TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2016) 40: 329-336
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doi:10.3906/vet-1502-68

The effects of probiotics, organic acid, and a medicinal plant on the immune system and gastrointestinal microflora in broilers challenged with *Campylobacter jejuni*

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Received: 18.02.2015 • **Accepted/Published Online:** 31.01.2016 • **Final Version:** 07.04.2016

Abstract: *Campylobacter jejuni*, a zoonotic bacterial pathogen with worldwide distribution, infects about 400 million humans in the world annually. In order to reduce *C. jejuni* colonization in the gastrointestinal tracts of broilers and make chickens less susceptible to colonization, four commercial products based on organic acid, probiotics, and medicinal plants were used. In this experiment, 210 one-day-old male broiler chicks (Ross 308) were assigned to 7 treatment groups randomly with 3 replications and 10 birds in each pen. Birds were challenged on day 21 by 1 mL of 6×10^7 CFU/mL *C. jejuni* live suspension and samples were collected on days 28 and 42. The immune system's efficiency was evaluated by lymphoid organ development assessment and two specific and nonspecific immune system tests. The cecal contents and liver were considered for *C. jejuni* enumeration. According to the results, all treatments except one showed a significant difference from the control in terms of cecal colonization ($P \leq 0.001$). Probiotic and *Echinacea purpurea* treatments could significantly increase the immune system's efficiency ($P \leq 0.001$). In general, in this study we provide evidence that some commercial feed and water additives can reduce chickens' susceptibility to *C. jejuni* colonization and also can effectively increase immune system efficiency.

Key words: Broilers, *Campylobacter jejuni*, colonization, *Echinacea purpurea*, organic acid, probiotics

1. Introduction

Campylobacter spp., mainly *Campylobacter jejuni* and *Campylobacter coli*, are the most widely reported bacteria around the world that cause human foodborne infections (1). These bacteria are responsible for intestinal infections in developed countries. Additionally, *C. jejuni* is the most commonly known prior infection in patients with Guillain–Barré syndrome, a severe inflammatory polyneuropathy. Epidemiological studies have proved that poultry and poultry products are the main source of human infection by *Campylobacter* (2). Accordingly, it becomes essential to control the viable numbers of *Campylobacter* in poultry products. Seemingly, hygiene and biosecurity efforts aimed at minimizing *Campylobacter*-contaminated poultry are not very effective. The most interesting aspect of *Campylobacter* pathogenesis is its capability to invade chicken immune responses, although it heavily colonizes (up to 10^9 bacteria/g) chickens' intestinal mucosa (3). On the other hand, antibiotic abuse causes a serious medicinal problem and increase in the incidence of resistant *Campylobacter* strains significantly impairs the process of combating campylobacteriosis by prolonging therapy or rendering it ineffective (4). Therefore, several studies and experiments have been implemented to reduce the quantity of *Campylobacter* in poultry through nonantibiotic approaches over the past few years. These include vaccination (5), feeding chickens bacteriophages and bacteriocins, and various efforts to reduce *Campylobacter* by applying feed and water additives such as organic acids and their derivatives, medium-chain fatty acids, and probiotics. Although there is no specified or reliable plan to control *Campylobacter* colonization in the poultry gastrointestinal tract, there are signs of hope that reduction of *Campylobacter* colonization in the poultry gastrointestinal tract can reduce probability of contamination of poultry meat during slaughter and dressing. It is expected that a 2-log decline in *C. jejuni* populations on poultry carcasses could cause about a 30-fold reduction in human campylobacteriosis cases (6). Among other alternatives, applications of organic acids

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and especially medium-chain fatty acids, probiotics (7), and medicinal plants have been shown to seemingly be an efficient approach to control *Campylobacter*.

