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The Role of Synbiotic and Organic Acids on Ifn-Y , Igg Titer and the Mpn in the *Salmonella Enteritidis* Infected Layers Chicken

Fadhil Jaafar Hashim *[1], Sameer Mezher Abdullah[2]
*[1] Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

ABSTRACT

This study investigates the effects of synbiotics and organic acids (BioAcid Ultra) on immune response and bacterial load in layer chickens infected with *Salmonella enteritidis*. A total of 100 chickens were divided into four groups: a control positive, control negative, treatment groups receiving synbiotics and organic acids groups. Blood samples were collected at 15, 25, and 35 days post-infection to measure interferon-gamma (IFN-γ) levels and IgG titers using ELISA. Additionally, the Most Probable Number (MPN) of *Salmonella* was assessed from feces samples using selective enrichment and culture methods. Results indicated significant increases in IFN-γ levels and IgG titers in the treatment groups at day 15, suggesting enhanced immune activation. However, both IFN-γ and IgG levels declined by days 25 and 35. Notably, the MPN of *Salmonella* was significantly lower in treated groups compared to the control, highlighting the effectiveness of synbiotics and organic acids in reducing bacterial load. These findings demonstrate that the use of synbiotics and organic acids can effectively enhance immune responses and mitigate *Salmonella enteritidis* infections in layer chickens, offering potential strategies for improving poultry health and production.

Key words: antibodies, bacteria , bacterial account, prebiotic

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INTRODUCTION

Salmonella enteritidis is a significant pathogen in poultry, particularly affecting layer chickens, which can lead to substantial economic losses in the egg production industry (Jung *et al*., 2022). This bacterium not only compromises animal health but also poses a serious risk to food safety, as contaminated eggs can serve as a transmission route to humans (Góchez *et al*., 2019). Several approaches have been investigated to counteract this, such as the utilization of organic acids and synbiotics. Synbiotics, a combination of prebiotics and probiotics, have garnered attention for their potential to enhance gut health and improve immune responses in animals (Atiyah and Hamood, 2021). Since they can be treated as one of the alternatives to the use of antibiotics in poultry production, they affect the health of birds, biochemical blood indicators, and can modulate the immune system, possibly leading to increased production of cytokines such as Interferon gamma (IFN-γ), which plays a crucial role in the immune response against intracellular pathogens like *Salmonella*, also limit the negative impact of high density in the building on the organism of birds (Żbikowski *et al*., 2020). Using a synbiotic with a water disinfectant increased the number of immune cells, such as B and T lymphocytes (Chechet *et al*., 2022). These substances can modify the intestinal microbiota by increasing the number of beneficial bacteria and the concentration of lactic acid in the intestines (Śliżewska *et al*., 2020). Organic acids is another feed additive with a health-promoting and immunostimulant effect (Sugiharto *et al*., 2019). They are classified as organic chemical compounds that contain the carboxyl group –COOH in their

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structure (Vera-Álava *et al*., 2023). In poultry nutrition, formic, acetic, propionic, malic, fumaric, and citric acids are used primarily (Ali *et al*., 2020). These substances are an additive that has a positive effect on feed hygiene through their preservative impact (Feye *et al*., 2021), and inhibition of the development of pathogenic microorganisms as a result of lowering the pH of the gastrointestinal tract, creating an unfavorable environment for pathogenic bacteria (Lund *et al*., 2020; Pope *et al*., 2022). They may also influence the immune response by increase in the number of lymphocytes in the spleen, antibody titers against Gumboro disease, contributing to improved health outcomes in infected animals (Zhang *et al*., 2024). This study aims to evaluate the effects of synbiotics and Bio acid Ultra on IFN-γ levels, IgG titers, and the Most Probable Number (MPN) of *Salmonella* in layer chickens infected with *Salmonella enteritidis.* Understanding these interactions could provide valuable insights into effective strategies for enhancing immune responses and controlling infections in poultry, ultimately contributing to improved animal health and food safety.

