Pakistan J. Zool., pp 1-6, 2022. DOI: https://dx.doi.org/10.17582/journal.pjz/20210809100837

Evaluation of Antimicrobial Activity of Organic Acids against *Campylobacter jejuni* in Broilers

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Article Information
Received 09 August 2021
Revised 07 October 2021
Accepted 19 October 2021
Available online 04 February 2022 (early access)

Authors' Contribution
FG, MR, AG and HM conceived and designed study. FG executed the experiments. FG and MR analyzed the data. FG and MR prepared the manuscript. The manuscript is critically revised by all authors and approved this final version of manuscript.

Key words
***Campylobacter jejuni*, Organic acid, Broiler chicken, Feed water, Bacteria, Food safety, Poultry**

ABSTRACT

Campylobacteriosis is a collective term used for the infection caused by the members of *Campylobacter* species. The causative agent is *Campylobacter* that asymptomatically colonizes broilers during development and contaminates it during slaughter. Outbreaks mostly start from the ingestion of contaminated poultry products or infected water. Reducing colonization of *Campylobacter jejuni* in the gut can be useful in decreasing the contamination of the poultry. Different organic acids display potential as a substitute of antibiotics. These not only improve poultry performance by modifying the pH of the gastro-intestinal tract of bird, but also change the composition of its microbiome and ultimately protecting the chicken from pH-sensitive pathogens. The purpose of this study was to define the bactericidal action of organic acids on *Campylobacter jejuni*, individually and in combination. Total 120 broiler chickens were randomly distributed in ten groups. The groups included negative and positive control, pure organic acid group and commercial organic acid formulation group. Excluding negative control group, all other groups were orally challenged with 0.1 ml of the 6-Log 10 CFU/ml of the *Campylobacter jejuni* culture in normal saline via oral route. Cloacal samples were collected for *Campylobacter* count, body weight (BW) and feed conversion ratio (FCR), which were determined weekly and cumulatively for 35 days. The birds of a specific treatment group were given organic acid on daily basis for 6-8 h. Excluding the negative control group, all groups were tested with fresh culture of *Campylobacter jejuni* on 14, 21, 28 and 35 day of age. Bacterial count was performed at 6, 8, 13, 15, 20, 22, 27, 29, 34 and 36 day of age. The results suggest synergistic actions of a mixture of organic acids are effective for decreasing *Campylobacter jejuni* colonization *in vivo.* Moreover, our study also suggests that there is no direct impact of organic acids on weight gain and FCR of the birds statistically.

INTRODUCTION

Campylobacter jejuni is recognized to be the prominent cause of human intestinal ailments globally (Gharib *et al*., 2012). Poultry and poultry products are identified to be the main reservoir of this bacterium (Jørgensen *et al*., 2002). *Campylobacter* infections may be fatal in children, aged and immuno-compromised patients (Beier *et al*., 2020). An infective dosage of only around 500-800 of *C. jejuni* are required for infection (Kothary and Babu, 2001). Using replacement ways and means to prevent colonization of these bacteria in the intestinal tract of flocks may help regulate the spread of these bacteria from food to human (Rosenquist *et al*., 2006).

Certain organic acids have long been used as food condiments and for extending the shelf life of perishable food constituents. Fatty acids have been described to possess antimicrobial activities against a wide range of microorganisms (Hermans *et al*., 2011). The mechanism of organic acid inhibition is presumed to be principally dependent on pH (Nannapaneni *et al*., 2009) or the un-dissociated arrangement of the organic acids (Fernández and Pisón, 1996), which are thought to penetrate the lipid membrane. Nevertheless, the precise mechanism by which organic acids constrain bacteria is not known (Beier *et al*., 2020). It has furthermore been stated that using organic acids in drinking water lessens *C. jejuni* population in crop and carcasses (Byrd *et al*., 2001).

The purpose of this study was to examine the bactericidal effects of different organic acids, either single on in a mixture, in reducing cecal colonization and excretion of *C. jejuni* if given through drinking water. Body weight gain and feed conversion ratio were also monitored.