The beneficial properties of probiotics and their effects on animal health and reduction of pathogens in the food chain have increasingly been highlighted in the past years. It has been reported that bacteria, and especially lactobacilli, could possess inhibitory characteristics against *Campylobacter* spp. (7). Short-chain fatty acids have been widely used to prevent pathogenic bacteria in food products. Fatty acids diffuse the bacteria in the undissociated form and dissociate in the protoplasm, causing intracellular acidification (8). *Campylobacter*'s susceptibility to the acidic condition has been shown in several studies and this characteristic has been used in different approaches to reduce the gastrointestinal tract's pH in order to control *Campylobacter* colonization. Medicinal plant extracts have antimicrobial, antiviral, anthelminthic, and antioxidative properties and they are known to aid the endocrine and immune systems (9). Accordingly, the dual actions of *Echinacea purpurea* (EP) in the eradication of bacteria and reversal of their antioxidative activities would assist in combating infections (10). Overall, EP has shown a strong capability to stimulate the immune system, this stimulation leading to production of more immune system cells (11). Hence, in this study, we evaluate four commercial products based upon organic acids, probiotics, and a medicinal plant to see their effects on broilers' cecal microbial populations and immune response and especially their capability to reduce *Campylobacter* colonization in the broilers' gastrointestinal tract.

2. Materials and Methods

2.1. Birds and experimental design

A total of 210 one-day-old male broilers (Ross 308) were obtained from a local commercial hatchery (Kosar, Karaj, Iran). Chicks were reared on wood shaving litter in a completely randomized design in separated pens at the Poultry Research Center of the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. Feed and nonchlorinated water were supplied ad libitum throughout the study and all birds received the same amount of a certain diet based on corn and soybean. Birds were assigned to 7 treatment groups with 3 replicate pens/treatment and 10 birds/pen. The transmission of *Campylobacter* between pens was largely prevented by the hygienic procedures during the experiment. The treatments were as follows: organic acid (Selko-pH®) in drinking water (1 mL in 1 L for 2 weeks, and 7–8 h for the rest of the growth period) (Group OA); organic acid (Selko-pH®) in drinking water for 12 h before slaughter (1/1000 (v/v)) (Group OA_2); protected organic acid and butyric acid glycerides (Baby C_4®) at 2.5/1 g kg^{-1} mixed in the feed 12 h before slaughter (Group POA); water-soluble and mix-in probiotic feed (Primalac®, Star Labs, Clarksdale, MO, USA) (in drinking water: 120/1 g/L until day 14, mixed in feed: 454/1000 g/kg until day 28 and 225/1000 g/kg for the rest of the growth period) (Group PM); *Echinacea purpurea* alcoholic extract in drinking water (1 mL in 1 L for 2 weeks, and 7–8 h for the rest of the growth period) (Group EP); and two groups considered as a negative control (NC, not challenged) and a positive control (PC, bacterially challenged).

2.2. Bacteria preparation and *Campylobacter* challenge

The bacterial isolate *C. jejuni* RTCC 1097 was used for experimental challenge (provided by the Razi Vaccine and Serum Research Institute, Karaj, Iran). This strain was originally isolated from the cloaca of a local commercial broiler chicken. The bacterium was cultured onto *Campylobacter* selective agar (Oxoid, UK) with 10% sheep blood and incubated at 42 °C in microaerophilic conditions (7% CO_2, 10% H_2, and 80% N_2, produced by the Anaxomat System, Mart Microbiology, Lichtenvoorde, the Netherlands) for 48–72 h. After 48 h of incubation at 42 °C, the majority of *C. jejuni* strains produced gray, moist, flat, and spreading colonies on the agar. The bacteria were harvested and diluted in PBS to the specific viable concentration (6×10^7 CFU/mL) (12). The inoculum concentration was estimated by MacFarlane tubes. The inoculum was kept on ice for less than 1 h previous to oral gavage (inoculation in crop) of chicks. On day 21, except for the negative control group that received 1.0 mL of sterile PBS, the rest of the birds were orally challenged with a 1.0-mL dose of the inoculum (12).

2.3. Sampling

On days 28 and 42, six birds per treatment (two per pen) were euthanized by cervical dislocation and then cecal contents and blood and liver samples were collected and the relative weights of lymphoid organs (bursa of Fabricius, spleen, and liver) were measured. Cecal contents (1 g) were suspended in 9 mL of PBS. Serial dilutions were prepared in saline from 10^{-1} to 10^{-7}. Afterwards, 10 µL of the last dilutions were spread onto 9-cm plates contain *C. jejuni* specific medium (*Campylobacter* Agar Base, Oxoid) with 10% sheep blood according to standard bacteriological procedures. Plates were incubated under microaerophilic conditions (microaerophilic conditions were created with the Anaxomat System, 7% CO_2, 10% H_2, and 80% N_2) for 24–48 h, until colonies were visible and large enough to be counted. To evaluate the cecal quantity of lactobacilli and total bacteria, 0.1 mL of the last dilution was also cultured on selective lactobacilli medium (Rogosa Agar, Oxoid) and trypticase soy agar (Oxoid) by pour plate method, and then both of them were incubated for 72 h and 48 h, respectively.