MATERIALS AND METHODS

Sampling: Samples were taken from the digestive tract and fresh feces of layer chickens of varying ages. The research was conducted between November 1st and December 1st. All isolates obtained by intestinal cotton swabs from Al-Sink Laboratories for Diagnosing Poultry Diseases (Iraq, Baghdad) were transported using non-selective Peptone water broth. Every specimen was moved right away to the lab for examination while being cooled.

Experimental design: A total of 100 layer chickens, aged 250 days, were randomly divided into four groups (25 chickens per group):

Control negative group (G1): Received standard feed and water, not infected with *Salmonella enteritidis*

Control positive group (G2): Received standard feed and water, infected with *Salmonella enteritidis*

Synbiotic group (G3): Received standard feed supplemented with a synbiotic formulation, infected with *Salmonella enteritidis*

BioAcid Ultra Acids group (G4): Received standard feed supplemented with organic acids, infected with *Salmonella enteritidis*

Infection protocol: After 5 days of treatment acclimatization, all groups except the control were infected with a standardized dose of *Salmonella enteritidis* ($1.5*10^8$ CFU/mL) (Zamora and Pérez-Gracia, 2012) via drinking water Post-infection, all chickens were monitored for clinical signs of illness.

Synbiotic and bioacid ultra acids preparation: Synbiotics: A commercial formulation under name (Triple P®- 500) Mixture of Bacillus based probiotic and prebiotic yeast cell walls as shown in (table 1). It was prepared to the dosage: 250 – 500 g / ton final feed mixed (12.5 gm/ 50 kg feed). The group of chicken received the diet mixed with the 2261ymbiotic from the (1-35) days of breeding.

Bio acid Ultra: A mixture of commonly used (lactic acid, formic acid/ammonium formate, propionic acid/ammonium propionate, citric acid and acetic acid) was prepared to the Dosage:1kg / ton final feed mixed (50 gm/ 50 kg feed). The group of chicken received the diet mixed with the Bio acid Ultra from the (1-35) days of breeding.

Table 1. Synbiotics commercial formulation

Microorganism	Microbial contents cells
Bacillus licheniformis (DSM 5749)	1.3×10^9 CFU
Bacillus subtilis (DSM 5750)	1.3×10^9 CFU
TechnoMos (Biological active materials from the cell wall fractions of *Saccharomyces cerevisiae* rich in 1,3 β-Glucans &Mannans)	up to 1 g
Contains:	
Mannans	> 20 % (> 200 mg)
Total Glucans	> 20 % (> 200 mg)

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Sampling and immune parameter measurement: Blood Collection: Blood samples (5 mL) were collected from the wing vein and jugular vein of four groups, each corresponding to breeding durations of 15 days, 25 days, and 35 days.

Serum Preparation: Blood samples were centrifuged at 2000 rpm for 10 minutes, and serum was stored at -20°C until analysis (Dai *et al.*, 2023).

Measuremen of IFN-γ and antibody levels: IFN-γ Detection: Serum levels of IFN-γ were measured using an enzyme-linked immunosorbent assay (Chicken Interferon γ, IFN-γ ELISA Kit) specific for chicken IFN-γ, following the manufacturer's instructions.

IgG Measurement: Antibodies against *Salmonella enteritidis* were quantified using a commercial (*Salmonella* Group B and D Antibody ELISA Test Kit) designed for avian species.

Most probable number (MPN/g) of *salmonella* in chicken feces: On days 15, 25, and 35 following inoculations, aseptically obtained fecal samples from individual chicks in plastic caps were used to count the *salmonella*. Five randomly chosen birds from each group's feces were sampled and placed in sterile screw-capped vials before being immediately sent to the lab for *S. enteritidis* quantification and *Salmonella* enumeration using the most probable number (MPN) technique (Chenu *et al*., 2013). One gram of feces was weighed into sterile plastic tubes, and then 9 ml of buffered peptone water (BPW) (1:10) was added. The tubes were then homogenized for one minute. Decimal dilutions of 0.1 mL were streaked on *Salmonella-Shigella* agar. The plates underwent a 24-hour inculating period at a temperature of 37 °C. The original sample was counted to determine the number of *Salmonella* colonies per gram.