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0030-9923/2022/0001-0001 $ 9.00/0

MATERIALS AND METHODS

Animal housing

A total of 120 broiler chicks were acquired from a commercial hatchery on the day of hatch and raised for 35 days in the experimental chamber of animal shelter, Institute of Microbiology, UVAS, Lahore. The primary weight of the hatchlings was 40.0–41.35 g. The cloacal swabs in normal saline were taken and grown on CCDA under microaerophillic conditions to confirm the chicks are *Campylobacter* free. The chicks were kept on floor pens with wood shavings. The composition and nutrient value of the basal diet is described in Table I. Feed and water were accessible *ad libitum* for the 35-day trial.

Table I. The composition and nutrient values of basal diet (%).

Ingredients	Starter (1-17 Days)	Grower (18-35 Days)
C.P	22.7	21.2
Fat	4.1	4.6
Ash	3.6	4.0
M.E	2900	2980
Ca	0.9	1.0
P	0.52	0.46
D. Lysin	1.2	1.16
D. Methionine	0.5	0.464
D. Threonine	0.79	0.76

Microbial strain

C. jejuni strain ATCC 33291, used for inoculation of the birds, was kept frozen at -80˚C in 80% glycerol solution. The innoculum was prepared for the challenge trial by culturing it on Charcoal-Cephoperazone-Deoxycholate-Agar (CCDA) and incubating for 48 h at 42˚C under microaerophillic conditions (5% O_2, 10% CO_2 and 85% N_2). The bacteria were harvested and diluted in normal saline solution to the precise viable concentration of 6-Log 10 CFU/ml according to the technique explained by (Lamb-Rosteski*et al*., 2008). Inoculum concentration was assessed by 0.1 MacFarland tubes. The inoculum was kept on ice for less than 1 h before oral administration of chicks. Except the negative control group, the rest of the birds received 1 ml dose of the inoculum in normal saline at day 7 of the trial (Table II).

Organic acids as an alternate therapy

Four pure organic acids (Formic Acid, Propionic Acid, Acetic Acid and Lactic Acid) and four commercially available preparations i.e. Multiacid (EWABO, The Hygiene Company, Wietmarschen, Germany) composed of formic acid, acetic acid, propionic acid and lactic acid, SELKO-pH (Selko Feed Additives, Tilburg, Netherlands) made up of ammonium formate, formic acid and acetic acid, Acid Punch (Herbavita Feed Supplements, ZS Biotech, Pakistan) consisted of formic acid, propionic acid, acetic acid and lactic acid and Lipto-Safe L (Forward Solutions, Pakistan) contained formic acid, propionic acid, citric acid and lactic acid, were used in treatment groups to check the reduction in microbial count. The pH of all acids and formulations were adjusted at level 4.

Experimental design

The chicks were randomly distributed to eight treatment groups containing 10 birds in respective groups. The birds of the particular group were given respective organic acid on daily basis in drinking water. *C. jejuni* lives as a commensal in chicken gut. Therefore, the microbial load of this pathogen was estimated by taking cloacal swab in normal saline and growing on CCDA under microaerophillic conditions, a day before administering the fresh culture of this bacterium. The initial count was noted down. Except the negative control group, all treatment groups were tested with fresh culture of *C. jejuni* (0.1 MacFarland) on 14, 21, 28 and 35 day of age. The bacterium was allowed 24 h to colonize the gut of the bird. After 24 h, the microbial load was again calculated by taking the cloacal swab in normal saline and growing the sample on CCDA under microaerophillic conditions, a day after administering the innoculum. The reading was again noted down and compared with the initial count. The study plan is presented in Table II. Chickens of all groups were vaccinated against New Castle Disease (NDV) vaccine according to the routine vaccination program of the broilers which contains administration of live virus "LaSota" vaccine via eye drop route at day 5 followed by booster dose at day 15.

Weight gain analysis on weekly basis

The results of different organic acids, affecting the body weight gain (BW) of the birds of all groups, were obtained on weekly basis that is on day 1, 7, 14, 21, 28 and 35. Whereas, feed intake and water consumption were checked on daily basis. For 35-day trial, 2kg feed was given to all groups for 24 h every day. Feed conversion ratio (FCR) was determined by the intake of the feed vs weight gain of the bird.