Liver samples were stored in sterile petri dishes and transferred to the laboratory for microbiological analysis. The middle part of the liver after scorching the surface of samples was used for bacterial analysis and then 1 g of the internal part was macerated, transferred into 9 mL of PBS, and completely homogenized by vortexing. Serial dilutions were prepared in saline from 10^{-1} to 10^{-3} and then sample culturing and enumeration were conducted as mentioned above.

2.4. Sheep red blood cell (SRBC) injections

On days 24 and 31, a SRBC suspension (5% V:V in sterile PBS), which was provided in the laboratory (Tarbiat Modares University, Faculty of Agriculture, Tehran, Iran), was injected into the breast muscles of six birds (2 from each pen). One week later, blood samples were taken via wing vein on day 38. Thereafter, antibody titration against SRBC was done by hemagglutination inhibition (HI) test. Antibody titers were expressed as the log-2 of the reciprocal of the highest serum dilution giving complete agglutination.

2.5. Heterophil to lymphocyte ratio (H/L)

Blood samples were collected from wing veins within 1 min after capturing each bird and then transferred into test tubes containing ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. A blood smear was prepared using May–Grünwald–Giemsa stain and the number of heterophils (H) to lymphocytes (L) was counted for a total of 100 cells (13). The counting results were converted to a ratio of H/L and introduced as a percent.

2.6. Statistical analysis

The experimental design was a completely randomized design including 7 treatments and 3 replications. All data were subjected to SPSS 17 (SPSS Inc., Chicago, IL, USA) for analyzing variances and Duncan's multiple range test was used for comparison of means.

3. Results

As a result of this study (Figures 1 and 2), the number of lactobacilli was significantly increased in the PM and OA treatments ($P \leq 0.05$). At day 28, the number of lactobacilli in the PM treatment was significantly higher than in the positive control ($P \leq 0.05$). This enhancement was not seen at day 42.

The data of enumeration of *Campylobacter* cecal content obviously showed that the number of *Campylobacter* in the OA, PM, POA, and EP treatments was significantly decreased ($P \leq 0.001$) (Figures 1 and 2). The positive control and PM treatment (probiotics) had the highest and lowest *C. jejuni* cecal and liver colonization, respectively (Figure 3). *Campylobacter* content in the liver also showed that all treatments could not significantly reduce the quantity of *Campylobacter* in the liver (Figure 3).

The improvement of antibody concentration in response to SRBCs and gain in spleen, bursa of Fabricius, and liver weights of broilers are presented in Figure 4 and the Table, respectively. Our findings showed that the relative weight of the spleen and bursa of Fabricius was only higher in the EP treatment compared with the