Statistical analysis: The Statistical Analysis System- (SAS ,2018) program was used to detect the effect of difference factors in study parameters. Least significant difference-LSD was used to significant compare between means (ANOVA/ One way) in this study.

RESULTS AND DISCUSSION

Significant variations in serum IFN-γ titers were seen across all groups at 15, 25 and 35 days after the challenge with *Salmonella enteritidis* (P < 0.05) in order to examine the impact of synbiotics and organic acids on interferon-gamma (IFN-γ) levels in infected layer hens. Interestingly, at 15 days, IFN-γ titers increased in all groups, with Group 2 (G2) having the highest mean levels and Group 3 (G3) following. Group 4 (G4), on the other hand, had the lowest IFN-γ titers in contrast to the negative control group (G1). IFN-γ levels dropped dramatically (P < 0.05) at 25 days after infection in all groups, especially in G4 and G3, but G2 still had the highest blood titers. At 35 days after infection, G4's IFN-γ levels were still much lower than those of G2, which had the highest titers, while G3's levels were slightly higher as shown in (Table 2).

Table 2. Effect of Synbiotic and B.I.O Acid ULTRA on IFN-γ in Chicken

Group	Mean ±SE of IFN-γ conc. (pg/mL)			L.S.D. value
	15 days	25 days	35 days	
G1	9.05 ±1.67Aa	12.42±2.04Aa	9.93±1.06Aa	4.960 NS
G2	106.29±1.91Ba	86.21±3.01Bb	72.65±1.28Bc	6.972 *
G3	65.15±4.43Ca	53.11±2.09Cb	32.42±2.14Cc	9.085 *
G4	37.31±1.34Da	29.69±0.79Db	17.15±0.72Dc	2.914 *
L.S.D.	8.103 *	6.331 *	4.365 *	---

-* (P≤0.05), NS: Non-Significant.
- Small letters represent the comparison in the same row
- Capital letters represent the comparison in same column

In investigating the IgG level, (Table 3) shows that at 15, 25, and 35 days after the challenge, there were extremely significant differences (P ≤ 0.05) in specific IgG antibodies against *Salmonella enteritidis* across all groups. When compared to the negative control group (G1), Group 2 (G2) had the greatest mean levels of specific IgG antibodies at 15 days, followed by

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Group 4 (G4) on the other hand, had the lowest mean titer. All groups had significant (P ≥ 0.05) decreases in IgG titers by 25 and 35 days post-infection, with G4 showing the biggest drops. During these time intervals, however, G2 continued to have the highest serum titers.

Table 3. Effect of Synbiotic and B.I.O Acid ULTRA on IgG in Chicken in different period

Group	Mean ±SE of IgG conc. (pg/mL) 15 days	25 days	35 days	L.S.D. value
G1	5.47 ±0.87Da	6.82±1.04Da	6.03±1.2Da	1.821 NS
G2	86.48±5.21Aa	71.34±11.2Ab	58.32±8.12Ac	9.253 *
G3	62.72±14.03Ba	51.23±10.9Bb	40.33±8.71Bc	7.427 *
G4	44.24±7.81Ca	28.46±6.28Cb	16.92±2.54Cc	8.764 *
L.S.D.	12.211 *	13.214 *	10.905 *	---

-* (P≤0.05), NS: Non-Significant.
- Small letters represent the comparison in the same row
- Capital letters represent the comparison in same column

Salmonella levels in chick feces were measured using the most probable number (MPN/g) method. The results showed significant differences between the groups at 15, 25, and 35 days post-challenge (P≤0.05) (Table 4). When compared to the negative control group (G1), all groups showed higher MPN/g values at 15 days, with Group 2 (G2) having the highest mean and Groups 4 (G4) having the lowest means. At 15, 25, and 35 days following infection, MPN/g values significantly decreased in all groups (P≤0.05), with G4 showing the largest drop.