Microbial count

Microbial load for *C. jejuni* was evaluated from each bird by collecting the cloacal swab in normal saline at 6, 8, 13, 15, 20, 22, 27, 29, 34 and 36 day of age. The samples were diluted serially ten-fold and counted on CCDA under microaerophillic conditions. Bacterial colonies were counted and CFU/gram was converted into log_{10} values. The mean ± standard deviation (S.D) of log_{10} values were calculated and compared among groups. Log reduction of plate count was calculated by subtracting log values of day post infection (DPI) from day before infection (DBI).

Statistical analysis

The data was transferred to the spreadsheet using MS Excel 2016 and the results were evaluated through Statistical Package for the Social Sciences (SPSS) version 16.0. Enumeration data was presented as Mean± S.D log_{10} CFU/mL and compared by one-way ANOVA followed by Tukey's multiple comparison test at $p< 0.05$ level of significance by SPSS.

RESULTS AND DISCUSSION

The control strategies on-farm, for the decrease of *Campylobacter*, have been comprehensively studied as a significance of well-established association of

Table II. Treatment group description (organic acid/distilled water) at pH 4.

Groups		Treatment
Control groups		
A	Negative control	No treatment
B	*C. jejuni*	*C. jejuni* treatment weekly
Pure organic acid model		
C	Formic acid (0.1mL/1L)	Daily+ *C. jejuni* treatment weekly
D	Acetic acid (0.1mL/100mL)	Daily+ *C. jejuni* treatment weekly
E	Propionic acid (0.1mL/100mL)	Daily+ *C. jejuni* treatment weekly
F	Lactic acid (0.1mL/130mL)	Daily+ *C. jejuni* treatment weekly
Commercial organic acid model		
G	Acid punch (0.25mL/1000mL)	Daily+ *C. jejuni* treatment weekly
H	Lipto-Safe L (0.1mL/250mL)	Daily+ *C. jejuni* treatment weekly
I	SELKO-pH (0.1mL/180mL)	Daily+ *C. jejuni* treatment weekly
J	Multiacid (0.1mL/200mL)	Daily+ *C. jejuni* treatment weekly
K	Antibiotic (Ciprofloxacin)	Antibiotic formulation+ *C. jejuni* treatment weekly

Table III. Antibacterial activity of organic acids against *Campylobacter jejuni.*

Groups	6 (BC) Count	Day 7		Day 14		Day 21		Day 28		Day 35	
		Count	L.D	Count	L.D	Count	L.D	Count	L.D	Count	L.D
A	3.41	3.44	-	3.33	-	3.31	-	3.77	-	3.66	-
B	3.37	6.55	-	6.45	-	6.27	-	6.22	-	6.15	-
C	3.05	4.16	3.19	3.88	2.17	3.23	2.82	3.61	2.44	3.77	2.28
D	3.18	4.32	2.23	3.32	2.9	3.19	2.31	3.17	3.08	3.05	2.6
E	3.15	5.07	1.48	5.01	1.54	4.77	1.45	4.92	1.63	4.61	1.94
F	3.35	4.72	1.83	4.61	1.94	4.38	1.84	4.05	1.45	4.01	2.63
G	3.33	3.93	2.12	3.77	2.28	3.75	2.3	3.61	2.44	3.23	2.82
H	3.27	4.01	2.23	4.06	2.16	3.82	2.45	3.75	2.4	3.61	2.54
I	3.39	4.38	1.84	4.05	2.17	4.12	2.1	4.24	2.83	4.16	2.26
J	3.33	4.61	1.94	4.77	1.45	3.93	2.34	3.82	2.45	3.77	2.38
K	3.29	4.88	1.67	4.86	1.54	4.06	2.16	3.82	2.45	3.75	2.4

Count, Mean ± SEM log^{10}; LD, log^{10} reduction of *C. jejuni*.

Campylobacter and poultry meat (Santini *et al*., 2010; Gharib *et al*., 2012; Neal-McKinney and Konkel, 2012; Nishiyama *et al*., 2014) Studies designate that adding organic acid to the drinking water aids in the reduction of pathogens in the water and the crop/ proventriculus, to control gut micro-flora, to intensify the digestion of feed and to increase growth performance (Byrd *et al*., 2001; Açıkgöz *et al*., 2011; Hamed and Hassan, 2013). However, the exact mechanism(s) by which organic acids prevent bacteria are not known (Kim *et al*., 2019).