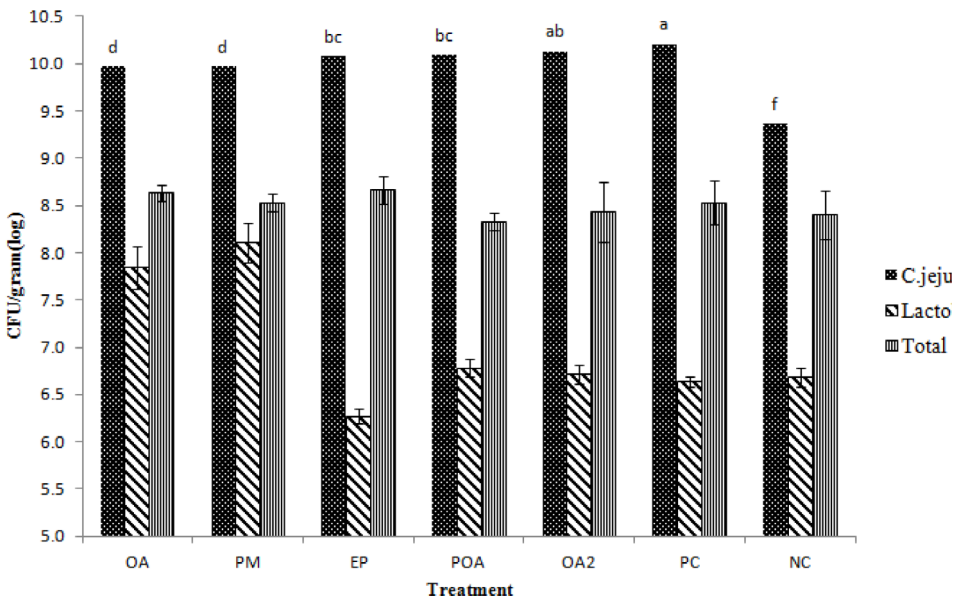

Figure 1. The number of *Campylobacter jejuni*, *Lactobacillus*, and total count of bacteria in the cecum at the first sampling point (day 28). OA: Organic acid (Selko-pH®) in drinking water; PM: water-soluble and mix-in feed probiotic (Primalac®); EP: *Echinacea purpurea* alcoholic extract in drinking water; POA: protected organic acid and butyric acid glycerides (Baby C_4®); OA_2: organic acid (Selko-pH®) in drinking water for 12 h before slaughter; PC: positive control (bacterially challenged); NC: negative control (not challenged).

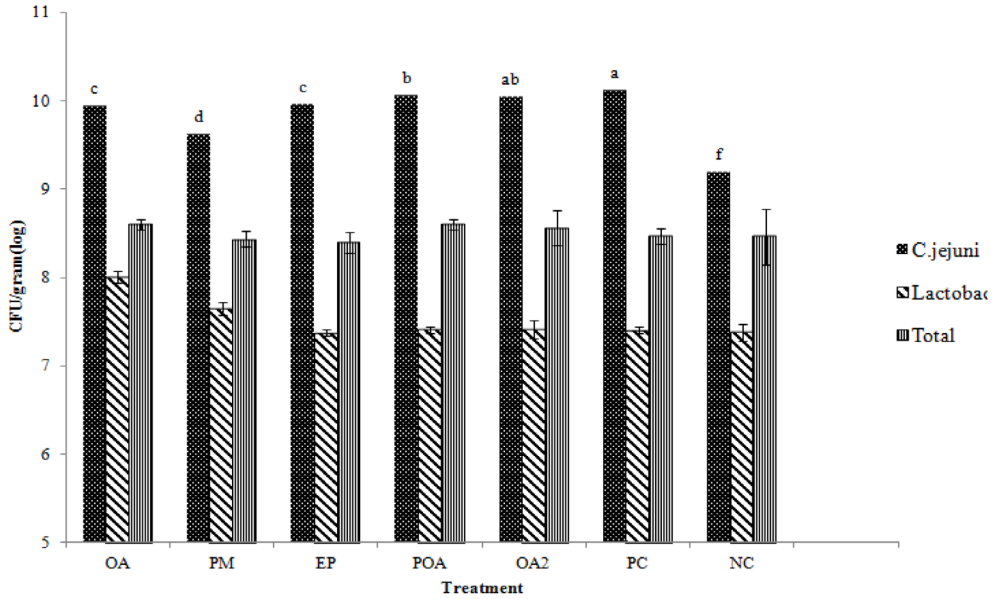

Figure 2. The number of *Campylobacter jejuni*, *Lactobacillus*, and total count of bacteria in the cecum at the first sampling point (day 42). OA: Organic acid (Selko-pH®) in drinking water; PM: water-soluble and mix-in feed probiotic (Primalac®); EP: *Echinacea purpurea* alcoholic extract in drinking water; POA: protected organic acid and butyric acid glycerides (Baby C_4®); OA_2: organic acid (Selko-pH®) in drinking water for 12 h before slaughter, PC: positive control (bacterially challenged), NC: negative control (not challenged) ($P \leq 0.001$).

