the best results as it returned leukogram, biochemical and immunological parameters nearly to their normal levels and decreased bacterial colonization in poultry.

Keywords: *Salmonella* Typhimurium, Propionic acid, Formic acid, Kanamycin, Broilers

Introduction

Salmonella Typhimurium is enteric bacteria colonizes the chicken`s intestine causing salmonellosis [1]. Addition of an organic acid in diet can control the most common enteric bacterial diseases as *Salmonella*, campylobacter and *Escherichia coli* that affect the intestine of poultry [2, 3]. The first symptoms of salmonellosis is growth performance reduction due to the decrease feed intake, which caused by mucosal damage and diarrhea [4, 5].

Since long period, the antibiotics are used as feed additives in animal and poultry due to their growth-promoting effects and perfect

therapeutic efficiency [6]. Furthermore, the heavy use of antibiotics caused residues in food animals and bacterial resistant to drugs [7]. Kanamycin is an aminoglycoside antibiotic; it is bactericidal *in vitro* against both of Gram-negative and Gram-positive bacteria causing inhibition to protein synthesis of the bacterium, which essential for its growth [8].

Because of the adverse effect of antibiotics on the human health, there has been a great move towards the use of organic acids as alternative to antibiotics in the diet as growth promoters. Organic acids and their salts when used in poultry diets and drinking water improve the growth performance. By means of dietary acidification that made inhibition of pathogenic bacteria competing with the host for available nutrients, and decrease harmful bacterial metabolites resulting in the improvement of the performance of birds and enhancing its immunity [9].

Feed supplemented with organic acids causes damaging the bacterial cytoplasmic membrane thus disrupting metabolic and replication functions of pathogenic bacteria, such as the *Salmonellae enterica* species [10]. Propionic acid (PA) was recorded to control fungi and bacteria in stored grains and hay, because of its fungicide and bactericide effects. Formic and propionic acids are particularly effective and used as food preservation [11].

European Union (EU) documents verified PA as a good grain preserver and effective in limiting *Salmonella*. Recently, it is used as a feed additive in poultry because these are generally considered safe, so it is allowed in poultry production [12]. High bacteriostatic property of PA is due to its pH reduction activity both in feed and gastrointestinal tract through the action on microflora. The use of mixture of formic and propionic acid, was effective against *Salmonella* without effect on performance [13, 14]. Formic acid considered an effective antimicrobial, which limit *Salmonella* species. Once, it is consumed with feed or through adding in drinking water in poultry [15].

Therefore, the objective of this study was to demonstrate the effects of continuous feeding of organic acids and antibiotic supplemented diets on some immunological and biochemical parameters in healthy and *Salmonella* Typhimurium experimentally infected broiler chickens.

Material and methods

Bacterial strain

S. Typhimurium standard strain (ATCC14028) was purchased from serology department, animal health research institute, Doki, Giza to be used for experimental infection

Antibiotic susceptibility test

The antibiotic susceptibility was executed for the obtained strain by Kirby Bauer disc diffusion procedure [16] using the following antimicrobial agents: amoxicillin (AX), gentamicin (CN), kanamycin (K), ciprofloxacin (CIP), norofloxacin (NOR), nalidixic acid (NA) and erythromycin (E). The inhibition zones` diameters were interpreted according to the criteria published by Clinical and Laboratory Standards Institute guidelines **[**17].

Drugs

Propionic acid 99% and Formic acid 85% were purchased from Mix acid® Nanjing Weite Veterinary Co., Ltd, China. Kanamycin 25%: It was obtained from Alfasan, Holand.

Experimental design

One hundred and twenty one-day-old broilers (Ross 308) from local hatchery were divided to 6 groups, each group contained 20 chicks. The chicks were vaccinated at the 7^{th} and 17^{th} days against Newcastle virus (Intervet Boxmeer Company, Boxmeer, Netherlands); and at 11^{th} and 22^{th} days against Gumboro in drinking water using Holland Gumboro vaccine (Rhone-Merieau Company, France). The experiment was done from one day to the 35^{th} day. The chicks were reared on floor rearing. The temperature was adjusted according to chick's age; these chicks were subjected to drinking water and feed ad-libitum according to NRC [18]. Addition of propionic acid and formic acid from day one to

the end of the experiment was performed. Kanamycin was used after infection for 5 successive days. At day 14 of age, all birds except the control -ve group (G 1) were infected by an oral dose of 1 x10^8 CFU of *S.* Typhimurium [19]. The beginning of collecting samples was at 21 days (1st period) and 35 days (2nd period) from beginning of the experiment.

