IMMUNOLOGICAL STATUS OF *SALMONELLA PULLORUM*-INFECTED BROILERS TREATED WITH *BACILLUS SUBTILIS*, ORGANIC ACIDS AND CIPROFLOXACIN

Abeer I. Abdulwahhab **M. T. Al-Zuhariy**
Researcher **Assist. Prof.**
College of Veterinary Medicine, University of Baghdad
mushtaq.t@covm.uobaghdad.edu.iq

ABSTRACT

The current study was aimed to ascertain the impact of ciprofloxacin, an acidifier acid, and a *Bacillus subtilis* on immunological function and growth efficiency after a starter-phase challenge with *Salmonella pullorum*. 250 broiler chicks (Ross 308) were put into five groups of fifty each, all groups except the fifth group, orally challenged with *S. pullorum* at one day old, and provided with a supplement in drinking water after three days as follow G1: treated with *B. subtilis* (2×10^7 CFU/g) (250 g/1000 L). G2: treated with acidifier acid (0.5-1 ml/L). G3: treated with ciprofloxacin 10% (0.5 ml/L). G4: only infected with *S. pullorum*. G5: control negative. The findings revealed extremely significant variations in the means antibodies (IgG) and IFN-γ titre against *Salmonella pullorum* in serum which was increased significantly at the level ($P\leq0.05$) in all groups at 7 days, particularly in G4 and G1, in comparison with G5, but, at 21 and 35 days the results of IgG and IFN-γ titers showed a significant decrease ($P>0.05$), the results of immunity matching with most probable number (MPN/g) of *S. pullorum* in feces. Additionally, the highest significantly differences at the level ($P\leq0.05$) in growth performance across all groups. We conclusion, the organic acid has biggest role in protection in comparison with ciprofloxacin that have immunosuppression also, the *B. subtilis* not recorded any protection.

Key words: ELISA, IgG, IFN-γ, MPN, growth efficiency, challenged, acidifier.

الحالة المناعية لفروج اللحم المصاب *Salmonella pullorum* والمعالج *Bacillus subtilis* والأحماض العضوية والسيبروفلوكساسين

عبير إبراهيم عبد الوهاب مشتاق طالب بكر
باحثة أستاذ مساعد
كلية الطب البيطري، جامعة بغداد

المستخلص

الهدف من الدراسة الحالية هو تحديد مدى تأثير السيبروفلوكساسين، والاحماض العضوية، و*Bacillus subtilis* على الحالة المناعية وأداء النمو للطيور المصابة *Salmonella pullorum* في المرحلة الأولى من النمو. تم تقسيم 250 فرخة للاحم (روس 308) الى خمس مجموعات كل مجموعة مكونة من خمسين فرخة، كل المجاميع ما عدا المجموعة الخامسة، بعمر يوم واحد أصيبت بالسالمونيلا بللورم عن طريق الفم، ومن ثم عولجت بالمكملات بعد ثلاثة أيام عن طريق ماء الشرب كما يلي G1: عولجت *B. subtilis* (2×10^7 CFU/غم) (250 غم/1000لتر). G2: عولجت بالحمض العضوي (0.5-1مل/لتر). G3: عولجت بالسايبروفلوكساسين (0.5 مل/لتر) 10%. G4: تم اصابتها *S. pullorum* ولكنها لم تعالج. G5: اعتبرت مجموعة سيطرة. أوضحت النتائج أن هناك اختلافات كبيرة للغاية في متوسط الأجسام المضادة المحددة (IgG) وكذلك معيار IFN-γ في المصل ضد *S. pullorum* وزادت بشكل ملحوظ عند المستوى ($P\leq0.05$) في اليوم 7 لكل المجاميع المصابة، وخاصة في المجموعة الرابعة والاولى، مقارنة بالمجموعة الخامسة, لكن في اليوم 21 و35 أظهرت نتائج معيار IgG و IFN-γ انخفاضًا معنويًا ($P>0.05$)، وتطابقت نتائج المناعة مع نتائج الرقم الأكثر احتمالا للبكتريا في الفضلات التي كانت الأكثر عند اليوم 7 وانخفضت في 21 و 35. بالإضافة الاختلافات المعنوية العالية عند مستوى ($P\leq0.05$) في أداء النمو لجميع المجموعات. توصلنا إلى استنتاج ان الحمض العضوي له الدور الأكبر في حماية دجاج اللاحم عند تعرضه باليوم الأول *S. pullorum* بالمقارنة مع السايبروفلوكساسين الذي كان له اثباط مناعي بينما *B. subtilis* لم يسجل دور فعال في الاصابة.

