Effect of Dietary Formic and Propionic Acids Mixture on Limiting S*almonella pullorum* in Layer Chicks**

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ABSTRACT : This investigation was conducted to study the effect of dietary formic acid (FA) and propionic acid (PA) mixture on inhibitory effect of *Salmonella pullorum* in layer chicks. Nine groups of one day-old layer chicks in addition to positive and negative controls, were fed with acids treated feed containing mixture of different acids concentrations, from 0.5% and 0.5% up to 1.5% and 1.5% FA and PA, respectively. Positive and negative controls were fed untreated feed. Groups except the negative control were challenged orally on day three with 10^4 cfu/ml *S. pullorum*. Cloacal swabs were taken at three successive days and at 7, 14 and 21 days of challenge. After 1, 2 and 3 weeks after challenge, 4 chicks from each group were sacrificed and crop and cecal contents were examined for *S. pullorum* and pH. The numbers of *S. pullorum* positive culture from the excretion of all treated groups except groups treated with mixture of 0.5% and 0.5%, 1% and 0.5%, 0.5% and 1% FA and PA decreased significantly ($p<0.05$) as compared with the positive control. The mortality rates of all treated groups except the group treated with 0.5% FA and 0.5% PA were decreased significantly ($p<0.05$) as compared with the positive control. The treatment significantly ($p<0.05$) lowered the pH of the crop and cecal contents in all groups except the group treated with 0.5% FA and 0.5% PA as compared with the control. Also, the treatment significantly ($p<0.05$) lowered the pH of the crop and cecal contents in all groups after three weeks of treatment compared to the first and second weeks. The treatments significantly ($p<0.05$) lowered the frequency of *S. pullorum* recovery from crop and cecal contents in six groups treated with 1.5 and 0.5, 1 and 1, 1.5 and 1, 0.5 and 1.5, 1 and 1.5, 1.5% and 1.5% FA and PA respectively. These results indicate that addition of FA and PA mixture in a total concentration of 2 % or more to the diet of newly hatched infected layer chicks significantly decreases the crop and cecal colonization by *S. pullorum* and significantly decreases *S. pullorum* fecal excretion and reduced the chick mortality rate. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 1 : 77-82)***

Key Words : *Salmonella Pullorum*, Formic Acid, Propionic Acid, Layer Chicks, Dietary

INTRODUCTION

Salmonella pullorum is one of the major pathogens of concern to the industry in the early days of poultry production intensification (Barrow, 1993; Alshawabkeh and Yamani, 1996). The three major sources of Salmonella infections in poultry are the introduction of infected stock, the environment and the contaminated feed (Gage, 1911).

Many methods have been used to reduce the level of salmonellae infection including the use of antimicrobial agents e.g. sulfonamide (Anderson et al., 1948), nitrofurans (Smith, 1954), vaccination (Silva et al., 1981), competitive exclusion (Rantala and Nurmi, 1973; Alshawabkeh, 1995) and some feed additives as certain carbohydrates (Oyofo et al., 1989; Alshawabkeh, 1998; Barnhart et al., 1999). Also, lactic acid producing bacteria which control the unwanted pathogenic bacteria by reducing gut pH and addition of organic acids such as formic acid (FA) and propionic acid (PA) have been reported to control salmonellae and reduce gut pH in broiler chickens (Iba and Berchieri Jr., 1995; Alshawabkeh and Tabbaa, 2002). All of the above mentioned methods were used with varying success. The antibacterial activity of these organic acids is achieved by influencing the cell structure and the cell metabolism as a result of reducing the pH in the alimentary tract below the growth range of the pathogenic bacterial cells (Thompson and Hinton, 1997).

FA and PA incorporation had a disinfecting effect on contaminated feed and had sufficient antibacterial effect in the alimentary tract (Iba and Berchieri Jr., 1995). In addition, it is effective in preventing intestinal colonization of the Salmonella organisms from naturally or artificially contaminated feed.