Experimental groups

G1: It was kept as control without any treatment (control-ve), and provided with balanced ration.

G2 (control positive): It was infected with *S.* Typhimurium by a dose of 0.5 mL of 1 x10^8 CFU of *S.* Typhimurium at day 14 of age and was provided with basal diet without treatment.

G3: It was infected with *S.* Typhimurium and treated with kanamycin 0.1 mL I/M injection for 5 successive days.

G4: It was administered with propionic acid by a dose of 2 kg/ ton diet [20], and then infected with *S.* Typhimurium.

G5: It was administered with formic acid by 1 mL/ L in drinking water then infected with *S.* Typhimurium.

G6: It was administered with propionic acid by a dose of 2kg/ ton diet+ formic acid (1 ml/ L in drinking water) then infected with *S.* Typhimurium.

Samples

Blood samples were collected at day 21 and 35 day from beginning of the experiment. First blood sample was taken from 5 birds with anticoagulant (EDTA) to determine the total leukocytic count and its differential [21]. Second blood sample was taken from 5 birds without anticoagulant for serum separation to measure some biochemical and immunological parameters.

Biochemical studies

Test kits were used for colorimetric estimation of the following parameters using spectrophotometer. The liver transferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) activities were determined [22]. Serum uric acid was determined [23] and the serum creatinine was estimated [24]. Serum total protein was measured according to a previously published protocol [25]. Serum immunoglobulin (IgG, and IgA) were estimated using commercial ELISA kits (Kamiya Biomedical Company, USA) [26]. Total antioxidant capacity (TAC) was measured as previously described [27].

Bacteriological examination

Salmonella Typhimurium was re-isolated from experimentally infected chickens at age of 21 and 35 days (5 chickens in each group showing signs were selected to be sacrificed for salmonella re-isolation). Surface of target organs (liver and colon) was seared by hot spatula and a sterile loop was deeply introduced in the affected organ and cultured to brain heart infusion broth and incubated at 37°C/24h then a loopful was streaked to different isolation media according to international organization standardization (ISO 6549) [28].

Genotypic identification of S. Typhimurium strain ATCC14028

Bacterial DNA was extracted from obtained isolates by using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH, Catalogue no.51304). PCR amplification was performed in Step One™ Real-Time PCR System (Thermo FISHER, USA) in a final volume of 20 µL consisting of 2 µL of extracted DNA as template added to 18 µL of master mix (iQ™ SYBR Green Supermix; Bio-Rad, USA). The master mix contains 10 µl of iQ™ SYBR Green Super mix (2) add to 2 µL of forward and reverse primers and 6 mL of deionized water. Real-Time PCR evaluation assay was carried out after Josefsen *et al.* [30]. The cycling protocol used as following: one cycle of 95 C for 5 min, 40 cycles of 95 C for 10 sec and 60 C for 30 sec. The primers used are *Salmonella* 16S rRNA (Target gene) by nucleotide Sequence **(**forward CAG AAG AAG CAC CGG CTA ACT C, and reverse GCG CTT TAC GCC CAG TAA TT) [29].

Statistical analysis

The obtained data were statistically analyzed by one-way ANOVA (F-test) by

Duncan, using SPSS (Statistical package for Social Sciences) version 14 released on 2006 [31].

Results

This study was performed to demonstrate the effect of continuous feeding of organic acids and antibiotic supplemented diets on some immunological and biochemical parameters in healthy and in *Salmonella* Typhimurium experimentally infected broilers. The clinical signs, which observed in infected group with *Salmonella Typhimurium* (G2) loss of appetite, depression, diarrhea, fever and septicaemia in some cases with the mortality rate 20%**.** While group 3 infected and treated with kanamycin showed mild signs with mortality rate of 15 %. In addition, groups infected and treated with propionic acid and formic acid and its combination (G4, G5 and G 6, respectively) revealed mild clinical signs with mortality rate 10%. Table (1) showed significant increase in total and differential leukocytes in G2 infected by *S.* Typhimurium at the 2 period of the experiment compared to the control, while in G4 that infected and treated with propionic acid, there was significant increase in leukocytes in the 1st period, but in 2nd there was non-significant increase. In group 5 (infected then treated with formic acid) there was significant increase in 1st period in TLC, hetrophils, lymphocytes, and monocytes, where as non-significant increase of these parameters was observed in 2nd period. G3 (infected and treated with kanamycin) showed significant increase in total leukocytes, heterophils, lymphocyts and monocyts in the 1st and 2 nd period of the experiment compared to the control. The best improvement in leukocytes in g6 (infected +propionic+ formic acid) at the all period of the experiment.