الكلمات المفتاحية: الاليزا، IgG, IFN-γ, MPN, أداء النمو، تحدي، حامض عضوي.

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INTRODUCTION

With clinical signs like weight loss, decreased laying, diarrhea, sores, and reproductive system disorders, Pullorum disease (PD), which affects young birds primarily, causes acute septicemia (2, 4, 5). Despite being the focus of disease eradication programs in the majority of developed countries, a previous meta-analysis study (3, 6, 21) revealed that *S. pullorum* is still widespread, especially in poorer nations (32). An efficient way to both prevents and cure PD is with antibiotic therapy. Antibiotic resistance in microorganisms is one issue that has been brought up by the extensive use of antibiotics. At production stage the use of antibiotics is regulated in a number of nations worldwide. *Salmonella* infections are among the many bacterial illnesses that can be treated with Ciprofloxacin (CIP), a medication from the fluoroquinolone antibiotic class (14). When bacteria replicate DNA, they inhibit the actions of DNA topoisomerase and DNA gyrase (7). Salmonella ciprofloxacin resistance has been observed more commonly worldwide due to plasmid-mediated quinolone resistance in bacterial plasmids or chromosomes (11). In order to improve poultry production, probiotics (9), organic acids (30), enzymes (22), and medicinal herbs (12) are seen as alternatives to antibiotics. Organic acid is one such substitute with growth-promoting qualities that can keep animal performance high by balancing the usual gut flora (31). Because they are both bacteriostatic and bactericidal, organic acids (OA), which are mostly short and medium-chain fatty acids make up, hold promise as antibiotic substitutes in preventing the colonization of infections. The effectiveness of OA in reducing *Salmonella* colonization in turkeys, pigs, and broiler in an agricultural context as well as in preventing *Salmonella* contamination of meat and poultry products after slaughter has been investigated in a number of trials (13, 23). A probiotic called *B. subtilis* can broiler chickens increased output and innate immune response. It also works as a growth promoter (19). It is believed that *B. subtilis* promotes intestinal health by producing antibacterial compounds (25). It encourages the development of bacteria that produce lactic acid as well as other advantageous microorganisms in the intestines that actively and vehemently eliminate diseases (1). The goal of the current study was to compare the potential effects of *B. subtilis*, an organic acid, and Ciprofloxacin to lessen the negative effects in broiler chicken infected with *S. pullorum* in order to reduce the risk of *S. pullorum* by improving growth performance and the immune response.

MATERIALS AND METHODS

Experimental birds: In this experiment, 250 broiler chicks from the 308 Ross flock at one day old were employed. They came from the Al-Shukur Poultry Company (a local hatchery). 50 broiler chicks per group were randomly distributed into 5 equal groups.

Experimental design and feeding program

After three days, all groups were orally challenged with *Salmonella pullorum* 0.2 ml (25×10^4 CFU/ml) (OM988162.1) isolate at one day old. The exception of the fifth group, which was provided with a supplement as a follow: G1: exposed to *S. pullorum* and treated in drinking water with *Bacillus subtilis* (2×10^7 CFU/g) (250g/1000 L). G2: exposed to *S. pullorum* and treated with organic acid (0.5-1 ml/L) in drinking water. G3: exposed to *S. pullorum* and treated ciprofloxacin 10% (0.5 ml/L) in drinking water. G4: exposed to *S. pullorum* but not treated. G5: consider as control negative. The live Newcastle disease vaccine (NDV strain B1) eye drop was administered to the chicks in all groups with the exception of the G5 at one day. At 7 and 18 days old, a booster dosage of the NDV was administered by water. Additionally, they received an intermediate-strain IBDV vaccination when they were 14 days.