Table 4. *Salmonella* in feces counted using the most probable number (MPN/g) approach in different period

Group	Mean ±SE of MPN/g 15 days	25 days	35 days	L.S.D. value
G1	0 ±0Da	0 ±0Da	0 ±0Da	NS
G2	124±5.21Aa	89±11.2Ab	71±8.12Ac	11.022 *
G3	55±14.03Ba	39±10.9Bb	21±8.71Bc	10.571 *
G4	36±7.81Ca	20±6.28Cb	12±1.5Cc	7.201 *
L.S.D.	16.314 *	14.17 *	9.054 *	---

-* (P≤0.05), NS: Non-Significant.
- Small letters represent the comparison in the same row
- Capital letters represent the comparison in same column

Many immunological and intestinal disorders may be prevented and treated more successfully by the synergistic action of probiotics and prebiotics, or synbiotics, than by the two components acting alone (Bakheet *et al.*, 2020). The observed elevations in IFN-γ levels in hens given synbiotics and organic acids imply that the infection with different organisms has improved the cellular immune response . In order to activate macrophages and facilitate the removal of intracellular infections, such as *Salmonella*, IFN-γ is essential (Alspach *et al.*, 2018). This result is consistent with other research showing that synbiotics might alter immunological responses, possibly improving poultry resistance to disease (Abdulrahman, 2016). Prebiotics, probiotics, and synbiotics have been shown by researchers to have therapeutic potential for lowering inflammation, highlighting their promise as a modality of treatment for inflammatory illnesses. These supplements have an impact on a number of important processes, such as the growth of gut-associated lymphoid tissue, immune system education, integrity preservation of the intestinal mucosal barrier, modulation of the proliferation and differentiation of epithelial cells, regulation of angiogenesis, modification of enteric nervous system activity, and the vital role of nutrient extraction and processing (Al-Qayim, 2014). Certain bacteria in prebiotics can utilize fermentation products or intermediates like hydrogen, lactate, succinate,

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format, and ethanol, transforming them into end products that are then released into the gut lumen, such as short-chain fatty acids. This process significantly influences gut physiology (Shehata *et al*., 2022). The importance of organic acids as feed additives in animal production is becoming more widely acknowledged. Thus far, the primary focus has been on their potential to improve food safety, specifically by decreasing the prevalence of foodborne diseases in chicken and other livestock (Ricke *et al*., 2020). For use in monogastric animal feed, Bio acid Ultra is a semi-coated formulation that combines lactic, propionic, and formic acids with their salts. It effectively suppresses gram-negative bacteria, lowers pH and buffering capacity, and improves feed hygiene (Lee *et al*., 2021). The components of Bio acid have been the focus of previous research, especially formic acid, which has shown potent antibacterial qualities against Salmonella spp. and other pathogens in in vitro settings. *Salmonella* species and other pathogens appear to be inhibited by formic acid when consumed through feed or drinking water. But, as with propionate, formic acid's concentration is thought to drop in the lower gastrointestinal tract, meaning that its efficacy is mostly seen in the upper parts of the digestive system (Kovanda *et al*., 2019) . These findings align completely with the current results and suggest that they may be more effective than the use of Triple P-500 Synbiotic. An additional crucial indicator of the humoral immune response is IgG titers. The increased IgG levels in the treatment groups in our study suggest that organic acids and synbiotics may both help to boost the generation of antibodies against *Salmonella enteritidis*. Long-term immunity depends on this increased humoral response, which may also lessen the severity of infections (Männe *et al*., 2019; Dar *et al*., 2018). The current study found that after being exposed to a local strain of *Salmonella enteritidis* for seven days, the positive control group's antibody titers significantly increased in comparison to the negative control group. The results of earlier studies that assessed the bacterial infection, pathology, and immunological responses in hens following inoculation are in line with them. Increased serum IgG titers specific to *S. enteritidis* surface antigens and a rise in gut-associated T lymphocytes were the outcomes of the infection (Männe *et al*., 2019). Even with the activation of the immune system in chickens, previous research has demonstrated that oral infection with *Salmonella* fails to trigger immune responses that effectively control the infection (Ehrhardt *et al*., 2023). The MPN data showed that, in comparison to the control group, there was a notable decrease in the quantity of *Salmonella* in the feces of treatments groups. Due to its capacity to produce an environment that is unfavorable for pathogen growth in the gastrointestinal tract, Bio acid Ultra in particular seems to be particularly successful at reducing bacterial numbers. This result is in line with the body of research that shows how organic acids work to prevent harmful germs from growing (Chaney *et al*., 2022). This result is in line with previous research that, in the face of mounting demand to stop using growth-promoting antibiotics, found synbiotic formulations to be viable substitutes for antibiotics for a range of bacteria, including *Salmonella* spp. (Hassanzadeh *et al*., 2022). Using organic acids and synbiotics offers a viable way to treat *Salmonella enteritidis* infections in layer chickens. The overall health and productivity of poultry operations may be enhanced by these therapies, which also lower bacterial burdens and boost the immune response. Future research ought to examine the processes underlying these effects and take into account the long-term implications for food safety and animal welfare (Korver, 2023).