In Table (2), our results evoked significant increase in total protein in G3, 4, 5 and 6 at all the period of the experiment while non-significant in total protein in G2 at 1 st and 2nd period. In G2 (infected), there was significant decrease in albumin, and albumin/ globulin ratio. In the other groups there were significant increase in total globulin in G4,5,6 all over the experiment but in G3, 4, 5 and 6 in 1st and 2nd period of the experiment the albumin/ globulin ratio showed significant decrease. Creatinine was significant increase in G2 (infected) in the 2 period of experiment and G3 during 2nd period as well as G4 and 5 in 1st period, while other groups evoked non-significant increase in 2nd period of the experiment compared to the control and the best improvement in G 6. Uric acid revealed significant increase in G 2, 3& 4 in 1st period and non-significant increase in G3, 4, and 5 in 2nd period of the experiment compared to control. The group 6 showed non-significant increase all over the period of the experiment. Table (3) evoked significant increase in ALT and AST at G 2 (infected by *S.* Typhimurium), while other groups showed non-significant increase all over the experiment. Total antioxidant capacity (TAOC) revealed significant increase in G4 and 6 in the 2nd period of the experiment. However, G3 and 5 evoked significant increase in 2nd period of the experiment, but G2 showed non-significant decrease. The immunoglobulin IgG and IgA showed significant increase in all groups all over the experiment compared to the control. The best result was present in G6 (treated with propionic acid + formic acid).

Table (1) Effect of different dietary treatments on total and differential leucocytic count in broiler chickens at 21 and 35 days of age (mean ±SE) n=5

Group		Wbcs ($x10^3$/ mm^3)	Heterophils ($x10^3$/ mm^3)	Lymphocytes ($x10^3$/ mm^3)	Monocytes ($x10^3$/mm^3)	Eosinophils ($x10^3$/ mm^3)	Basophils ($x10^3$/ mm^3)
Control –ve G1	**1ST**	8.88± 0.36 ^{d}	3.36±0.09 ^{d}	5.25±0.26^{c}	0.22±0.01^{d}	0.02±0.01^{c}	0.03±0.003^{abc}
	2nd	9.06±0.17^{d}	3.44±0.05^{b}	5.36±0.14^{c}	0.22±0.01^{c}	0.02±0.001^{c}	0.02±0.003^{b}
G2 infected	**1ST**	28.45±1.76^{a}	6.77±0.09^{a}	20.45±1.56^{a}	1.12±0.09^{a}	0.06±.01^{a}	0.04±0.006^{ab}
	2nd	22.16±0.86^{a}	6.09±0.38^{a}	14.31±0.95^{a}	0.96±0.07^{a}	0.04±0.05^{a}	0.04±0.003^{a}
G3 infected +kanaamycin	**1ST**	12.52±0.72^{c}	4.85±0.17^{c}	7.19±0.55^{c}	0.39±.02^{c}	0.03±0.001^{c}	0.02±0.006 ^{bc}
	2nd	12.77±0.93^{b}	5.27±0.49^{a}	6.95±0.43^{b}	0.45±0.04^{b}	0.03±0.003^{c}	0.02±0.003^{b}
G4 propionic acid + infected	**1ST**	19.20±0.42^{b}	5.69±0.26^{b}	12.86±0.18^{b}	0.61±.01^{b}	0.03±0.001^{c}	0.03±0.003^{b}
	2nd	10.58±0.62^{cd}	4.09±0.23^{b}	6.16±0.29^{c}	0.33±0.04^{bc}	0.02±0.005^{c}	0.02±0.00^{b}
G5 formic acid+ infected	**1st**	11.79±0.41^{c}	4.54±0.41^{c}	6.89±0.02^{c}	0.32±0.02^{cd}	0.02±0.01^{c}	0.01±0.003^{c}
	2nd	11.52±0.55^{bc}	3.96±0.35^{b}	7.08±0.37^{b}	0.43±0.05^{b}	0.03±0.003^{c}	0.02±0.003^{b}
G6 propionic acid+ formic acid + infected	**1ST**	10.0±0.42^{c}	4.29±0.34^{c}	5.37±0.12^{c}	0.28±0.01^{cd}	0.02±0.01^{c}	0.02±0.00 ^{bc}
	2nd	9.07±0.31^{d}	3.58±0.31^{b}	5.16±0.03^{c}	0.27±0.03^{c}	0.02±0.003^{c}	0.02±0.003^{b}

1st means 21 day; 2nd means 35 days

Table (2): Effect of different dietary treatments on some biochemical parameters of liver and kidney in broiler chickens at 21 and 35 days of age (mean ±SE) n=5