S. pullorum could be transmitted either vertically and/or horizontally (Calnek et al., 1997). Therefore, the objective of this study was to determine the possible antibacterial effect of feeding FA and PA mixture on crop and cecal pH to reduce the colonization of

S. pullorum in these organs, to control *S. pullorum* fecal excretion and their effect on mortality rates, after challenge in layer chicks in Jordan.

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MATERIALS AND METHODS

Chickens

One-day old 140 Leghorn layer chicks free from Salmonella were obtained from a commercial hatchery in Jordan. The chicks were reared in cages and fed on a commercial balanced ration. The feed and water were cultured for the presence of Salmonella using a standard culture method (Cox, 1988; Quinn et al., 1994). Also, chicks were examined daily for the first three days of age for freedom of Salmonella by clinical observations and culture of cloacal swabs. In addition, eight chicks of one-day old were scarified and the visceral organs (liver, spleen, heart and intestine) were cultured and blood agglutination test using Salmonella colored antigens was performed (Anderw et al., 1978).

Salmonella

A primary isolate of *S. pullorum* originally isolated from a layer flock was obtained from Central Veterinary Laboratories, Ministry of Agriculture, Amman Jordan. The isolate was selected for its resistance to novobiocin and nalidixic acid and maintained on nutrient agar slant for the challenge. Media used to culture the *S. pullorum* isolate was Brilliant Green Agar (BGA) (DIFCO) containing 25 μg/ml novobiocin and 20 μg/ml nalidixic acid, to inhibit growth of other bacteria. Challenge inoculum was prepared from 24 h selenite F broth (DIFCO) culture, serially diluted to 4.7×10^4 cfu/ml in sterile normal saline. The viable cell count of challenge inoculum was confirmed by colony counts on duplicate BGA plates.

Experimental protocol

Groups were randomly assigned each of 12 layer one-day old chicks. Groups 1 to 9 feeds were treated as follows: Group 1:0.5 and 0.5, group 2:1 and 0.5, group 3:1.5 and 0.5, group 4:0.5 and 1, group 5:1 and 1, group 6:1.5 and 1, group 7:0.5 and 1.5, group 8:1 and 1.5 and group 9:1.5% and 1.5% FA and PA, respectively. The feed of positive and negative control groups was untreated. Feed contains FA and PA was prepared daily. The starting day of feeding with treated feed was on the third day of the chick age. Chicks of all groups (except negative control) were challenged orally on day three of the experiment "six hours after feeding of treated feed" with 10^4 cfu/ml of *S. pullorum* directly by crop injection (Barrow, 1993). Negative control group receives one-milliliter sterile normal saline directly into the crop of each chick. The treated positive and the negative control groups were placed in separate rooms.

Sampling

Cloacal swabs were collected at 1, 2, 3, 7, 14 and 21 days after challenge and examined for *S. pullorum*. On days 7, 14 and 21 after challenge four chicks from each group were euthanized by cervical dislocation at each time intervals. Mortile chicks during these time intervals were tested as if it's euthanized. Crop and cecum contents were examined for presence of *S. pullorum* and pH.

Bacteriological examination

Fecal swabs, crop and cecal contents were collected aseptically and added to 10 ml selenite F broth in tube and incubated for 24 h at 43°C. After incubation, the broth was streaked on brilliant green agar plates contains 25 μg /ml novobiocin and 20 μg /ml nalidixic acid. Plates were incubated at 37°C for 24 h and the suspected Salmonella pinkish colonies were identified by biochemical tests "triple sugar iron agar, indole, urease, lactose, sucrose, maltose and dulcitol" and serological test with Salmonella O antisera factors 1, 9, 12 (MUREX BIOTECH, England) (Quinn et al., 1994).