In this project, we compared the bactericidal effect of organic acids on load of *C. jejuni* at pH level 4. The outcomes of this experiment demonstrate that the acidification of the drinking water successfully reduced the number of *C. jejuni* in the guts of the experimental birds. The antibacterial activity of organic acids against *C. jejuni* at different weeks of 35 days trial is presented in the Table III. The effect of organic acids in reduction of *C. jejuni* is presented in Figure 1.

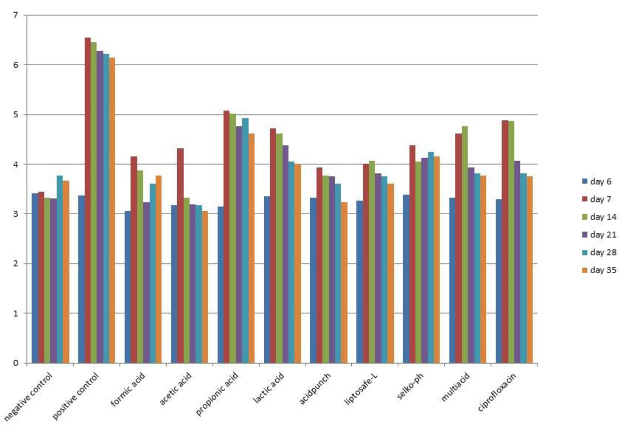

Fig. 1. Growth of *C. jejuni* on selective media (CCDA).

The main objective of our study plan was to evaluate the effect of organic acids on *C. jejuni* either already present in the chicken gut or administered orally. The experimental trial was designed to choose for an effective bactericidal method; whether it's the use of an individual organic acid as a better controlling measure or should it be a mixture of organic acids that obstructs the colonization of *C. jejuni* in the chicken gut.

Although all treatment groups show a decrease of *C. jejuni*, the treatment groups involving the combination of organic acids gave better results. Group G (Acid Punch) lower the load in the steadiest manner, followed by group H (LiptoSafe-L). No significant increase in the body weight of the birds was noted during the first 14 days of the trial. After the 2 weeks, positive control versus negative control showed differences in body weight gain.

Weekly average weight gains of birds throughout the 35 days trial is presented in Table IV, while weekly FCR of experimental groups is presented in Table V.

Table IV. Weekly average weight gain of birds in grams.

Groups	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
A	41.00	263.31	407.46	958.47	1421.37	1912.28
B	41.25	273.12	429.34	977.29	1431.27	1960.38
C	40.35	269.65	434.65	968.48	1464.17	1954.18
D	41.29	278.76	469.13	1023.48	1502.36	1983.37
E	40.00	259.46	434.64	996.39	1554.18	1918.19
F	41.30	252.43	465.21	1002.38	1535.26	1949.33
G	41.14	288.87	456.19	1050.36	1575.32	2017.28
H	41.35	265.69	446.45	960.10	1521.25	2048.19
I	40.16	282.43	432.32	1022.28	1536.18	1922.26
J	41.05	278.56	406.47	1087.36	1518.16	1951.66
K	40.26	269.47	442.34	1058.47	1671.14	2069.34

Table V. Weekly FCR of experimental groups.

Groups	Day 7	Day 14	Day 21	Day 28	Day 35
A	0.94	1.13	1.33	1.42	1.69
B	0.97	1.16	1.33	1.46	1.63
C	0.97	1.14	1.39	1.43	1.59
D	0.93	1.06	1.31	1.42	1.46
E	0.96	1.10	1.36	1.44	1.55
F	0.90	1.10	1.34	1.41	1.46
G	0.91	1.13	1.32	1.42	1.42
H	0.95	1.12	1.31	1.42	1.46
I	0.93	1.10	1.34	1.46	1.60
J	0.95	1.06	1.33	1.43	1.55
K	0.96	1.13	1.36	1.42	1.65

After the conclusion of 6 weeks, the weight gains varied considerably ($P < 0.05$) among treatments. The maximum weight gain (2069.3 g) was noted down for group K which includes antibiotic ciprofloxacin. Organic acid treatment groups, composed of individual acids and mixtures of these individual acids, are found to execute anti-microbial activities comparable to those of antibiotics (Wang *et al*., 2009). The K group was for comparison sake with the outcomes of organic acid treatment groups. The results of group K was followed by treatment group H containing commercial product LiptoSafe-L (2048.1 g) and group G comprising Acid Punch (2017.2 g), showing synergistic effect of the organic acids is more favorable than the use of individual acids. No significant difference in terms of body weight was observed in the negative control group A (1912.2 g) and positive control group B (1960.3 g).