Groups Parameters		Total protein g/dl	Albumin g/dl	Globulin g/dl	A/G ratio	Creatinine mg/dl	Uric acid mg/dl
Control –ve G1	**1st**	3.53±0.04^{b}	2.03±0.09^{a}	1.51±0.05 ^{c}	1.34±0.11^{a}	0.45±0.031^{c}	9.31±0.65 ^{b}
	2nd	3.48±0.11^{b}	2.05±0.11^{a}	1.43±0.11 ^{b}	1.43±0.15^{a}	0.43±0.035^{c}	8.83±0.44 ^{b}
G2 infected	**1st**	3.42±0.17^{b}	1.50±0.12^{c}	1. 92±0.23 ^{a}	0.78±0.12^{c}	0.65±0.102^{a}	10.73±0.86 ^{a}
	2nd	3.45±0.16^{b}	1.60±0.12^{b}	1.85± 0.24 ^{b}	0.89±0.31^{c}	0.74±0.09^{a}	12.70±0.91 ^{a}
G3 infected +kanaamycin	**1st**	3.99±0.31^{a}	2.11±0.24^{a}	1.88±0.07 ^{b}	1.12±0.09^{b}	0.50±0.043^{b}	9.77±0.77^{a}
	2nd	4.40±0.21^{a}	2.37±0.12^{a}	2.01±0.12 ^{a}	1.18±0.04^{b}	0.46±0.05^{c}	8.89±0.74 ^{b}
G4 propionic acid + infected	**1st**	3.99±0.31^{a}	2.11±0.24^{a}	1.88±0.07 ^{b}	1.12±0.09^{b}	0.50±0.043^{b}	9.77±0.77^{a}
	2nd	4.40±0.21^{a}	2.37±0.12^{a}	2.01±0.12 ^{a}	1.18±0.04^{b}	0.46±0.05^{c}	8.89±0.74 ^{b}
G5 formic acid+ infected	**1st**	3.94±0.15^{a}	2.01±0.23^{a}	1.93±0.15 ^{a}	1.04±0.16^{ab}	0.51±0.024^{b}	9.40±0. 36 ^{b}
	2nd	4.20±0.16^{a}	2.02±0.16^{a}	2.18 ±0.04 ^{a}	0.93±015^{c}	0.48±0.07^{c}	9.34±0.59 ^{b}
G6 propionic acid+ formic acid +infected	**1st**	3.96±0.28^{a}	2.04±0.29^{a}	1.92±0.02 ^{a}	1.06±0.086 ^{b}	0.49±0.072^{c}	9.71±0.79 ^{b}
	2nd	4.46±0.53^{a}	2.34±0.38^{a}	2.30±0.17^{a}	1.02±0.34^{b}	0.47±0.04b^{c}	8.26±0.57^{b}

1st means 21 day; 2nd means 35 days

Results are expressed as mean ±S.E.M.

A/G ratio= albumin and globulin ratio

Table (3): Effect of different dietary treatments on some liver enzymes and total antioxidant capacity and immunoglobulin of broiler chickens at 21 and 35 days of age (mean±SE) n=5

Groups / Parameters		ALT U/L	AST U/L	TAOC Mmol/dl	IgG mg/100ml	IgA mg/100ml
Control –ve	1st	13.56±0.48^{b}	194.13±3.29^{c}	2.40±0.29^{bc}	230.8±113.7^{c}	203.33±35.17^{d}
G1	2nd	13.53±0.55^{b}	180.0±5.77^{bc}	2.16±0.21^{c}	343.3±8.82^{b}	253.33±8.82^{d}
G2 infected	1st	42.30± 6.49^{a}	562.30±86.34^{a}	1.53±0.41^{bc}	298.0±107.76^{b}	222.33±13.86^{c}
	2nd	48.13±2.17^{a}	343.33±14.53^{a}	1.23±0.31^{c}	360.0±5.77^{a}	270.0±5.77^{c}
G3 infected +kanaamycin	1st	17.49±0.69^{b}	194.70±4.80^{c}	3.38±0.23^{b}	263.67±27.14^{b}	236.0±52.45^{c}
	2nd	15.35±2.83^{b}	217.27±17.14^{b}	3.05±0.60^{b}	353.67±54.27^{a}	292.33±55.36^{b}
G4 propionic acid + infected	1st	17.57±1.50^{b}	200.30±23.05^{c}	3.71±0.61^{a}	345.33±99.17^{a}	335.33±34.73^{b}
	2nd	14.77±1.64^{b}	218.0±12.77^{b}	4.85±0.52^{a}	480.67±84.95^{a}	366.0±44.2^{a}
G5 formic acid+ infected	1st	16.22±2.91^{b}	196.83±16.27^{c}	3.35±0.74^{b}	319.67±64.74^{a}	343.67±35.59^{a}
	2nd	14.81±1.04^{b}	197.93±17.80^{c}	4.31±0.67^{a}	474.0±99.58^{a}	356.0±23.00^{a}
G6 propionic acid+ formic acid + infected	1st	14.89±0.56^{b}	199.43± 11.72^{c}	4.95±0.41^{a}	385.33±124.24^{a}	281.0 ±27.20^{b}
	2nd	13.36±1.23^{b}	195.77±5.67^{c}	5.8±0.38b^{a}	492.67±112.76^{a}	376. 0±059.74^{a}