Experimental additives

Acidifier Commercial product (B.I.O.Acid Liquid): collection of organic acids, including formic acid, acetic acid, lactic acid, ammonium formate, propionic acid, and citric acid monohydrate. (Biochem, Germany), used as directed by the manufacturer (0.5–1 ml/L) through drinking water. *B. subtilis* (ATCC PTA-6737) viable spores (2×10^7 CFU/g) in a commercial probiotic product (BIO-SAC) (Clostat, Des Moines, Kemin Industries, USA), as directed by the manufacturer (250 g/1000 liters), used through drinking water. Use 0.5mL/L of the Ciprofloxacin

(Quinocycline) product through drinking water, as directed by the manufacturer.

Salmonella challenge protocol

Salmonella pullorum was used to test the chicks in this experiment. The strain had a reputation for effectively colonizing the broiler's gut. Before and after the inoculation, the bacteria's viability was confirmed. The bacteria were briefly kept at -20°C, doubled in retrieval and plating on Salmonella-Shigella agar (SS agar) at 37°C for 24 h. Into sterile, prewarmed Tetrathionat broth was added a single colony of the bacterium, which was then cultured at 41°C for 24 hours. The bacteria then made another plate on SS agar. To (25×10^4 CFU/ml), the challenge inoculum was diluted and adjusted. It was established that there were live bacteria both before and after inoculation.

Bird challenge: All groups of birds, with the exception of the fifth group, were challenged by oral gavages on day one. A 0.2 ml challenge inoculum was injected into the crop lumen of each bird.

Measurements: Growth performance : Each bird used in the experiment had a starting body weight that was recorded. The feed conversion ratio (FCR) and feed conversion efficiency were calculated based on the weekly tracking of each group's body weight, weight gain, and feed consumption. The daily feed intake (FI) during the post-infection phase was calculated by deducting the percentage of provided feed that was rejected. At the conclusion of the first, second, third, fourth, and fifth weeks, the total FI for each group was determined. Each group's FI and weight gain were modified in the case the birds perished. The feed conversion ratio (FCR) for each group was determined using the formula below: FCE = Weight Increase / FI was used to calculate feed conversion efficiency, while FCR is defined as FI/weight gain.

Immunity (IgG): Five chickens from each group were given blood samples at 7, 21 and 35 days of age for the ELISA test to identify *S. Pullorum* antibodies. Anticoagulant was not added to blood samples to help them clot. Centrifugation was used to separate the serum for 10 minutes at 3000 rpm. The manufacturer, BioChek (UK) Limited, specified the methods for detecting *S. Pullorum* antibodies in chicken using a fast serological test (ELISA).

Chicken IFN-γ level detection

A commercially available ELISA kit was used to measure the level of (IFN-γ) in chicken sera in order to determine the quantitative determination of IFN-γ concentrations in serum. The data were expressed in picograms per milliliter (Pg/ml), which have a detection range of 2.8 to 180 Pg/ml. the advice provided by the manufacturer. (SunLong Biotech Co.,LT).

Statistical analysis

Utilizing the Statistical Analysis System, all statistical analyses were carried out (SAS). The overall level for statistical significance was ($P\leq0.05$). The standard errors of the means were used to express all results (SEM).