Chicks of the group G (Acid Punch) displayed a significant improvement ($P < 0.05$) in terms of FCR (1.42) as compared with the chicks of group H (LiptoSafe-L), group F (Lactic acid) and group D (Acetic acid) with the same FCR (1.46). Why group H (LiptoSafe-L) did not gave better results in terms of FCR may be because *C. jejuni* cannot utilize the citric acid cycle to yield energy but it can use citric acid cycle intermediates, acetic acid and lactic acid to produce energy. The progress in the FCR in group G (Acid Punch) could be possibly because of the improved utilization of the nutrients causing increased body weight gain.

There was a statistically significant difference between the treatment groups at the $p < 0.05$ level of significance as demonstrated by one-way ANOVA ($F (10, 44)= 29.8$, $p= 0.00$) Details in supplementary data S1 A Tukey's post hoc test showed that there is significant difference in the group means of negative control group (Group 1) to positive control group (Group 2; sig = 0.00), propionic acid group (Group 5; sig = 0.00), lactic acid group (Group 6; sig = 0.10) and antibiotic group (Group 11; sig = 0.027). Positive control group (Group 2) to all groups (sig = 0.00). Formic acid group (Group3) to positive control group (Group 2; sig = 0.00) and lactic acid group (Group 5; sig = 0.00). Acetic acid group (Group 4) to positive control group (Group 2; sig = 0.00), propionic acid group (Group 5; sig = 0.00), lactic acid group (Group 6; sig = 0.03), Selko-pH group (Group 9; sig = 0.025), MultiAcid group (Group 10; sig = 0.028) and antibiotic group (Group 11; sig = 0.008). Propionic acid group (Group 5) to negative control group (Group 1; sig = 0.00), positive control group (Group 2; sig = 0.00), formic acid group (Group 3; sig = 0.00), acetic acid group (Group 4; sig = 0.00), Acidpunch group (Group 7; sig = 0.00) and Liptosafe group (Group 8; sig = 0.001). Lactic acid group (Group 6) to negative control group (Group 1; sig = 0.010), positive control group (Group 2; sig = 0.00) and acetic acid group (Group 4; sig = 0.003). Acidpunch group (Group 7) to positive control group (Group 2; sig = 0.00) and propionic acid group (Group 5; sig = 0.00). Lipto-safe group (Group 8) to positive control group (Group 2; sig = 0.00) and propionic acid group (Group 5; sig = 0.001). Selko-pH group (Group 9) to positive control group (Group 2; sig = 0.00) and acetic acid group (Group 4; sig = 0.025). MultiAcid group (Group 10) to positive control group (Group 2; sig = 0.00) and acetic acid group (Group 4; sig = 0.028). Antibiotic group (Group 11) to negative control group (Group1; sig = 0.027), positive control group (Group 2; sig = 0.00) and acetic acid group (Group 4; sig = 0.008). The statistical results show that there is no significant effect of organic acids on the WG of birds or FCR.

As the bacterial colonization is reduced, it gives a positive outcome on the health of the bird specified by good health, notable weight gain and acceptable FCR. These reductions, although appeared to be very small but important, can have serious impact on poultry industry.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this study displayed that synergistic actions of the combined organic acid supplementation (Acid Punch) presented higher decline rates of *Campylobacter* spp. than the single organic acids. It is not only effective in dropping the microbial count in an *in vivo* trial experiment, but also retains the general wellbeing by inhibiting the development of possible food borne pathogens.

ACKNOWLEDGEMENTS

Authors acknowledge Higher Education Commission (HEC) funded project# 4333/NRPU/R&D/HEC/14/278 entitled "Evaluation of anti- *Campylobacter* activity of indigenous probiotic *Lactobacilli* alone and in combination with different organic acids in poultry" for supporting this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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