1st means 21 day; 2nd means 35 days
ALT = alanine transferase, AST =Aspartate transferase, TAOC = Total antioxidant capacity, IgG = immunoglobulin, IgA = immunoglobulin A

Bacteriological isolation

The results of isolation and identification have revealed that *Salmonella* was re-isolated from examined tissue specimen as follow: (5 birds from each group were positive).

At day 21 : no bacteria was isolated from group 1 (0/5), 5 isolate from G2 (5/5) ,4 isolates from G3(4/5), 3 isolates from G4 (3/5), 3 isolates from G5(3/5) and 2 isolates from G6 (2/5).

At day 35, re-isolation of salmonella was not detected in G1 (0/5), 5 isolates from G2 (5/5), 3 isolates from G 3 (3/5), 2 isolates from G4 (2/5), 2 isolates from G5 (2/5) and 1 isolate from G6 (1/5).

The recovered isolates grows well on MacConkey`s agar producing pale colonies. On XLD media, it produced red colony with black center and identified by other biochemical tests according to ISO 6549.

The results of antibiotic sensitivity for *S.* Typhimurium isolate by disc diffusion method revealed that it was resistant to NA, AX and E (+ve), whereas the isolate exhibited moderate sensitivity to NOR and CIP (++ve). Meanwhile, it was more susceptible to K and CN (+++ve); thus Kanamycin was antibiotic of choice used in experimental design.

Genotypic identification

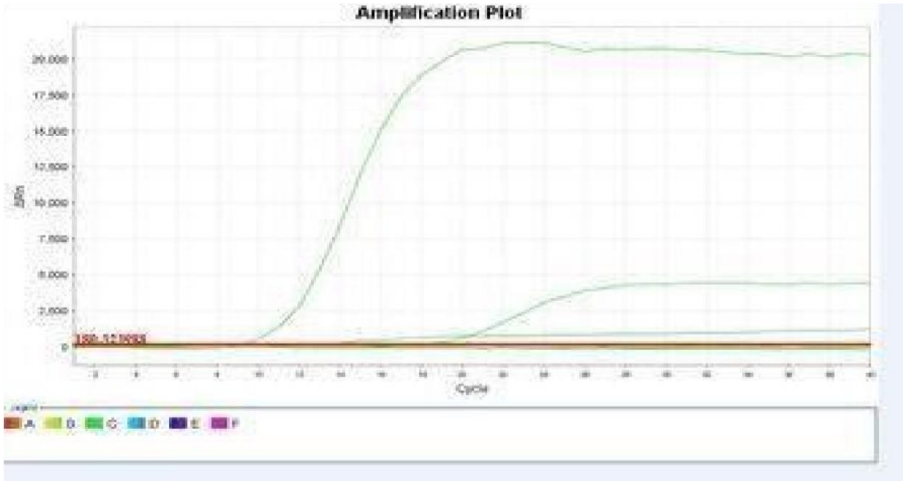

Figure1: Detection of *Salmonella* isolates by real time PCR

According to the results, 3 isolates were selected at day 35 to be confirmed by real time PCR from groups G2, G3 and G6. SYBR Green real-time PCR amplification plot revealed positive amplification for *16SRNA* gene of *Salmonella* in 3 isolates. Upper curve represents group 2 , second curve antibiotic treated group (G3), third curve represents G6 propionic and formic acid treated group and lower linear one represents control negative (G1)

At day 21

	G2	G3	G4	G5	G6
G1	**0.008 S**	**0.04 S**	0.16 NS	0.16 NS	0.44 NS
G2		0.99 NS	0.44 NS	0.44 NS	0.16 NS
G3			0.99 NS	0.99 NS	0.52 NS
G4				1 NS	0.99 NS
G5					0.99 NS