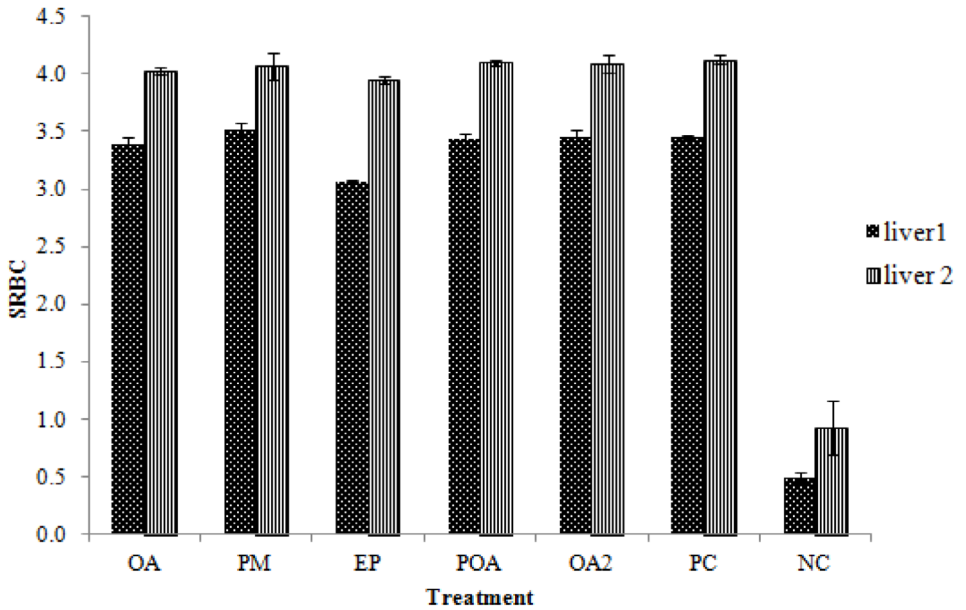

Figure 3. *Campylobacter jejuni* (CFU per gram of liver) in the liver on days 28 (liver 1) and 42 (liver 2).
OA: Organic acid (Selko-pH®) in drinking water; PM: water-soluble and mix-in feed probiotic (Primalac®); EP: *Echinacea purpurea* alcoholic extract in drinking water; POA: protected organic acid and butyric acid glycerides (Baby C_4®); OA_2: organic acid (Selko-pH®) in drinking water for 12 h before slaughter, PC: positive control (bacterially challenged), NC: negative control (not challenged) ($P \leq 0.001$).

positive control ($P \leq 0.001$). In addition, there were no significant differences among all treatments in terms of liver relative weight.

In order to evaluate the specific and nonspecific immune systems of the broilers, two immune assessment tests including SRBCs (a specific immune system indicator)

