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Effects of Dietary Supplement of Organic Acids Induced Protective Immunity against Coccidiosis

Research Article

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ABSTRACT

This study evaluated effects of three products of dietary organic acids (Acidomix® AFG; Activate® AD and Lacplus) on protective immunity against coccidiosis. Two hundred and eighty 1-day broiler chickens were randomly divided into 8 groups by 5 replications (7 birds per replicate), comprising 2 controls (negative=N; positive=P) and 6 supplemented organic acid groups (1% AFG, 1% DA, 1% Lacplus; 2% AFG, 2% DA and 2% Lacplus). With the exception of N control (uninfected), all 7 groups were orally challenged with 8×10^5 sporulated oocysts of *Eimeria tenella* at day 12 post-hatch. Samples of blood, spleen and ceca were collected on day 22 and 35 post-hatch. Body weight gain, fecal oocyst shedding, lesion score, mortality rate and pH of the ceca were assessed. ELISA was used to detect Cluster of T cell differentiation (CD4+, CD8+) molecules and reverse transcription polymerase chain reaction (RT-PCR) was employed for detection of pro-inflammatory chemokine (IFNγ-) and cytokines expression (IL-8, IL-15 and IL-17). The result revealed that supplementation of organic acids significantly increased body weight gain, improved feed conversion ratio (FCR), reduced lesion scores and oocyst index ($P<0.05$) compared to P control (infected non-supplemented). The mortality rate was higher in P control. The pH of ceca was significant ($P<0.05$) and maximum was observed in P control. Chicks fed 2% DA and 1% Lacplus had greater percentages of CD4 T-cell molecules, but had decreased CD8 T cell molecules, suggesting a protective function of these T-cell subsets in innate immune response against *E. tenella* infection. The cecal and splenic chemokine and cytokines mRNA expression encoding IFN-γ, IL-8, IL-15 and IL-17 showed higher levels of transcript compared to N and P controls, indicating the organic acid products might have exerted their protective effects by improving their production. It is concluded that the Activate® DA at 2% and Lacplus 1% levels at both 22 and 35-days post-hatch have shown best anticoccidial effects.

KEY WORDS coccidiosis, cytokines, immunity, organic acid, T-cells.

INTRODUCTION

Resistance to anticoccidials has been an issue for the commercial poultry industry for many years and it's a global problem. Avian coccidiosis is one of the most widely reported and economically important diseases disturbing commercial poultry production, with an estimated cost over $800 million USD annually in the United States and in excess of $3 billion USD worldwide (De Gussem, 2007; Sharman *et al*. 2010; Blake and Tomley, 2014).

The incidence of coccidiosis in commercial poultry can range from 5 to 70% (Du and Hu, 2004). To prevent the emergence of drug resistance, new drugs have been developed and administered on a rotational basis with existing drugs. However, this has resulted in the increased cost of poultry production.

Furthermore, drug or antibiotic residue in the poultry products is potentially hazardous to consumer. Therefore, alternative strategies are being sought for more effective and safer control of coccidiosis in chickens (Dalloul *et al*. 2006; Williams, 2005; Abbas *et al*. 2010; Abbas *et al*. 2011). Recent developments and an increasing focus on naturally derived molecules have suggested that there might be new ways to tackle avian coccidiosis. The present research has worked to this direction.

Dana *et al*. (2018) highlighted that acidic compounds comprising of organic acids show promise as antibiotic alternatives, because organic acids have established the capability to enhance poultry performance by altering the pH of the gastrointestinal tract (GIT) and accordingly changing the composition of the microbiome. Furthermore, organic acids, by altering the composition of the microbiome, protect poultry from pH-sensitive pathogens and enhance the morphology and physiology of the GIT and the immune system.

Richard Sygall (2003) worked on coccidiosis, and used organic acid blend and monoglyceride of butyric acid among treatment groups. The monoglyceride of butyric acid group showed significant decrease in lesion score, no mortality rather showed low clinical signs than the other infected groups.

Abbas *et al*. (2011) used different concentrations levels (1%, 2% and 3%) of acetic acid and amprolium at dose rate of 125 ppm given to the experimental groups in drinking water from 10-19th days of age; in another work the same authors used HCl; the results of these studies showed that, acetic acid and lower dose of HCl have the potential to be used as alternatives to chemotherapeutic drugs for *E. tenella* control.

Manzoor *et al*. (2013) evaluated the anticoccidial effects of formic acid in contrast to HCl in broiler chickens challenged with *E. tenella* infection in comparison with the amprolium (anticoccidial), the results had shown positive effects.

However, the researchers suggested further studies to determine the possible minimum safe levels of HCl and formic acid with least toxic effects to be used as anticoccidial. In present study three different organic products namely: AFG, DA and Lacplus at different levels were used to assess their protective efficacies against *Eimeria* infection and possible effects on immune response.