CONCLUSION

The present study demonstrated that dietary supplementation with synbiotics and organic acids effectively enhanced the immune response and reduced Salmonella enteritidis colonization in infected layer chickens. Birds receiving these supplements exhibited significantly higher IFN-γ concentrations and IgG antibody titers during the early stage of infection, indicating a rapid activation of both cellular and humoral immune responses. Although immune parameters gradually declined at later sampling periods, they remained associated with a marked reduction

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in the fecal Salmonella load, as evidenced by the lower Most Probable Number (MPN) values compared with the infected untreated group. These findings suggest that synbiotics and organic acids improve host resistance against S. enteritidis by modulating immune function and limiting bacterial shedding. Therefore, these feed additives represent promising alternatives to antibiotic growth promoters for enhancing poultry health, improving food safety, and reducing the risk of Salmonella transmission through poultry products.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR/S DECLARATION

We confirm that all Tables in the manuscript are original to us.

AUTHOR'S CONTRIBUTION STATEMENT

Fadhil Jaafar Hashim Conceptualization, methodology, data collection, laboratory analysis, statistical analysis, writing–original draft preparation.

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الدور الوقائي للسينبيوتك و الاحماض العضوية المناعية والعدد البكتيري الاكثر احتمالاً في الدجاج البياض المصاب بالسالمونيلا المعوية

فاضل جعفر هاشم [1] ، سمير مزهر عبد الله [2]
فرع الامراض وامراض الدواجن ،كلية الطب البيطري ،جامعة بغداد ،العراق

المستخلص

تهدف هذه الدراسة الى تحديد تأثيرات السينبيوتك والأحماض العضوية على الاستجابة المناعية والعدد البكتيري في الدجاج البياض المصاب بالسالمونيلا المعوية. تم تقسيم 100 دجاجة إلى أربع مجموعات: مجموعة تحكم إيجابية ، و مجموعة تحكم سلبية، ومجموعة العلاج التي تتلقى السينبيوتك ومجموعة العلاج التي تتلقى الأحماض العضوية. تم جمع عينات الدم بعد 15 و 25 و 35 يوما من الإصابة لقياس مستويات إنترفيرون جاما والجسم المضاد باستخدام تقنية الاليزا. بالإضافة إلى ذلك ، تم تقييم العدد البكتيري الأكثر احتمالا من السالمونيلا من عينات البراز باستخدام طرق الزرع الانتقائية. أشارت النتائج إلى زيادات كبيرة في مستويات إنترفيرون جاما والجسم المضاد في مجموعات العلاج في اليوم 15 ، مما يشير إلى تعزيز تنشيط المناعة. ومع ذلك ، انخفضت مستويات كل من إنترفيرون جاما والجسم المضاد بحلول اليومين 25 و 35.

الكلمات المفتاحية: الاجسام المضادة، بكتريا، العد البكتيري، البكتريا النافعة .