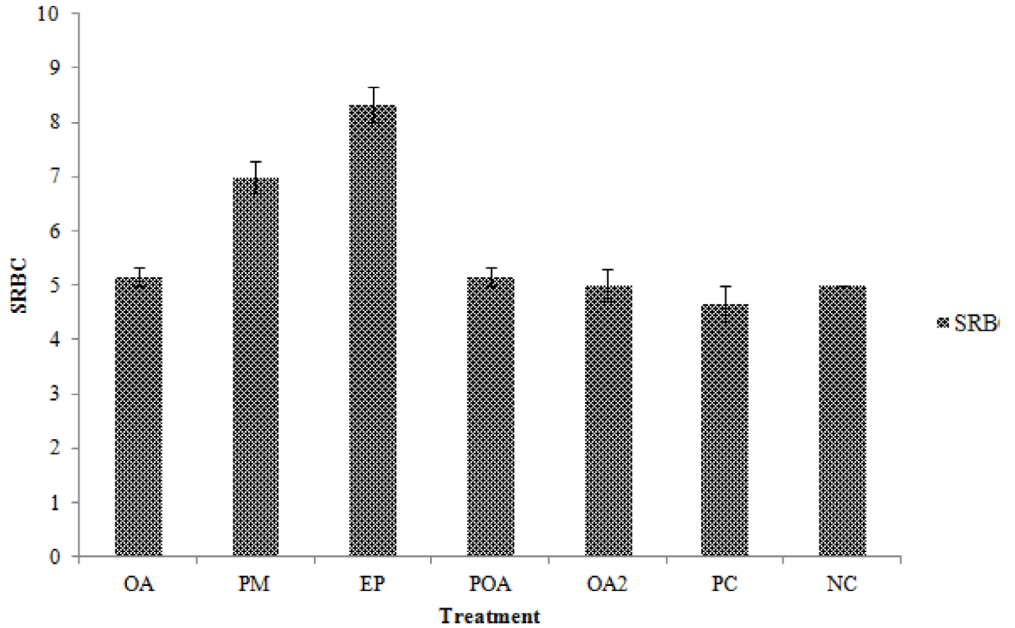

Figure 4. Titers of SRBC agglutinins, determined by doubling dilutions (log-2).
OA: Organic acid (Selko-pH®) in drinking water; PM: water-soluble and mix-in feed probiotic (Primalac®); EP: *Echinacea purpurea* alcoholic extract in drinking water; POA: protected organic acid and butyric acid glycerides (Baby C_4®); OA_2: organic acid (Selko-pH®) in drinking water for 12 h before slaughter, PC: positive control (bacterially challenged), NC: negative control (not challenged) ($P \leq 0.001$).

Table. The relative weight of lymphoid organs (spleen, bursa of Fabricius, and liver) on days 28 and 42.

Treatment	Spleen		Bursa of Fabricius		Liver	
	28	42	28	42	28	42
OA	0.11 ± 0.002	0.11 ± 0.001	0.16 ± 0.010	0.13 ± 0.004	2.42 ± 0.13	2.21 ± 0.12
PM	0.11 ± 0.002	0.11 ± 0.002	0.16 ± 0.014	0.13 ± 0.001	2.34 ± 0.10	2.20 ± 0.10
EP	0.14 ± 0.006	0.14 ± 0.003	0.19 ± 0.004	0.14 ± 0.004	2.50 ± 0.06	2.33 ± 0.16
POA	0.08 ± 0.006	0.10 ± 0.001	0.14 ± 0.009	0.13 ± 0.005	2.29 ± 0.09	2.23 ± 0.04
OA2	0.09 ± 0.007	0.10 ± 0.001	0.14 ± 0.009	0.13 ± 0.005	2.31 ± 0.10	2.24 ± 0.04
PC	0.09 ± 0.001	0.10 ± 0.001	0.15 ± 0.007	0.13 ± 0.004	2.36 ± 0.17	2.39 ± 0.12
NC	0.11 ± 0.001	0.11 ± 0.002	0.16 ± 0.016	0.12 ± 0.009	2.40 ± 0.10	2.21 ± 0.11

OA: Organic acid (Selko-pH®) in drinking water; PM: water-soluble and mix-in feed probiotic (Primalac®); EP: *Echinacea purpurea* alcoholic extract in drinking water; POA: protected organic acid and butyric acid glycerides (Baby C_4®); OA_2: organic acid (Selko-pH®) in drinking water for 12 h before slaughter, PC: positive control (bacterially challenged), NC: negative control (not challenged) ($P \leq 0.001$).

and heterophil and lymphocyte measurements were done. The antibody production (SRBC) was significantly higher in the EP treatment and PM in compared to the positive control ($P \leq 0.001$) (Figure 4).

Another factor that was measured to evaluate the immune response was the H/L ratio. The results indicated that the H/L ratio was significantly higher in the EP treatment and PM as compared to the positive control ($P \leq 0.001$) (Figure 5).