MATERIALS AND METHODS

Animal ethics

Animal welfare and experimental procedures were performed in accordance with the guide for the care and use of laboratory animals and were approved by the animal ethics committee of South China Agricultural University.

Organic acids

The three products of organic acids (from Novus and Greencore feed science and technology co., Ltd; China) are: Acidomix® AFG, Activate® DA and Lacplus. The Acidomix® AFG composition: {Ammonium formate, formic acid, Ammonium Propionate}; Activate® DA {2- hydroxyl -4- calcium butyrate [Ca (HMTBa) 2], fumaric acid, Benzoic acid} and Lacplus {lactic acid, citric acid, fumaric acid, phosphoric acid}. In this study, all the three products were used at two different levels (1% and 2%), to find out best level.

Infectious material

Institute of Animal Health, Guangdong Academy of Agricultural Science, Guangzhou China has donated *E. tenella* oocysts which were obtained from naturally infected chickens, passed through 2-week-old broiler chickens and stored in 2.5% potassium dichromate solution in a refrigerator (4 ˚C).

The sporulated oocysts was administered via 1ml syringe. An isolate may be defined as a population of oocysts obtained from a single farm at one geographical location. Usually it is necessary to propagate the oocysts at least once in un-medicated chickens in order to provide sufficient numbers for experiments.

Experimental animals

A total of 280 one day clinically healthy Huangshi (Yellow) broilers (Chinese breeds) were purchased from commercial hatchery. The birds were housed in cages on slat floors under conditions excluding further *Eimeria* infection. They were fed standard broiler diet (Chinese standard feeding requirement) without antibiotic growth promoters or coccidiostats, at starter and grower phases. Feeding and watering were given *ad libitum*.

Experimental design

Randomized complete block design (RCBD) was employed for experimental design. A total of forty pens of 7 birds per pen were selected for enrolment into 8 treatment groups with 5 replicates: T1 (uninfected, non-supplemented N control), T2 (infected, non-supplemented P control), T3 (Acidomix® AFG 1%), T4 (Activate® DA 1%), T5 (Lacplus

1%), T6 (Acidomix® AFG 2%), T7 (Activate® DA 2%) and T8 (Lacplus 2%). The chicks were allocated into treatment groups as per RCBD with blocking based on body weight on day ten. Hence, on day 12 seven of the eight treatment groups received a single challenge dose of 8×10^5 *E. tenella*. Lesion scoring on five birds from each group was done on day 22. Tissue samples (intestinal organs, spleen and blood) were taken from five birds in each group on days 22 and 35. Faecal oocysts counts were carried out on days 17-21 post-hatch. The study was terminated on day 35.

Parasitological studies

In order to determine parasitological parameters, body weight gains were calculated at 10- and 23-days post-infection (dpi) as described (Lee *et al*. 2007a). Lesion scores per bird were assessed according to Johnson and Reid (1970) and the oocyst index was measured (Cuckler, 1959).

The lesion score is one of the most widely used procedures for evaluating the efficacy of anticoccidial drugs. Oocyst counts (in droppings or litter) estimate the magnitude of infection in terms of parasite numbers. Oocyst counts can provide a useful measure of the ability of the parasites to reproduce in the host, providing low inocula are used. Oocyst shedding was assessed as described (Lee *et al*. 2007a).

Briefly, fecal droppings were collected daily (per replicate) between 5-10 dpi and pooled fecal material was suspended in 3 L of water. Two 35 mL samples were taken, diluted, and the number of oocysts was counted microscopically using a McMaster chamber. Figure 1 depicted some activities (pictures A-I) observed during the experiment.

Caecal pH determination

Samples of caeca contents of all groups were subjected to pH measurement with aid of pH meter.

Tissue samples

At 22 and 35 days of age, five birds per group were randomly selected for removal of ceca and spleen to detect cytokines and chemokine according to Lee *et al*. (2009); while blood was collected for detection of chicken cluster of differentiations (Lutz *et al*. 1994; Serge *et al*. 1999).

Determination of CD4/CD8 molecules by a cell marker ELISA

To determine concentrations of CD4 and CD8 molecules on T lymphocytes, we used the Chicken cluster of differentiation 4 and 8 (CD4, CD8) ELISA Kit (Wuhan Gene Technology Co., Ltd), based on the manufacturer's instructions.

Peripheral blood mononuclear cells were separated from blood by centrifugation on a density gradient (Lutz *et al*. 1994).

Determination of cytokine/chemokine mRNA levels

Cytokine and chemokine gene expression analysis was carried out using real-time RT-PCR as described (Hong *et al*. 2006a; Hong *et al*. 2006b). At 22 and 35 days of age, spleens were removed and placed in a Petri dish with 10 mL of Hank's balanced salt solution (HBSS) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Likewise, the intestinal ceca were removed, cut longitudinally, and washed three times with ice-cold HBSS containing 100 U/mL of penicillin and 100 µg/mL of streptomycin. The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 5% fetal bovine serum (FBS) and incubated for 20 min at 37 ˚C with constant swirling. Cells released into the supernatant were washed twice with HBSS and filtered by a syringe containing nylon wool.