At day 35

	G2	G3	G4	G5	G6
G1	**0.008 S**	0.16 NS	0.44 NS	0.44 NS	0.99 NS
G2		0.44 NS	0.16 NS	0.16 NS	**0.04* S**
G3			0.99 NS	0.99 NS	0.52 NS
G4				1 NS	0.99 NS
G5					0.99 NS

G1 = control – ve,
G2= control +ve (infected),
G3 = infected + kanamycin,
G4 = infected + propionic acid, G5 = infected + formic acid,
G6= infected + propionic acid+ formic acid

Figure 2: Effect of kanamycin and organic acids occurrence rate of *S.* Typhimurium in experimentally infected broilerchickens (N=5) with *Salmonella* Typhimurium analyzed by Fisher exact test

Our results showed a statistically significant difference between the studied groups in frequency of infection at both 1st and 2nd period. When comparing each two groups, it was found that the significance was between group 1 and both group 2 and 3 at day 21 and between group 2 and both group 1 and 6 at day 35 (2nd period).

There was a significant reduction of *Salmonella* occurrence in G6 (propionic acid and formic acid) on day 21; the percent of positive *S.* Typhimurium birds decreased from 100% to 40%, while at 35 days post infection the percentage decreased from 100% to 20% compared to the control group (Figure 2).

Discussion

Organic acids are recently used as alternatives to antibiotics in feed additives because of their safety and they have no side effects in birds or human health. In our investigation, one hundred and twenty-one-day old chicks were divided into six equal groups.

Group 2 infected by *S.* Typhimurium revealed loss of appetite, diarrhea, fever and septicemia in some cases. This result may be due to the bacterial endotoxin, which may reach the liver and lead to disturbance in the metabolic activity. These results agree with previously published articles [4, 5, 32] in which *Salmonella* infection causes endotoxemia due to stimulation the release of lipopolysaccharide (LPS) into blood circulation, which lead to organ dysfunction and death**.** The group 3 medicated with kanamycin and groups 4, 5 and 6 treated with organic acids showed mild clinical signs and decrease in mortality rate, which may be due to bactericidal action of kanamycin and inhibitory effect of organic acids by decreasing pH and lowering the harmful bacterial colonization of the intestine [8,10,13,14]. In the present work a significant increase in WBCs and differential leukocytes count in G2 and G3 was noticed at all the experimental period due to bacterial infection by *S.* Typhimurium and inflammation that lead to leukocytosis, heterophilia and monocytosis, which are responsible for phagocytosis of the infective microorganism and damaged cells **[**33]. *Salmonella* infection stimulates LPS, a bacterial endotoxin, constituent of the outer membrane of Gram-negative bacteria, which enhance the systemic inflammatory response by activating of monocytic cells and other leukocytes, stimulating the release of pro-inflammatory cytokines [32, 34].

In G 4, 5 and 6, improvement in the leukocytes and its differential were detected compared to non-treated group suggesting the efficacy of treatment. The non-significant increase in WBCs, heterophils, lymphocytes, monocytes, eosinophils and basophils in the 2nd period of the experiment was due to the effect of organic acids. Our results agreed with Hedayati [35] who stated that the microflora increase the acidity (lactic acid) in intestine can be used to improve the immune level and reduce the harmful effect of pathogenic bacteria of intestine (as *Salmonella*). This improvement was more obvious in G6 (mixture of propionic acid and formic acid). Also, our result matches with a previous report [36] who mentioned that organic acids reduce pH value in gastrointestinal tract, so increase effectiveness of the barrier function of the stomach against pathogens and increase the activity of digestive enzymes. The acidifiers can promote gastric acid secretion and lower pH of gastrointestinal tract, by enhancing the protease, lipase and amylase activity as well as improve serum calcium, phosphorus levels Zn and Cu. At the same time, intestinal acidic environment is also helpful in absorption of vitamins A and D. the present findings revealed hypoalbuminemia and significant increase in total globulin in group 2 infected by *Salmonella*. The hypoalbuminemia may be due to decreased feed intake, the loss through the intestine and the kidneys, liver failure to synthesize albumin or may be due to liver injury. Our results agree with a previous research [37] in which there were hypoalbuminemia and increase in globulin concentration after challenge with *Salmonella* on weeks 3 and 4 that indicated bacterial

challenge might increase the immune response in challenged birds. The treatment of salmonella with organic acids induce significant increase in total protein, albumin and globulinin G 4, 5 and 6 due to its immunostimulating effect, which was in harmony with a previously published data [38]. This result was clarified with those of Rahmani and Speer [39] who found that broilers given organic acids increase percentage of gamma globulin more than the control. The improvement of immune response accompanied with dietary acidification caused by their inhibitory effects against the pathogenic microorganisms in intestinal tract. In food animal production, propionic acid has approved to be an effective alternative to antibiotic growth promoters as result of antimicrobial effect, health enhancing and growth promoting. The mechanism of PA is through the decrease unfavorable bacteria by reducing gut pH, improving the rate of absorption and utilization of, amino acids, protein, minerals and vitamins [40].