Measurement of crop and cecal pH

Crop pH was measured in site by inserting the electrode into an incision in the crop before the contents were removed. Sample of 0.2 gm of cecal contents was suspended in 0.8 ml of sterile glass-distilled water and the pH was measured immediately with glass electrode (Nisbet et al., 1994).

Statistical analysis

Data were analyzed by the analysis of variance using the SPSS program. T-test of independent variables was used for separation of significantly different means. Two-tail Fisher's exact test was used to compare data of each group with the positive control. Probability value of 0.05 or less was considered significant.

RESULTS

Mortality

Challenge doses (4.7×10^4 cfu/ml) of *S. pullorum* caused mortality rates ranged from 0% in-group nine to 58.3% in the positive control (Table 1). The mortality rates of all groups "except the group treated with 0.5% FA and 0.5% PA" were decreased significantly ($p<0.05$) when compared to the untreated positive control. The negative control did not show any deaths through out the study period.

pH

Means of pH values of the crop and cecal contents of layer chicks receiving different treatments are shown in Tables 2 and 3. Data indicated that there were no significant ($p>0.05$) differences in the pH of the positive and negative control groups through out the experiment duration. The addition of FA and PA in feed significantly ($p<0.05$)

Table 1. Mortality rate of layer chick groups[1] after oral challenge with *S. pullorum*[2]

Groups	F&P acids conc. (%)[3]	Number of deaths[4] Days after challenge					Total		P. value[5]
		2	3	7	14	21	No.	%	
1	0.5+0.5	1	0	0	2	0	3	25.0	0.098
2	1+0.5	0	1	1	0	0	2	16.7	0.035[a]
3	1.5+0.5	1	1	0	0	0	2	16.7	0.035[a]
4	0.5+1	1	0	1	0	0	2	16.7	0.035[a]
5	1+1	0	2	0	0	0	2	16.7	0.035[a]
6	1.5+1	0	1	0	0	0	1	8.3	0.009[a]
7	0.5+1.5	0	0	1	0	0	1	8.3	0.009[a]
8	1+1.5	0	2	0	0	0	2	16.7	0.035[a]
9	1.5+1.5	0	0	0	0	0	0	0	0.000[a]
C+	Not treated	3	2	1	0	1	7	58.3	-[b]

1. Nine groups each of 12 chicks fed with acid treated feed.
2. Challenge dose is 4.7×10^4 cfu/ml of *S. pullorum*.
3. Formic and propionic acids concentrations.
4. Negative control group did not show any deaths through the whole period of the study.
5. P. values with superscript [a] are significantly ($p<0.05$) different from that of positive control with superscript[b].

Table 2. The effect of dietary formic and propionic acids on crop pH of layer chicks after challenged with *S. pullorum*[1]

Groups	F&P acids conc. (%)[2]	The mean of pH of the crop contents[3] Weekly intervals after challenge		
		1st	2nd	3rd
1	0.5+0.5	5.18±0.1	5.02±0.1	4.78±0.2
2	1+0.5	4.82±0.0[a]	4.74±0.0[b]	4.61±0.2[d]
3	0.5+0.5	4.63±0.2[a]	4.44±0.2[b]	4.31±0.1[d]
4	0.5+1	4.97±0.1[a]	4.77±0.2[b]	4.51±0.2[d]
5	1+1	4.90±0.1[a]	4.70±0.2[b]	4.27±0.1[d]
6	1.5+1	4.88±0.4[a]	4.52±0.2[b]	4.29±0.2[d]
7	0.5+1.5	4.94±0.1[a]	4.69±0.1[b]	4.41±0.1[d]
8	1+1.5	4.61±0.1[a]	4.51±0.1[b]	4.35±0.2[d]
9	1.5+1.5	4.51±0.2[a]	4.35±0.2[b]	4.07±0.1[d]
C+	Not treated	5.17±0.2[C1+]	5.16±0.1[C2+]	5.23±0.2[C3+]
C-	Not treated	5.21±0.2[C1-]	5.22±0.2[C2-]	5.10±0.2[C3-]

1. Chicks were challenged at 3 days old with 4.7×10^4 cfu/*S. pullorum*.
2. Formic and propionic acids concentrations.
3. Each mean of pH represents four readings of four chicks.
[a, b, d, C+1, C-1, C+2, C-2, C+3, C-3:] Means in the same column with different superscript lowered significantly ($p<0.05$) from that of the control groups. Also, means in the third column lowered significantly ($p<0.05$) from the means in the first and second column.