RNA extraction and cDNA synthesis

Total RNA was extracted from cecum and spleen tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) as described (Johnson and Reid, 1970). Five micrograms of RNA were treated with 1.0 U of DNase I and 1.0 mL of 10 reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 mL of stop solution was added to inactivate DNase I, and the mixture was heated at 70 ˚C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, 5.0 mg of RNA were combined with 10 first-strand buffer, 1.0 mL of oligo (dT) primer (5.0 mg/mL), 0.8 mL of dNTP mix (25 mM each), and RNase-free water to a total volume of 19 mL. The mixture was incubated at 65 ˚C C for 5 min, cooled to room temperature, 50 U of StrataScript reverse transcriptase was added, the mixture was incubated at 42 ˚C for 1 h, and the reaction was stopped by heating at 70 ˚C for 5 min (Lee *et al*. 2008; Lee *et al*. 2009).

Quantitative RT-PCR

Quantitative RT-PCR oligonucleotide primers for chicken interferon-γ (IFN-γ), interleukin-8 (IL-8), interleukin-15 (IL-15), interleukin-17 (IL-17) and GAPDH control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from cecum and spleen tissues using the M×3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using log10 diluted cDNA from pooled infected and non-infected total RNA.

Figure 1 Activities observed during experimental period
A: Healthy chicks before challenge with *E. tenella*; **B**: Infected chicks after challenge with *E. tenella*; **C**: Blood oozing from cecum of infected chick; **D**: Fecal hemorrhage; **E**: *E. tenella* Infected dead chickens; **F**: Post mortem to investigate *E. tenella* in the caecum; **G**: Confirmed cecal hemorrhage, lesion score 4; **H**: Intestinal structure of uninfected chick and **I**: Cecum of uninfected chick

To normalize individual replicates, the logarithmic scaled raw data unit cycle threshold (CT) was transformed into the linear unit of normalized expressions and then calculating means and S.E. for the references (CT reference, mean and S.E. CT reference, mean) and individual targets (CT target, mean and S.E. CT target, mean). To correct for differences between RNA levels between samples within the experiment, the difference factor for each sample was calculated by dividing the mean threshold cycle (Ct) values for GAPDH from all samples in that experiment (Livak and Schmittgen, 2001; Lee *et al*. 2009).

Statistical analysis

The data collected were analyzed using Microsoft office 2013 and Statistix 8 software. The ANOVA by Duncan range test was used to test for differences between the groups. The data were expressed as mean ± SEM. Results were considered significant when $P < 0.05$ (Çilek and Tekin, 2005).

RESULTS AND DISCUSSION

Body weight gain and FCR during experimental coccidiosis

Mean body weight gain (g) of uninfected and infected groups on normal diet without organic acid (N and P control) and on diet with organic acids (3-8) were calculated over the 10- and 23-days' post infection period. Table 2 shows that weight gain was significantly ($P<0.05$) reduced in *E. tenella* infected group compared with uninfected birds at both 22- and 35-days' post-hatch. In contrast, among infected *E. tenella* groups 2% DA recorded maximum weight than other organic acid groups; and all organic acids groups were higher than P control birds. The results of FCR in Table 2 revealed that the FCR values of 1% DA, 1% Lacplus and 2% DA groups were numerically lower compared with infected P control group at 22-day post-hatch, but only 1% Lacplus and 2% DA at 35 days were numerically low; although a statistical comparison could not be made due to group feeding.

Table 1 Oligonucleotide primers used for quantitative RT-PCR of chicken IFN-γ, IL-8, IL-15, IL-17 and GAPDH (Lee *et al*. 2009)

RNA target	Primer sequences (5'→3')[1]	PCR product (bp)	Accession number
IFN-γ	F: AGCTGACGGTGGACCTATTATT R: GGCTTTGCGCTGGATTC	259	Y07922
IL-8	F: GGCTT GCTAG GGGAAATGA R: AGCTGACTCTGACTAGGAAACTGT	200	AJ009800
IL-15	F: TCTGTTCTTCTGTTCTGAGTGATG R: AGTGATTTGCTTCTGTCTTTGGTA	243	AF139097
IL-17	F: CTCCGATCCCTTATTCTCCTC R: AAGCGGTTGTGGTCCTCAT	292	AJ493595
GAPDH	F: GGTGGTGCTAAGCGTGTTAT R: ACCTCTGTCATCTCTCCACA	264	K01458

[1] F and R: represent sense and antisense primer respectively.