RESULTS AND DISCUSSION

Humoral immunity (IgG): The results of maternal immunity against *Salmonella Pullorum* at one day before divided into groups for ten serum samples were (285±24). Fig. (1) demonstrats highly significant differences at level ($P\leq0.05$) in specific antibodies (IgG) against *Salmonella Pullorum* among all groups at (7, 35) days old chicks. The results of ELISA at 7 days showed a highest means of specific IgG antibodies was given in G4 and G1 flowed by G2 but the lowest mean titer was recorded in G3 in comparison to control negative group G5. While the results of IgG titer show decrease significantly at ($P>0.05$) at 21- and 35-days post infection in all groups especially in G3 and G2 respectively, in comparison with G4 and G1 that revealed higher titer in serum.

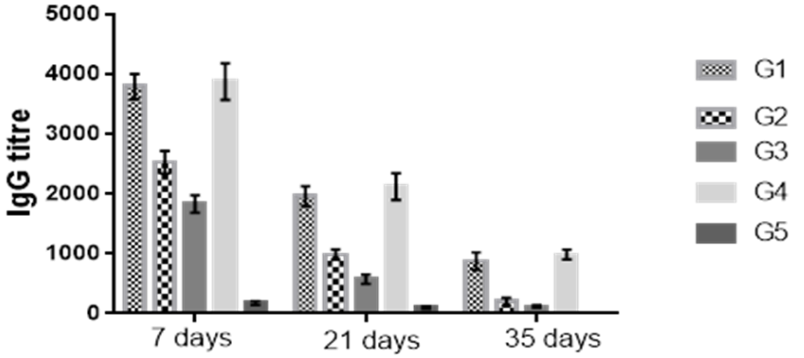

Figure 1. Specific IgG antibodies in serum against Salmonella Pullorum

Cellular immunity (IFN-γ)

The lowest mean of IFN-γ titre was recorded in G3 compared to control negative group G5, although the ELISA results at 7 days show evolution of IFN-γ titre in serum against *Salmonella Pullorum* in all groups, predominantly in G4 and G1 that given a higher means, coming after G2 As opposed to G4 and G1, which had larger serum titers, the findings of the means of IFN-γ titre show a significant drop after 21 days after infection in all groups, particularly in G3 and G2. In compared to G4, which had a larger serum titer, the means of IFN-γ titre in G3 exhibited a significant reduction at (P≤0.05) in 35 days after infection, whereas G2 and G1 were considered to have medium means.