The enhancing the weight gain and performance occurred due to less bacterial fermentation associated with decreased level of toxic bacterial metabolites, causing an improvement in the protein and energy digestibility [41]. On the same ground with Abdel-fattah et al. [42] who found that supplemented organic acids improve immune response as it resulted in higher globulin values and lower A/G ratio. In broilers, the use of organic acid mixture significantly decreases the total bacterial counts [43]. All dietary supplements reduce total pathogenic bacterial count (*E. coli* and *Salmonella*). Organic acids were infiltrating the lipid membrane of bacteria and interrupt the normal physiology by disrupting DNA and protein synthesis [44, 45].

Significant increase of uric acid and creatinine were observed in G2 as the degeneration of renal tubules of infected birds prevented excretion of uric acid and creatinine leading to increase of their levels in serum [46]**.** *Salmonella* infection causes endotoxemia by stimulating production and release of proinflammatory cytokines also releasing of LPS into blood circulation, leading to organ dysfunction and death [33, 34] . The non – significant changes in uric acid in group 6 due to the improve digestibility and utilization of amino acid and protein. As uric acid is the main end product of protein metabolism. [38], The improvement of liver and kidney function in-group 6 is due to mixture of organic acids. Data presented in Table (**3**) showed significant increase in AST and ALT in group 2, which is similar to a previous article [47] that documented that the increase in liver enzyme activities in the blood was attributed to colonization of *Salmonella* in organs. While non-significant change in other groups was due to the antimicrobial effects of the organic acids. These results agree with previous studies [36, 39] in which organic acid had no significant effect on AST and ALT. In addition, another study [48] documented that acidifier had no significant effect on AST in broiler. On the contrary, our results disagree with those verified previously [49]. Regarding to total antioxidant capacity (TAOC) it showed decrease in G2 while there were significant increase in IgG & IgA all over the period of experiment in all groups due to effect of organic acid administration and its immunostimulating effect. Our result was in agreement with Savage *et al.* [50] who found that IgA increased numerically when fed with organic acid through the rate of IgA, which comes from plasma and also into intestine from bile duct. Similar results were obtained by Dar *et al*.**)** [51] who found significant increase in IgG in infected group with S. typhimurium. Also, the increase in IgG and IgA antibody levels after inoculation with *S.* Typhimurium occurred from 1 to 4 weeks post infection in chickens [52]. In group 3, an improvement was noticed by Kanamycin, which acts by binding to the bacterial ribosome, causing inhibition to protein synthesis of the bacterium, which essential for its propagation and cause death of the bacteria **[**8]. Antimicrobial susceptibility testing against salmonella showed more susceptible to gentamycin and kanamycin (+++ve), and resistance to naldixic acid (+ve). This result

agreed with Saad *et al.* [53] who recorded that *Salmonella* appeared resistant to naldixic acid in contrast gentamycin and kanamycin had the basic effect on variability of salmonella. Many investigations on salmonella colonization were performed until now to evaluate the effect of feed additives PA acids in chickens. These results were inconstant with Hinton [54] who recorded that formic acid was very effective at reduction of *S*almonella occurrence rate where 50% of control were positive for *Salmonella*. In this investigation, reduction of *S.* Typhimurium re-isolation rate was observed in group 6 (propionic and formic acid mixture) after day 21 and 35 of experimentally infected, which agreed with a previous study [55] which documented that the animal feed on organic acid reduces the number of some pathogenic bacteria.

Our results showed that no significance different between groups 3, 4, and 5 in *S.* Typhimurium recovery rate after (1^{st}) 21 and (2^{nd}) 35 days of experimental infection. These finding are in accordance to Hajat [20] who stated that organic acid have an antimicrobial activity as antibiotic by the acids can penetrate bacterial cell wall and change the normal actions of bacteria including salmonella spp. Therefore, in animals fed on organic acid, reduction in numbers of normal intestinal bacteria as well as pathogenic bacteria could be noticed [56]. Treatment of *Salmonella* infection with kanamycin, propionic acid and formic acid alone or their combination (propionic acid and formic acid) resulted in ameliorating the severity of infection and decrease colonization of the bacteria in intestine of birds as well as a significant improvement in some immunological and biochemical parameters.