Table 2 Comparative values of the mean weight gain (WG), feed conversion ratio (FCR), lesion score, oocyst index, and mortality percentage

Treatments	WG at 22 days (g)	WG at 35 days (g)	FCR at 22 days*	FCR at 35 days*	Lesion score	Oocyst index	Mortality (%)
N control	459.77^{a}	822.75^{a}	1.33	1.98	0.00^{e}	0.00×10^{5g}	0.0
P control	271.70^{d}	616.50^{b}	1.47	2.00	2.75^{a}	13.31×10^{5a}	7.1
1% AFG	348.32^{bc}	799.00^{a}	1.41	1.83	1.50^{bc}	10.08×10^{5e}	4.3
1% DA	327.55^{bd}	779.25^{a}	1.14	1.85	1.75^{b}	10.26×10^{5d}	5.0
1% Lacplus	359.05^{bc}	809.00^{a}	1.13	1.67	1.00^{cd}	9.55×10^{5f}	2.9
2% AFG	315.90^{cd}	735.25^{a}	1.22	1.98	2.00^{b}	11.45×10^{5b}	6.8
2% DA	387.27^{b}	816.25^{a}	1.19	1.63	0.75^{d}	9.49×10^{5f}	2.9
2% Lacplus	323.27^{bd}	745.00^{a}	1.30	1.88	1.75^{b}	10.99×10^{5c}	5.7
SEM	22.73	30.70	-	-	0.25	-	-

* Statistical analysis was not possible because of group feeding of chicks.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Organic acid supplemented groups showed better FCR compared with N and P control groups. Among organic acid groups, the lowest FCR was observed in the group supplemented with 1 and 2% DA followed by the groups supplemented with 1% Lacplus.

Lesion score, oocyst index and mortality rate during experimental coccidiosis

The results of the lesion scores are indicated in Table 2. The uninfected N control group showed zero score. All the groups supplemented with organic acids showed significantly (P<0.05) lower lesion scores than infected non-supplemented group. Among all organic acid groups, the lowest lesion scores were shown by the group supplemented with 2% DA and 1% Lacplus.

The results of the oocyst index (Table 2) revealed a pattern relatively similar to that of lesion scores across different groups. The oocyst index were lower (P<0.05) in supplemented groups compared with infected non-supplemented group.

And it was maximum in 2% DA and 1% Lacplus groups followed by 1% AFG, 1% DA, 2% Lacplus, and 2% AFG organic acid groups, respectively. The results revealed that all the organic acid groups have shown anticoccidial effects in terms of oocyst index.

The mortality percentage (Table 2) was higher in the infected non-supplemented P control group compared with organic acid groups. Among organic acid groups, the mortality was numerically lower in 2% DA and 1% Lacplus groups followed by 1% AFG, 1% DA, 2% Lacplus, and 2% AFG organic acid groups, respectively. Figure 1 has depicted pictures of infected chicks, bloody feces, died chicks due to *E. tenella* infections and cecal hemorrhage.

The pH of the ceca during experimental coccidiosis

The results on the pH of cecal contents in different experimental groups are shown in (Table 3). Within the periods (10 and 23 days post), a significant difference (P<0.05) was observed among intestinal pH of the organic acid groups, and both N and P controls. A maximum pH was observed in the infected non-organic acid (P control).

Percentages of CD4/CD8

Determination of percentages of CD4 T-cell molecules by ELISA in Figure 2 shows that the percentages of CD4 at both 22- and 35-days' post-hatch were increased in all infected groups compared with uninfected control group, and there were significant differences between organic acids treatment groups and controls (P<0.05).

Table 3 Effect of organic acids treatment on mean (n=5) pH of caecal contents (on 22 and 35 days' post hatch) in broiler chickens

Treatments	pH at 22 days	pH at 35 days
N control	6.71^{ab}	6.25^{ab}
P control	7.17^{a}	6.67 ^{a}
1% AFG	6.26^{bc}	6.27^{ab}
1% DA	6.26^{bc}	6.27^{ab}
1% Lacplus	6.05^{c}	6.28^{ab}
2% AFG	6.18^{c}	6.24^{ab}
2% DA	6.09^{c}	5.83^{b}
2% Lacplus	6.12^{c}	6.13^{b}
SEM	0.17	0.17

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

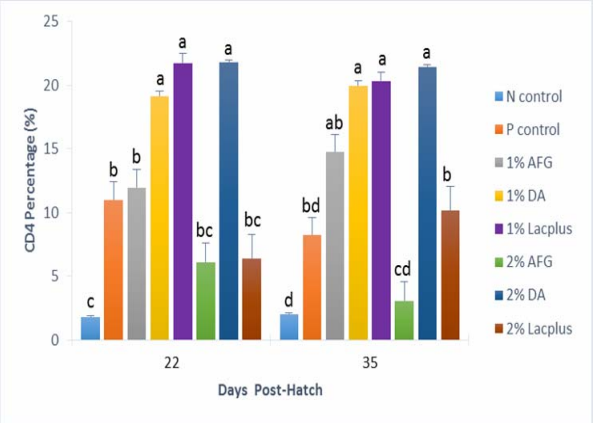

Figure 2 Effects of organic acids on the percentages of CD4 T-cell molecules by ELISA
The tissues were isolated at 22 and 35 d post-hatch
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