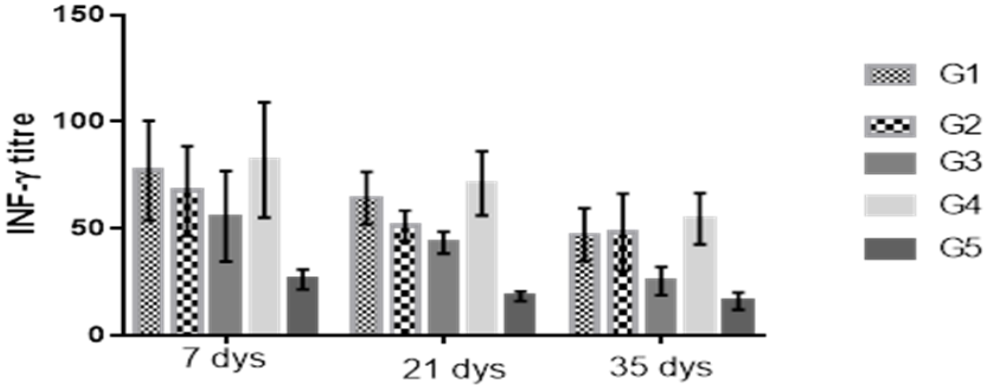

Figure 2. IFN-γ titre in serum against Salmonella Pullorum

According to a study, the drop in Ab titre in G3 when ciprofloxacin treatment was resumed compared to G2 resulted in decreased antibody titres against *Salmonella pullorum* in broiler chicks. A related conclusion was also previously reported (15). demonstrates that enrofloxacin changes the way that birds' immune systems react by lowering humoral immunity, which includes the production of antibodies, and enhancing a cellular response, which involves T cells as the main effector cells (18). There is data that suggests several antibiotics, particularly enrofloxacin, may have a negative impact on hens' humoral defense systems while positively influencing their cell-mediated immunity (10, 20). The distinguishing feature of Th1 immune responses is interferon (IFN-γ), whereas the cytokines that characterize Th2 immune responses include IL-4 and IL-13. IL-18, an inflammatory cytokine with IFN-γ inducing characteristics, is released by activated macrophages (Th1 immunological mechanism). Levels of IL-18 were raised by *S. Pullorum* (24), that would imply that between 7 and 35 days after ingestion, *S. pullorum* promotes the Th1 immune system. The findings support He *et al.* (16) findings that activated cells cause the seldom IL-18 to be produced. This might be because lymphocytes

at day 7 following infection release adherent macrophage cells into the cell medium along with them. Our study's recorded the lowest IFN-γ concentration in G3 is consistent with Williams *et al.* (28), who observed that the quinolones ciprofloxacin and moxifloxacin, which are equivalent to enrofloxacin, inhibit the release of IL-4 and IFN-γ. The main determinants of whether defensive processes are directed toward type Th1 (IFN-γ) or Th2 cells are these cytokines (IL-4). The blood level of IgG antibodies in hens taking enrofloxacin is consistent with our data suggesting that blocking IL-4 release may cause a considerable reduction in antibody production. When chickens were given enrofloxacin at ages 7, 21, and 35, their levels of this immunoglobulin were at their lowest. Perhaps it has to do with this antibiotic's ability to block the expression of IL-4 in Th2 cells (26, 28).

Most probable number (MPN/g)

Additionally, the results showed that all groups of 7, 21 and 35-day-old when utilizing the most probable number (MPN/g) method to count the amount of Salmonella in the chicks' feces, there were extremely noticeable differences at level (P≤0.05). At 7 days, the results showed that MPN/g means were higher in all groups, with G4 and G1 having the highest means and G2 and G3 having the lowest means relative to the control negative group, G5. In contrast to G4 and G1, which have larger MPN/g means, all groups show a substantial drop in MPN/g at 21 and 35 days after infection (P≤0.05), primarily in G3 and G2, respectively.

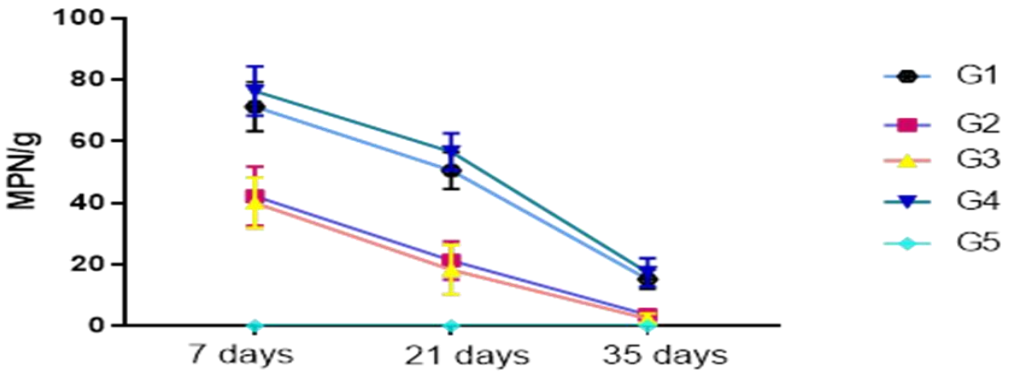

Figure 3. Salmonella in feces counted using the most probable number (MPN/g) approach at different peroids