Conclusion:

The combination of propionic acid and formic acid showed the best results as it returned all (Total leukocytes and differential), biochemical and immunological parameters to their relatively normal levels and decrease number of bacterial colonization in poultry.

Conflict of Interest

The authors declare no conflict of interest

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الملخص العربي

المتلازمة الأيضية فعالية الاحماض العضوية و الكاناميسين كاضافات اعلاف لتحسين الحالة المناعية لدجاج التسمين المصاب با لسا لمونيلا

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تم إجراء هذا البحث لتوضيح تأثير التغذية المستمرة للأحماض العضوية والمقارنة بالمضادات الحيوية على بعض المتغيرات المناعية والكيميائية الحيوية في دجاج التسمين السليم والمعدي بالسالمونيلا. تم تقسيم مائة وعشرون كتكوت سلالة روس 308 بعمر يوم واحد إلى ست مجموعات متساوية.
المجموعة الاولي: استخدمت كمجموعة ضابطة بها 20 كتكوت غير مصابة .
المجموعة الثانية : بها 20 كتكوت مصابة بالسا لمونيلا تيفيميوريم بجرعة 10^8 x 1ميكروب لكل مل .
المجموعة الثالثة : بها 20 كتكوت ومصابه بالسا لمونيلا تيفيميوريم بجرعة 10^8 x 1ومعالجة بالكاناميسن 0.1 ملي/ 1كجم وزن حي حقن في العضل لمدة 5 أيام متتالية.
المجموعة الرابعة : بها 20 كتكوت تم إعطائها الحمض العضوي البروبيونك بجرعة 2 كجم / طن علف ثم تمت اصابتها بالسالمونيلا تيفيميوريم بجرعة 10^8 x 1 .
المجموعة الخامسة : بها 20 كتكوت تم إعطائها الحمض العضوي الفورميك بجرعة 1 ملي/لتر في ماء الشرب ثم تمت اصابتها بالسالمونيلا تيفيميوريم بجرعة 10^8 x 1 .
المجموعة السادسة : 20كتكوت تم إعطائها حمض البروبيونيك بجرعة 2 كجم / طن دايت + حمض الفورميك بمقدار 1 مل / لتر فى ماء الشرب ثم تمت اصابتها بالسا لمونيلا تيفيميوريم بجرعة 10^8 x 1 .

تم اخذ عينات دم علي مانع تجلط في اليوم 21 واليوم35 لقياس العدد الكلي والنوعي لكرات الدم البيضاء و اخذت عينات دم اخري بدون مانع تجلط لفصل السيرم لقياس البروتين الكلي والالبيومين وانزيمات الكبد والكرياتينين وحمض اليورك بالاضافة الي الاجسام المناعية IgA وIgG و تم قياس القدرة الكلية المضادة للاكسدة total antioxidant capacity (TAOC)

أظهرت النتائج ان الدجاج المصاب بالسالمونيلا تيفيموريوم يعاني من فقدان الشهية والاسهال والخمول وارتفاع في درجة الحرارة وكانت نسبة النفوق 20% . اما الطيور المعالجة بالكاناميسين فكانت نسبة النفوق 15 % وبينما الطيور المعالجة بالاحماض العضوية ظهرت عليها اعراض اكلينيكية خفيفة وكانت نسبة النفوق 10% .

كما لوحظ ان بدارا النسمين المصاب بالسالمونيلا تيفيموريوم يوجد بة زيادة معنوية في عدد الكريات الدم البيضاء ،و ا لخلايا لمتعادلة وخلايا monocytes،(الوحيدات) بالإضافة إلى زيادة معنوية في نشاط حمض البوليك ، والكرياتينين ، وأنزيمات الكبد ، و الاجسام المناعية IgA و IgG أيضًا انخفاض معنوي في الألبومين و TAOC.

اما المجموعات المعاجة بالكاناميسين ، والأحماض العضوية وحدها أو مجتمعة (حمض البروبيونيك وحمض الفورميك) ادت إلى تأثير إيجابي في العلاج ، مما يخفف من حدة العدوى ويحسن معنويًا في بعض المتغيرات المناعية والكيميائية الحيوية. نستخلص من الدراسة أن استخدام الأحماض العضوية اعطي أفضل النتائج حيث أعادت Leukogram (خلايا الدم البيضاء) والقيم المناعية والبيوكيميائية إلى مستوياتها الطبيعية و قللت من وجود البكتيريا الضارة بالامعاء .