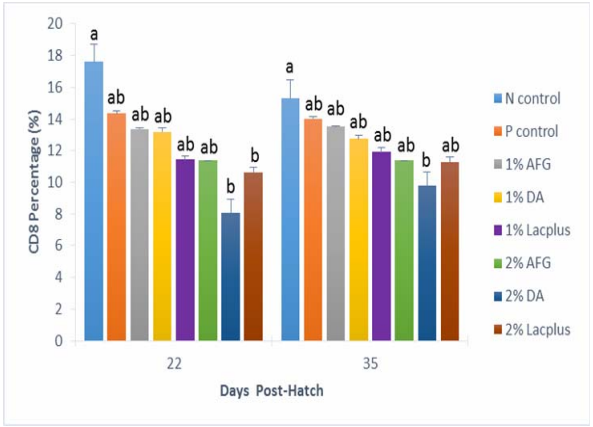

Figure 3 Effects of organic acids on the percentages of CD8 T-cell molecules by ELISA
The tissues were isolated at 22 and 35 d post-hatch
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

Higher percentages were observed in 1% of DA, 1%Lacplus and 2% DA compared to infected and uninfected groups. Determination of percentages of CD8 T-cell molecules by ELISA in Figure 3 shows that the percentages of CD8 at both 22- and 35-days' post-hatch, there were significant difference between organic acids treatment groups and N control (P<0.05). At 22-day, lower percentages were observed in 2% DA and 2% Lacplus; while only 2% DA was lowest at 35-day compared to non-infected group.

Cecum chemokine and cytokines productions

At 22 days' post-hatch, the transcript levels of IFN-γ were significantly higher in 1% Lacplus and 2% DA groups compared with other organic acids and control groups. But only 2% DA was significantly (P<0.05) differed with N control at 35 days' post-hatch as shown in Figure 4. While both at 22- and 35-days' post-hatch, the transcript levels of IL-8, IL15 and IL-17 were significantly (P<0.05) higher in 2% DA groups compared with other organic acids and control groups as depicted in Figures 5, 6 and 7.

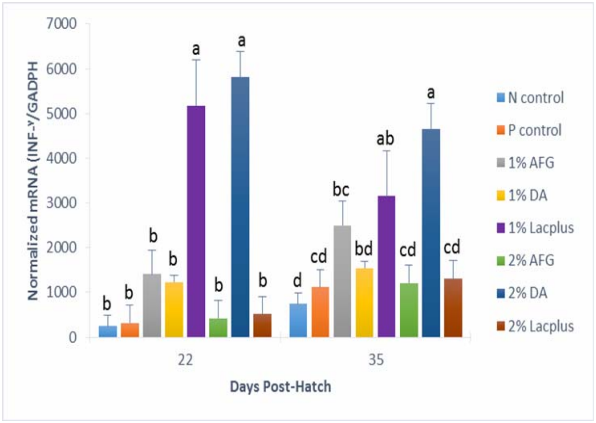

Figure 4 Effects of organic acids on the level of IFN-γ mRNA transcript in cecum epithelial tissue of Yellow broilers
The tissues were isolated at 22 and 35 d post-hatch, and the levels of IFN-γ transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

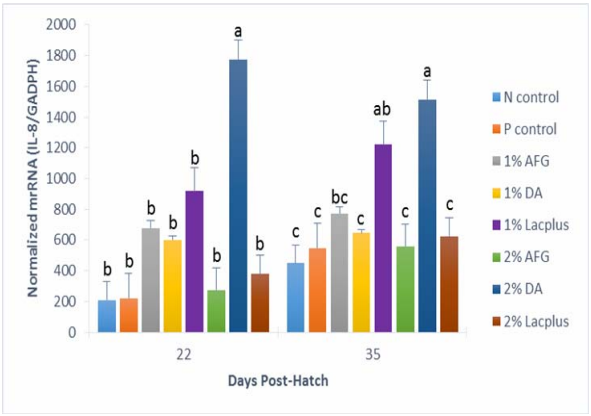

Figure 5 Effects of organic acids on the level of IL-8 mRNA transcript in cecum epithelial tissue of Yellow broilers
The tissues were isolated at 22 and 35 d post-hatch, and the levels of IL-8 transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5) Bars with different letters differ significantly (P<0.05)