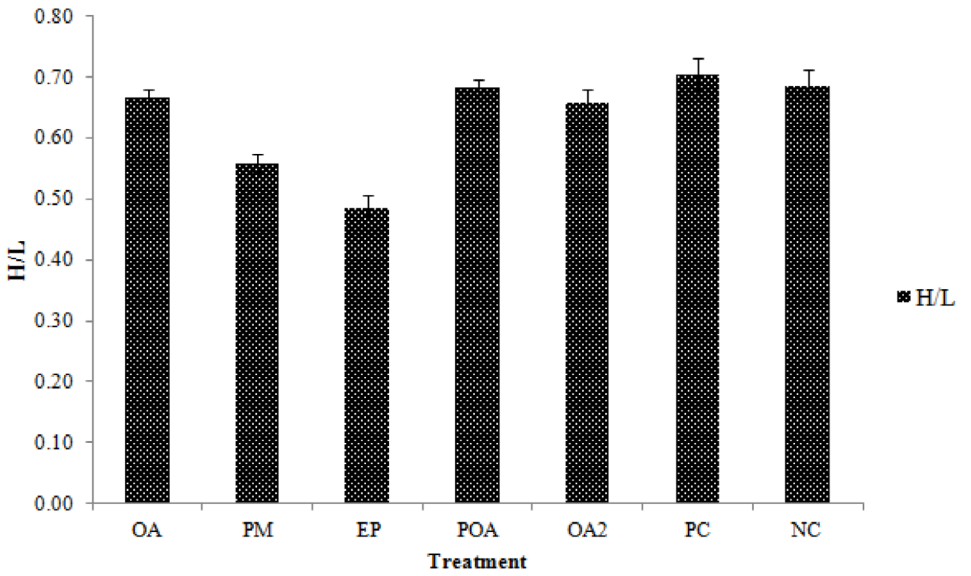

Figure 5. Heterophil to lymphocyte ratios.
OA: Organic acid (Selko-pH®) in drinking water; PM: water-soluble and mix-in feed probiotic (Primalac®); EP: *Echinacea purpurea* alcoholic extract in drinking water; POA: protected organic acid and butyric acid glycerides (Baby C_4®); OA_2: organic acid (Selko-pH®) in drinking water for 12 h before slaughter, PC: positive control (bacterially challenged), NC: negative control (not challenged) ($P \leq 0.001$).

4. Discussion

The intestinal microbial flora of poultry is composed of different varieties of bacteria. The majority of this flora are lactic acid bacteria known as advantageous bacteria by their production of organic acid. According to our results PM and OA could significantly affect the number of lactobacilli by day 42 but PM treatments did not show a regular increase. This might be due to the fact that the number of bacteria reached saturation level as birds matured. This was observed while the OA treatment indicated higher numbers of lactobacilli bacteria at both day 28 and 42. It also could be due to lactobacilli exclusively excelling at colonization in low pH conditions compared with other kinds of competing bacteria.

All treatments except OA_2 could effectively reduce the number of *Campylobacter* in the cecum but they had no effect on the *Campylobacter* content of the liver. This can be a result of the feed additives' direct and limited effect only on the gastrointestinal contents. It has been shown that there is a direct correlation between the intestinal contents of *Campylobacter* and the amount of translocation of bacterium to the spleen and liver, so we expected to see a significant decrease in the liver as well.

The main bacteria used in the probiotic (Primalac) included *Lactobacillus* and *Bifidobacterium*. The results from the in vitro experiment showed that *Lactobacillus* and *Bifidobacterium* strains and also some strains of *Enterococcus faecium* have antagonistic action against *C. jejuni*, and noticeable decrease has been seen in the amount of *C. jejuni* with inhibitory and antimicrobial activity (7). The bactericidal effect against *Campylobacter* probably results from the production of organic acids, as already evidenced. Other studies indicated that this inhibitory activity might be due to the production of a proteinaceous molecule (13). Results from this study are in agreement with the results of experiments using similar probiotics in order to control *Campylobacter* colonization (14). Many in vitro studies have shown that *Campylobacter* spp. have a hypersensitivity to low pH conditions (15). The host animal might be less susceptible to *Campylobacter* colonization due to acidification of the drinking water. The basis of this is that the gastrointestinal tract of chickens becomes more acidic when the animals receive acidized drinking water. This might reduce the number of bacteria that reach the lower parts of the gastrointestinal tract. It is also possible that the acid used in drinking water changes the intestinal microflora, similar to a pre- or probiotic, decreasing enteric *Campylobacter* content. A recent study showed that using organic acids in drinking water can reduce bacteria in the fecal content of pigs (16). In another study, it was shown that chickens fed with acidized feed are less susceptible to infection with *Campylobacter* (17). It has been shown that the use of acetic, lactic, or formic acid in the drinking water of market-age broilers significantly reduced crop pH and decreased the total quantity of *Salmonella* and *Campylobacter* in the broiler carcasses at