The most protective isolates only tenfold enhanced the chicken's resistance to *Salmonella enterica serovar Enteritidis* following challenge, despite the fact that none of the examined individual bacterial isolates on their own effectively protected chicks against *Salmonella enterica serovar*. Gram-negative bacteria's capacity to colonize lends credence to earlier studies that showed specific bacterial species colonized the chicken caecum after being administered caecal extracts (27, 29) or following interaction with an adult hen, the isolates did not require the co-colonization of any other microbiota members in order to successfully colonize the caeca of chicks. These did not depend on the colonization of other microbiota members and colonized the chicken caecum on their own. The other microbiota members' capacity to influence *S. enteritidis* colonization is further indication of their rather minor impact. While several of the isolates reduced *S. enteritidis* levels by one log, colonization with diverse microbiota lowers *Salmonella* counts by five logs, therefore this reduction must be viewed as insignificant (29). According to one theory, adding organic acid greatly decreased the amount of *Salmonella* present by creating an acidic environment in the gut that encourages pH lowering, inhibits the growth and multiplication of harmful bacteria, and increases the growth of good bacteria (31).

Growth performance

The average body weight of all groups at one day old was 45g, and analyses of average weight gain showed significant differences between the probiotic, organic acid, and ciprofloxacin groups and the control group at

the level of (P≤0.05) among all groups compared to the control group. The second group of organic acids had the highest significant differences at level (P≤0.05) in the final body weight comparisons after 35 days, followed by the third group, while the first and fourth groups had the lowest body weights, in contrast to the fifth group of controls (Tab.1). The results of feed conversion efficiency, on the other hand, revealed findings that were consistent with the feed conversion ratio and revealed a significant difference at level (P≤0.05) in feed conversion efficiency across all groups, where (second, third, fifth, first, and fourth) groups, respectively.

Table 1. Results of different groups in feed intake, feed conversion ratio, and feed conversion efficiency (Mean±SE)

Groups	final weigh	Feed consumption	FCR	FCE
G1	2137±321 D	4970.4±389.2 A	2.32±0.02 A	0.42±0.002 C
G2	2784±245 A	4544.3±231.3 B	1.63±0.03 C	0.61±0.003 A
G3	2578±321 B	4600.7±259.7 AB	1.78±0.04 B	0.56±0.001 B
G4	2104±259 D	4899.6±377.3 A	2.32±0.02 A	0.42±0.003 C
G5	2465±354 C	4377.2±389.3 C	1.77±0.01 B	0.56±0.001 B

Five samples total. Significant differences at the level of (P≤0.05) are denoted by capital letters

Since the use of antibiotics in animal feed was outlawed, the current trend in poultry is to employ more feed additives non-antibiotic to improve growth and feed utilization (25). Despite the fact that it has been discovered that using antibiotics as a feed additive at a level below the subtherapeutic range is beneficial for the growth of chicken, their use has been outlawed due to the likelihood of antibiotic resistance and the presence of their residues in meat products. As a result, following the delivery of a single dose, neither *Lactobacillus*, *Enterococcus*, nor *Bacillus* colonized the chicken digestive tract. In terms of growth performance and feed utilization, the findings of the present study showed that the addition of an organic acid mix was equally as successful as the addition of an antibiotic, suggesting their potential usage as substitutes. Similarly, Abudabos *et al.* (1) It was found that when confronted with germs like *Salmonella*, broilers fed organic acids outperformed in terms of growth performance and feed consumption when given antibiotics. However, it was done in this study to evaluate an organic acid blend with an antibiotic alone or in combination with a probiotic, which has probably never been done previously. The probiotic bacteria's mechanism of action entails lowering the pH of the digestive tract, suppressing pathogenic bacteria by producing organic acids, preventing bacterial colonization by competitive exclusion, producing antibacterial mucin, boosting the immune system, and producing antibacterial enzymes (β-glucosidase), has been attributed to the enhanced performance in birds treated with them (17). Additionally, it has been shown in the past that Broiler performance was enhanced by the addition of organic acid without causing any microbiological issues (31). Furthermore, an acidic environment encourages the production and release of pepsin, gastrin, and cholecystokinin, all of which are essential for nutrient uptake, subsequent growth performance, and feed effectiveness (8).

CONCLUSION

The authors came to the conclusion that organic acid, as opposed to ciprofloxacine, might be employed successfully to maintain the metabolic profile and growth of broilers exposed to *S. pullorum.*

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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