Table 3. The effect of dietary formic and propionic acids on cecal pH of layer chicks after challenged with *S. pullorum*[1]

Groups	F&P acids conc. (%)[2]	The mean of pH of the ceca contents[3] Weekly intervals after challenge		
		1st	2nd	3rd
1	0.5+0.5	5.84±0.2	5.66±0.1	5.64±0.2
2	1+0.5	5.72±0.3[a]	5.33±0.2[b]	4.95±0.2[d]
3	0.5+0.5	4.70±0.2[a]	4.23±0.3[b]	4.11±0.2[d]
4	0.5+1	5.43±0.1[a]	5.28±0.2[b]	5.21±0.2[d]
5	1+1	5.38±0.2[a]	5.19±0.2[b]	5.07±0.1[d]
6	1.5+1	4.95±0.3[a]	4.48±0.3[b]	4.20±0.2[d]
7	0.5+1.5	5.41±0.2[a]	5.16±0.2[b]	4.92±0.2[d]
8	1+1.5	4.47±0.4[a]	4.23±0.2[b]	4.12±0.2[d]
9	1.5+1.5	4.33±0.2[a]	4.15±0.3[b]	4.04±0.1[d]
C+	Not treated	5.87±0.3[C1+]	5.84±0.2[C2+]	5.75±0.1[C3+]
C-	Not treated	6.0±0.1[C1-]	5.93±0.1[C2-]	5.90±0.2[C3-]

1. Chicks were challenged at 3 days old with 4.7×10^4 cfu/*S. pullorum*.
2. Formic and propionic acids concentrations.
3. Each mean of pH represents four readings of four chicks.
[a, b, d, C1+, C1-, C2+, C2-, C3+, C3-:] Means in the same column with different superscript lowered significantly ($p<0.05$) from that of the control groups. Also, means in the third column lowered significantly ($p<0.05$) from the means in the first and second column.

decreased the pH of crop and cecal contents in all treated groups except group number one compared with the control groups. Also, the pH of crop and cecal contents on the third week of all treated groups were significantly ($p<0.05$) lowered than the pH of the first and second week.

Salmonella recovery

Fecal excretion : In Table 4, *S. pullorum* was isolated from the cloacal swabs after 24 h of challenge from all groups except group nine. On day 14 and 21 after challenge, all feed treated groups showed significant ($p<0.05$) decreased of *S. pullorum* excretion compared to excretion on days 1, 2 and 3 post challenge. Number of *S. pullorum* positive cultures excreted in feces was significantly ($p<0.05$) decreased in all treated groups except groups number one, two and four compared to positive control. The positive control group excretes *S. pullorum* after 24 h of challenge through the whole period of the experiment while negative control remained healthy and did not show any *S. pullorum* excretion.

Crop and cecal colonization : The numbers of *S. pullorum* positive culture of the crop and cecal contents at different time post challenge in all treated groups except groups number one, two and four were significantly ($p<0.05$) decreased compared with the positive control (Tables 5 and 6). No *S. pullorum* isolates was recovered from crop or cecal contents or cloacal swabs of the negative control, through out the period of the experiment.