processing (18). Between two acidic feed (POA) and water additive (OA_2) treatments that were administrated at 12 h before slaughter, only POA significantly reduced the number of viable *Campylobacter*. *Campylobacter* is able to modulate gene activities, which allows quick adaptation of the microorganism to new environmental conditions (19). Results also suggest that the ability to create biofilms might be a key factor for chicken colonization (20). Using a cross-sectional approach to reduce the number of viable *Campylobacter* thus seems to be feasible. Regarding the fact that *Campylobacter* spp. have high sensitivity to acidic conditions, using acidified nutrition a few hours before slaughter could be an effective way to reduce the number of viable *Campylobacter* in the gastrointestinal tract. The antimicrobial properties of n-butyric acid and its derivatives against *Salmonella* Typhimurium and *Clostridium perfringens* were studied and it was revealed that n-butyric acid and its derivatives (monobutyrin and a combination of mono-, di-, and triglycerides of butyric acid) were able to reduce pathogenic bacteria (21). It seems that the *C. jejuni* reduction in the POA treatment was related to effective transmission of n-butyric acid and its derivatives in the gastrointestinal tract by protection. According to the positive effect of OA on the reduction of *Campylobacter*, the nonsignificant results of OA_2 could be due to the short time of administration and the weakness of this product in reducing intestinal pH.

Echinacea spp. contains four major groups of compounds that could possibly be responsible for an enhancement of the immune response (22) Glycoproteins, polysaccharides, caffeic acid conjugates (caftaric acid, cichoric acid, and echinacoside) as well as alkyl amides (2-ene and 2,4-dienes) are commonly found in *Echinacea* species (23). Most of these components have been investigated for their immunomodulating potential, and many have been found to enhance macrophage activity (24). These components induce some alterations in NFκB levels. NFκB is a nuclear transcription factor that stimulates the expression of several genes including key components of the inflammatory response such as TNFα, IL-1, chemokines, adhesion molecules, and cyclooxygenase-2 (25). Many studies have been conducted in order to investigate the antibiotic activity and it was generally found that *Echinacea purpurea* has antibiotic and immunostimulant properties (22). In the case of poultry, studies have mentioned that *Echinacea purpurea* can promote poultry's immune system efficiency (26,27). It has been shown that using EP root extract as a dietary supplement significantly decreased lesions in the intestines of poultry contaminated with the protozoan parasite *Coccidia*. It also enhanced the health of the birds in comparison with controls (26).

The immunostimulant property of *Echinacea* spp. has been mentioned in many studies and this characteristic probably made the lymphoid organs more active. Furthermore, it has been proven that administration of *Echinacea purpurea* can increase spleen cells' angiogenic activity (28).

Because of the specific function of the lymphoid organs, they are susceptible to oxidative injury, and the antioxidant activities of EP probably protected these organs from oxidative injuries and may allow antibody-producing organs to do their functions more efficiently. Allen found that EP can promote the immune response to live vaccination and may provide protective immunostimulation in the incidence of the natural coccidian inhabitants in the litter (26). Böhmer et al. showed same results in the case of ascending antibody titers against SRBCs in broilers using EP for immune system enhancement (10). Moreover, it has been indicated that lactobacilli possess the capability to persist in the intestinal tract and can act as adjuvants to the humoral immune response (29). This could be related to the increasing activities of antibody-producing cells, which might have been stimulated by the probiotic organism. Khaksefidi et al. reported that antibody production against Newcastle disease virus in the 50 mg/kg probiotic-supplemented group increased significantly at 10 days after immunization compared to the control. They also showed that the probiotic had a positive effect on production and durability of antibodies in response to SRBC antigens (30).

Several strains of live lactobacilli have been shown to provoke the release of proinflammatory cytokines, TNFα, and IL-6, and they can stimulate nonspecific immunity (30). In addition, direct-fed microbial supplements increased spontaneous as well as antigen-specific spleen lymphocyte proliferation in chickens, a surrogate marker of increased cellular immunity. As mentioned before, *Echinacea* spp. has positive effects on the entire immune system and can be traceable both in specific and nonspecific immune responses. This finding of the current study is consistent with those of Böhmer et al., who found that ethanolic juice of *Echinacea* increased the number of lymphocytes and total leukocytes in hens and pigs significantly (10).

In conclusion, in this study we provide evidence that a specific probiotic, organic acid, and *Echinacea purpurea* as a medicinal plant can reduce chickens' susceptibility to *C. jejuni* colonization and can also effectively reduce the extent of *C. jejuni* colonization by their effect on microflora and chickens' immune system efficiency.

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