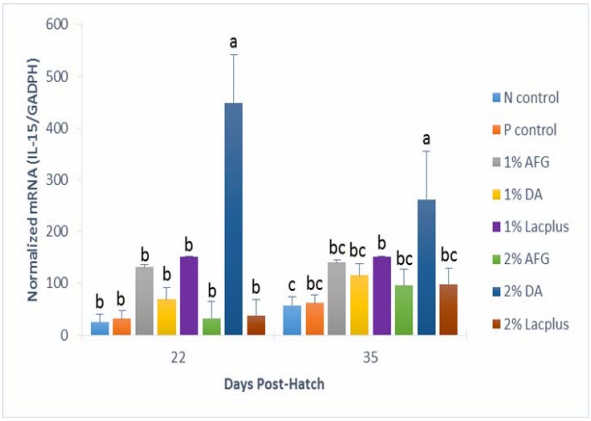

Figure 6 Effects of organic acids on the level of IL-15 mRNA transcript in cecum epithelial tissue of Yellow broilers
The tissues were isolated at 22 and 35 d post-hatch, and the levels of IL-15 transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

Splenic chemokine and cytokines production

The mRNA expression levels of IFN-γ in the spleen of organic acids and control groups are shown in Figure 8. Both at 22- and 35-days' post-hatch, 2% DA differed (P<0.05), higher than other organic acid and 2 control groups. While the mRNA expression levels of IL-8 in the spleen of organic acids and control groups are shown in Figure 9. At 22-day post-hatch, 1% Lacplus and 2% DA differed (P<0.05), higher than other organic acids and 2 control groups, but only 2% DA at 35-day post-hatch was highest. The mRNA expression levels of IL-15 in the spleen of organic acids and control groups are shown in Figure 10.

Both at 22- and 35-days' post-hatch 2% DA differed (P<0.05) higher than other organic acid and 2 control groups. The mRNA expression levels of IL-17 in the spleen of organic acids and control groups are shown in Figure 11. At 22-day post-hatch 2% DA was higher than other organic acids and N control groups.

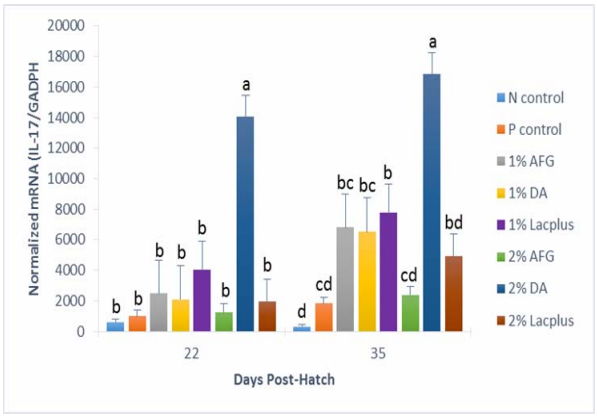

Figure 7 Effects of organic acids on the level of IL-17 mRNA transcript in cecum epithelial tissue of Yellow broilers
The tissues were isolated at 22 and 35 d post-hatch, and the levels of IL-17 transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

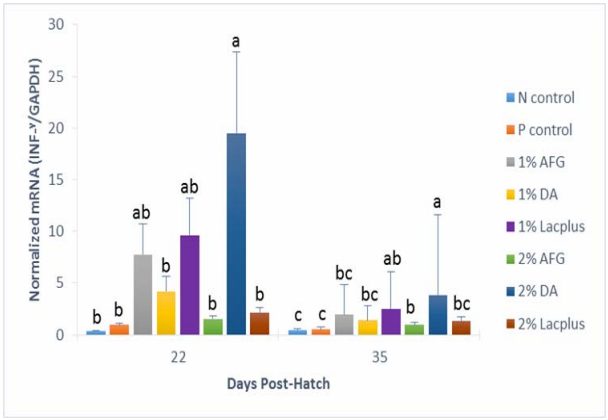

Figure 8 Effects of organic acids on the level of IFN-γ mRNA transcript in cecum epithelial tissue of Yellow broilers
The tissues were isolated at 22 and 35 d post-hatch, and the levels of IFN-γ transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

However, 1% AFG, 1% Lacplus and 2% DA were significantly (P<0.05) different from N control at 35-day post-hatch.

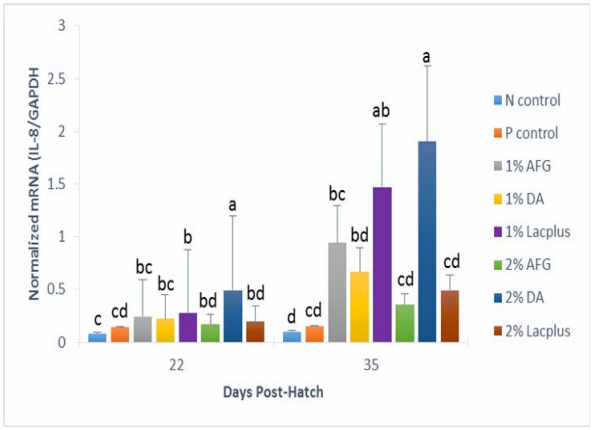

Figure 9 Effects of organic acids on the level of IL-8 mRNA transcript in spleen tissue of Yellow broilers
The spleen was isolated at 22 and 35 d post-hatch, and the levels of IL-17 transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