Table 4. Frequency of *Salmonella pullorum* isolation from cloacal swabs of layer chicks[1] fed formic and propionic acids after different intervals after oral challenge[2]

Groups	F&P acids conc. (%)[3]	Numbers of infected chicks[4] Days after challenge						Total	P. value[5]
		1	2	3	7	14	21	No.	
1	0.5+0.5	10	7	1	0	1	1	20	0.162
2	1+0.5	7	8	6	3	0	1	25	0.350
3	1.5+0.5	3	7	2	3	0	0	15	0.033[a]
4	0.5+1	3	5	8	2	2	1	21	0.143
5	1+1	3	4	4	2	2	1	16	0.039[a]
6	1.5+1	4	3	1	0	0	0	08	0.004[a]
7	0.5+1.5	4	4	6	4	2	1	21	0.097
8	1+1.5	6	5	6	2	1	1	21	0.091
9	1.5+1.5	0	3	4	2	1	0	10	0.007[a]
C+	Not treated	5	8	8	6	4	2	33	-[b]

[1] Nine groups each of 12 chicks fed with acids treated feed
[2] Challenge dose is 4.7×10^4 cfu/ml of *S. pullorum*
[3] Formic and propionic acids concentrations
[4] Negative control chicks did not excrete any *S. pullorum* through out the experiment.
[5] P. values with [a]superscript are significantly ($p<0.05$) different from that of positive control with [b]superscript.

Table 5. Frequency of *Salmonella pullorum*[1] isolation from crop of dead or sacrificed layer chicks treated with dietary formic and propionic acids after different intervals of oral challenge

Groups	F&P acids conc. (%)[2]	Number of positive crop Days after challenge[4,5]					Total		P. value[3]
		2	3	7	14	21	No.	%	
1	0.5+0.5	1	3	1	0	1	6	50.0	0.094
2	1+0.5	2	1	2	0	0	5	41.7	0.089
3	1.5+0.5	2	0	1	1	0	4	33.0	0.012[a]
4	0.5+1	0	1	1	1	2	5	41.7	0.089
5	1+1	0	0	0	0	0	0	00.0	0.000[a]
6	1.5+1	0	0	1	1	0	2	16.6	0.001[a]
7	0.5+1.5	0	0	0	0	0	0	00.0	0.000[a]
8	1+1.5	1	1	0	0	0	2	16.6	0.001[a]
9	1.5+1.5	0	0	0	0	0	0	00.0	0.000[a]
C+	Not treated	3	2	0	3	2	10	83.3	-[b]

[1] Challenge dose was 4.7×10^4 cfu/ml of *S. pullorum.*
[2] Formic and propionic acids concentrations.
[3] No significant difference between isolation rate at different interval times, but P. values with [a]superscript are significantly ($p<0.05$) different from that of positive control with [b]superscript.
[4] No *S. pullorum* recovered after 24 h after challenge.
[5] Negative control group did not show *S. pullorum* positive culture through the whole period of the study.

DISCUSSION

To our knowledge, no previous study using such wide range of dietary short chain fatty acids (SCFA) concentrations, *S. pullorum* and layer chicks had been done in an experiments using of dietary SCFA in controlling salmonellae.

Feed treatment with FA and PA showed a significant ($p<0.05$) decrease in the overall mortality rate (1-21 days) in all treated groups except group one received 0.5% FA and 0.5% PA compared with acid untreated positive control. These results were in agreement with Berchieri Jr. and Barrow (1996) and with Junior and Barrow (1996) who found that the bio-Add™ treatment at 0.68% reduced mortality rate from 77% in untreated chicks to 33% in treated ones.