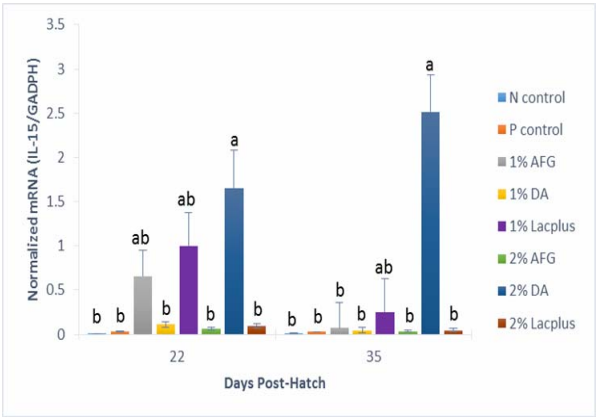

Figure 10 Effects of organic acids on the level of IL-15 mRNA transcript in spleen tissue of Yellow broilers
The spleen was isolated at 22 and 35 d post-hatch, and the levels of IL-17 transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

In previous years, antibiotics were used to prevent disease and improve the performance of poultry, but its overuse caused bacterial resistance and consequently its ban in Europe and other developed countries; thus, increased the urgency to find suitable alternatives to antibiotics (Khan *et al*. 2012; Okoro *et al*. 2016; Alhidary *et al*. 2016; Khan *et al*. 2016a; Mahanta *et al*. 2017). Organic acids are among alternative growth promoters that have been shown to stimulate growth performance in poultry (Abudabos and Al-Mufarrej, 2014), Specific organic acids reduce the pH of poultry feed and lead to the buffering capacity of feed to control intestinal microbial population (Dhama *et al*. 2014; Abudabos *et al*. 2017).

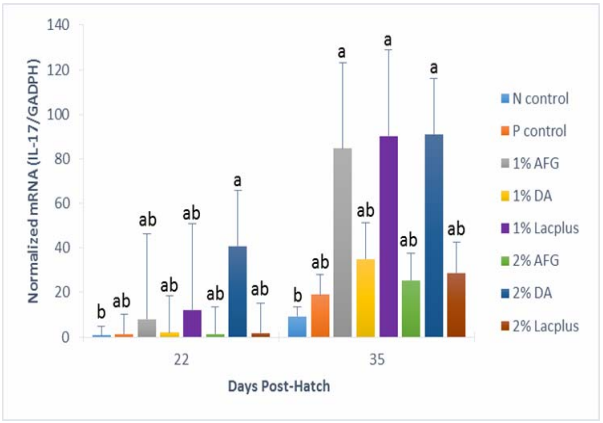

Figure 11 Effects of organic acids on the level of IL-17 mRNA transcript in spleen tissue of Yellow broilers
The spleen was isolated at 22 and 35 d post-hatch, and the levels of IL-17 transcripts were quantified by RT-PCR and normalized to the levels of GAPDH transcripts
Each bar represents the Mean ± SE value (n=5)
Bars with different letters differ significantly (P<0.05)

Ghazvinian *et al*. (2018) reported that diets including organic acids enhanced the microbial population of broiler gut, improve productive traits and health status in broiler chickens. Fathi *et al*. (2016) worked on feed supplementation with increasing levels of organic acids, the results revealed that supplementation of broiler fed with 0.5% of formic, propionic acids or ammonium salts showed the best results in body weight gain, feed conversion rate and final body weight which influenced the productivity traits. In our present study, weight gain was significantly (P<0.05) reduced in *E. tenella* infected group compared with uninfected birds at both 22- and 35-days' post-hatch.

The first effect of organic acids in animal agriculture is related to feed preservation, likewise the activity of organic acids toward gut microflora is very similar. In both cases, the acid changes the microbial populations in accordance with its antimicrobial spectrum of activity (Dibner and Buttin, 2002).

In Pakistan, hydrochloric acid [HCl] and formic acid are being used extensively at various dose rate as anticoccidial agent in the local poultry industry. Manzoor *et al*. (2013) had proposed that lower doses of HCl and formic acid have the potential to be used as alternative to chemotherapeutic drugs for *Eimeria tenella* control.

But the exact mode of action against *Eimeria* species is not clear; therefore, the authors suggested further studies to determine the possible minimum safe levels of HCl and formic acid and to find out the exact mode of action of these acids against coccidia.

The current study has worked toward this direction, however different organic acid products (AFG, AD and Lacplus) were added to broiler diet at 1% and 2% level fed to chicken infected with *E. tenella*; and considerable attention are paid to immune response. Previous studies were reflected in using individual organic acid; nonetheless, this experiment was designed to use blend of organic acids by anticipating to boost synergistic effects.

The severity of *Eimeria* infection is normally assessed by reduced body weight gain and fecal excretion of oocysts (Idris *et al*. 1997). Though it is desirable to see the combined positive effects of increased weight gain and reduced oocyst shedding as indicators of host resistance to coccidiosis, however, direct correlation between these two parameters has not always been observed (Lee *et al*. 2007a; Lee *et al*. 2007b; Dalloul *et al*. 2005). Nonetheless, the present results demonstrated both increased weight gain and lowered parasite excretion in infected chickens fed organic acids especially the 2% DA and 1% Lacplus groups. A series of studies were conducted to investigate how these 3 products of organic acids supplemented to diet would influence two particular parameters of chicken inflammation on protective immunity T-cell subpopulation of CD4 and CD8, and spleen and cecum cytokine gene expression during experimental coccidiosis.

Changes in immune related genes expression and intestinal T cells subpopulation after *E. tenella* infection and treatment of chicken with organic acids were examined to determine protective immunity. It was reported that $CD4^+$ and $CD8^+$ cells are involved in different phases of host immunity (Lillehoj *et al*. 2004).

The result of this study showed that greater percentages of CD4 T-cell molecules and decreased CD8 T-cell molecules were observed in infected birds fed 2% DA at both 22- and 35-days' post-hatch; also 1% DA and 1% Lacplus were greater percent in CD4 and lower in CD8 compared to N control at both periods, suggesting that a protective function of these T-cell subsets in innate immune response against *E. tenella* infection. This finding is in agreement with the work of Yun *et al*. (2003) and Lee *et al*. (2009); though in our experiment $\alpha\beta$-TCR^+ and $\gamma\delta$-TCR^+ were not detected, because Capcellia assay was employed. It is ELISA, an alternative method to enumerate CD4/CD8 T lymphocyte, with low cost and less need for specially trained personal for cytofluorometry which is helpful in developing countries (World Health Organization, 1992; Carriere *et al*. 1994; Nicholson *et al*. 1994; Pinilla Moraza *et al*. 1997; Serge *et al*. 1999).

Accordingly, current results revealed that the cecal and splenic chemokine and cytokines mRNA expression encoding IFN-γ, IL-8, IL-15 and IL-17 showed higher levels of transcript compared to N and P controls, suggesting that these organic acid products might have exerted their protective effects by improving their production. Similar results were reported in previous literatures (Lee *et al*. 2007c; Lee *et al*. 2009). $CD4^+$ cells are the major cells producing the proinflammatory cytokine IFN-γ in response to antigen challenge (McSorley *et al*. 2000); and IFN-γ plays an important role in protective immunity to experimental coccidiosis (Lillehoj and Choi, 1998). Lillehoj *et al*. (2001) reported that chickens vaccinated with 3-1E DNA in combination with IL-8 or IL-15 shed significantly fewer fecal oocysts compared with chickens vaccinated with 3-1E alone. Additionally, *in ovo* co-vaccination with 3-1E plus IL-15 or IL-17 reduced oocyst output beyond that diminished by 3-1E alone. The present work demonstrated that organic acids promote protective immunity against coccidiosis by motivating proliferation of local lymphocytes and production of cytokines. Among the three products of organic acids used in this experiment, all organic acids groups have exacted their effects compared to non-organic acids (N and P control) groups. However, in comparison by all parameters detected, 2% DA, 1% Lacplus and 1% DA groups appeared more prominent, followed by 1% AFG, 2% Lacplus, and 2% AFG organic acid groups, respectively. The immune-protective effects were most evident by inclusion of 2% DA and 1% Lacplus in the standard chicken diet. Thus, it might be due to synergistic effects of organic acid component of Activate® DA and Lacplus. Generally organic acids could serve as effective alternatives which would be equally efficacious and have no negative impact on animal welfare and consumer health, as ban was imposed on antibiotic growth promoters (AGPs) in livestock and poultry diets in some countries.

A study carried out in Thailand by Lückstädt and Kühlmann (2013) demonstrated that including water acidification in broiler production has beneficial effects on the performance of broilers and may be considered as a low-cost option to improve production parameters in general. Abbas *et al*. (2011) highlighted that with the escalating cost of anticoccidial drugs, how nice it would be to replace expensive anticoccidial drugs with dilute hydrochloric acid, the cost of which is nil.

CONCLUSION

Conclusively, all the organic acids used in this study have shown anticoccidial effects especially Activate® DA at 2% levels and 1% Lacplus. Thus, they could serve as effective alternatives which would be equally efficacious and have no negative impact on animal welfare and consumer health. Further research to better understand immuno-modulatory effects of organic acids against other *Eimeria* species is advocated. It is the first report that highlight on immuno-

protective effects, mechanism and action mode of dietary organic acids supplementation in broiler chickens against *E. tenella* infection. It is found that T-cell subpopulations CD4/CD8, and inflammatory chemokine and cytokines IFN-γ, IL-8, IL-15 and IL-17 have played important roles.

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