This study demonstrated a significant reduction in the pH of the crop contents in eight groups when the acid concentration in the diet increased. The addition of dietary FA and PA in concentrations >0.5% and 0.5%, respectively, lowered significantly ($p<0.05$) the pH of the layer chick crops. This indicates that the crop pH decreased as the FA and PA level increased. Also, the addition of dietary FA and PA in all groups except group treated with 0.5% FA and 0.5% PA, significantly ($p<0.05$) lowered the pH of the layer chick cecal contents. Such lowered pH significantly ($p<0.05$) reduced the frequency of *S. pullorum* isolation from crop and ceca of layer chicks in groups treated with dietary FA and PA in a total concentration mixture of ≥2%. This indicates that degradation or absorption of such acids occurred in the intestine is not sufficient to exclude its inhibitory effect on cecal pH. This is in agreement with

Table 6. Frequency of *Salmonella pullorum*[1] isolation from cecum of dead or sacrificed layer chicks treated with dietary formic and propionic acids after different intervals of oral challenge

Groups	F&P acids conc. (%)[2]	Number of positive ceca					Total		P. value[3]
		Days after challenge[4,5]							
		2	3	7	14	21	No.	%	
1	0.5+0.5	0	0	1	3	2	6	50.0	0.068
2	1+0.5	0	2	0	3	2	7	58.3	0.154
3	1.5+0.5	1	0	0	2	1	4	33.0	0.012[a]
4	0.5+1	0	0	2	1	2	5	41.7	0.089
5	1+1	0	0	1	2	0	3	25.0	0.001[a]
6	1.5+1	0	1	0	1	1	3	25.0	0.001[a]
7	0.5+1.5	0	0	1	1	1	3	25.0	0.001[a]
8	1+1.5	0	0	1	1	1	3	25.0	0.001[a]
9	1.5+1.5	0	0	1	0	1	2	16.6	0.000[a]
C+	Not treated	3	2	0	3	3	11	91.7	-[b]

[1] Challenge dose was 4.7×10^4 cfu/ml of *S. pullorum.*

[2] Formic and propionic acids concentrations.

[3] No significant difference between isolation rate at different interval times, but P. values with [a]superscript are significantly ($p<0.05$) different from that of positive control with [b]superscript.

[4] No *S. pullorum* recovered after 24 h after challenge.

[5] Negative control group did not show *S. pullorum* positive culture through the whole period of the study.

Hume et al. (1993) who reported that addition of PA decreased crop pH and increased the bactericidal level. Also, it's in agreement with McHan and Shotts (1993) and Thompson and Hinton (1997) who found that FA and PA were likely to be antibacterial since they lowered the cecal pH in a concentration-dependent manner. The significant reduction in the number of *S. pullorum* in the crop, ceca and fecal excretion by adding FA and PA in the diet of layer chicks indicates a strong potential for the use of these acids in the diet to reduce infection of *S. pullorum* in layer flocks.

The results of this study indicated that the antibacterial activity of FA and PA is a concentration-dependent. These results were in agreement with Thompson and Hinton (1997) who found that addition of Bio-add™ to diet reduced the number of *S. enteritidis* from the crop of hens. Also, it was agreed with Hinton and Linton (1988) and Oliveira et al. (2000) who showed high bactericidal activity of FA in the crop that inhibit the bacterial cells to grow and suggest that FA and PA mixture is active in the chicks digestive tract. Also, the results of this investigation were paralleled with the earlier work by Izat et al. (1990) who found that the buffered PA reduced the number of *S. typhimurium* from broiler chickens, and were partially in agreement with Hume et al. (1993) who found that the PA - treated feed resulted in a Salmonella negative small intestine of broiler chickens after one week, but did not eliminate them from the intestinal tract after three weeks.

In this study, the addition of FA and PA mixture to the diet of experimentally infected layer chicks in a concentration ≥2%, gradually inhibited *S. pullorum* colonization to crop and ceca and decreased its fecal excretion. This is in agreement with the report of Iba and Berchieri Jr. (1995) who used FA-PA mixture (Bio-Add™) in the diet of broiler that resulted in no recovery of Salmonella from infected broiler chickens.

In conclusion, dietary formic and propionic acids mixture in a total concentration of 2% or more was effective to limit *S. Pullorum* colonization in crop and ceca and decreased *S. pullorum* fecal excretion and reduced the mortality rate of infected layer chicks.

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