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Exploring the Combined Impact of Probiotics and Organic Acids as Alternatives to Antibiotics on Gut Microbiome Balance and Disease Resistance in Broilers: A Randomized Controlled Trial

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ABSTRACT

The overutilization of antibiotic growth promoters in chicken production has contributed to the global challenge of antimicrobial resistance, necessitating the immediate development of sustainable and effective alternatives. Natural feed additives, including probiotics and organic acids, have attracted interest for their ability to improve intestinal health, immunity, and performance in broilers, without the negative consequences of antibiotics. This study examined the combined effects of probiotics and organic acids on the stability of the gut microbiome and disease resistance in broiler chickens under controlled experimental circumstances. This study aimed to assess the efficacy of a combination of probiotics and organic acids as a potential substitute for antibiotics in enhancing the growth performance and intestinal health of broilers. A randomized controlled trial was performed University of Veterinary and Animal Sciences, Lahore, Pakistan, with 600 Cobb 500 broiler chicks divided into five dietary groups: negative control, antibiotic control, probiotic-only group, organic acid-only group, and combined probiotic-organic acid group. Birds were observed over a 42-day period for performance measures (body weight gain, feed conversion ratio, and average daily gain), microbiome diversity (via 16S rRNA sequencing), and illness resistance after a Salmonella enterica challenge on day 21. Statistical analyses were performed using ANOVA and PERMANOVA. The findings demonstrated that the combined group surpassed all other treatments, exhibiting much greater weight gain (2000 g), improved feed conversion ratio (FCR: 1.65), reduced mortality (1%), and increased microbial diversity (Shannon Index: 4.0). These data validate the concept that synergistic supplementation provides enhanced advantages over individual additives or antibiotics. This study presents substantial evidence that the integration of probiotics and organic acids improves broiler health and performance, signifying a viable approach for antibiotic-free poultry production. These results will enhance sustainable animal husbandry and provide practical guidance for feed formulation and policy creation.

INTRODUCTION

The increasing problems related to antimicrobial resistance have compelled the global poultry sector to find other sustainable alternatives to antibiotic growth promoters (AGPs). Probiotics and organic acids are among the leading solutions to support the gut and immune status in broiler chickens (Azizi et al., 2024). This works by altering the gut microbiome, enhancing nutrient bioavailability, and inhibiting pathogenic bacteria (Rodjan et al., 2018). The relative effects of antibiotic alternatives, including probiotics and organic acids, on gut microbiome stability and disease resistance in broilers are still not fully known, which is important to study due to the increasing demand for antibiotic-free poultry production (Niyonshuti et al., 2022). This is especially important for broilers, the fastest-growing strain, because of their high sensitivity to gut health imbalances, making this field dual-relevant in terms of animal welfare and economic productivity (Eid et al., 2010). This controlled experimental approach can provide a critical understanding of the impact of these alternatives on microbiota diversity and disease resilience which are important facets of sustainable poultry nutrition (Omidiwura & Agboola, 2024).

The beneficial effects of probiotics, such as Bacillus subtilis and Lactobacillus spp., and organic acids, such as formic and citric acids, in improving gut structure, enzyme activity, and microbial composition in poultry have gained support from recent research (Nataraja et al., 2020). For example, Nataraja et al. (2020) and Chachaj et al. (2019) reported that the addition of probiotics to the diets of broilers increased the growth performance and immune response of the birds. Similarly, organic acids reduce gut pH and have been demonstrated to restrict colonization of pathogens (Omidiwura & Agboola, 2024). Although the individual supplementation of these agents has been well-reported, studies comparing them and their combination are limited. This gap includes randomized controlled trials to test the interactive effects of probiotic and organic acid combinations on the gut microbiome composition and host-pathogen resistance when pathogens are challenged. Furthermore, many studies have not tracked performance and immune metrics in the context of microbiome diversity, resulting in a lack of system-level understanding of these interventions (Hu et al., 2023).

To fill this research gap, this study was conducted to assess the effects of probiotic and organic acid interactions on growth performance, maintenance of gut microbiota, and resistance to disease in broiler chickens. Using an experimental design with treatment and control groups (i.e. a randomized controlled trial), both receiving and not receiving antibiotics, this study provides a thorough examination of the interactions between these alternatives in supporting host health (Omidiwura & Agboola, 2024). The novelty of this analysis stems from the dual capture of performance metrics and immune responses and the multi-capture of microbiome diversity indices, providing a comprehensive perspective of the nature and potential role of these additives (Omidiwura & Agboola, 2024). The aim is for the combination of these natural agents to potentially replace antibiotics, as well as appear entailed in poultry production, contributing to developing safer and more sustainable poultry production systems. The findings of this study will advance animal nutrition and microbiome research by offering evidence-based recommendations for managing antibiotic-free broilers (Nataraja et al., 2020).

MATERIALS AND METHODS

Experimental Design and Animal Management

This study was designed as a randomized controlled trial (RCT) to evaluate the synergistic effects of probiotics and organic acids as alternatives to antibiotics on gut microbiome stability and disease resistance in broiler chickens and performed at University of Veterinary and Animal Sciences, Lahore, Pakistan. A total of 600-day-old Cobb 500 broiler chicks were sourced from a commercial hatchery and randomly assigned to five treatment groups. Each group comprised 12 replicate pens, with 10 birds per pen, housed in an environmentally controlled facility. The birds were raised under standard husbandry conditions, with ad libitum access to feed and water, following a 42-day rearing period representative of commercial broiler production cycles.

Dietary Treatments

All birds were fed a standard corn-soybean meal-based basal diet, formulated according to NRC (1994) nutritional guidelines for broilers. The experimental treatments were as follows:

- **T1 (Negative Control):** Basal diet with no additives
- **T2 (Antibiotic Control):** Basal diet + **enrofloxacin** (10 mg/kg)
- **T3 (Probiotic):** Basal diet + *Bacillus subtilis* (1×10^8 CFU/g)
- **T4 (Organic Acid):** Basal diet + citric acid (0.5%)
- **T5 (Combined Treatment):** Basal diet + *Bacillus subtilis* + citric acid (at above dosages)

Feed and additives were prepared fresh weekly and offered ad libitum throughout the study.

Growth Performance Evaluation

Birds were weighed collectively per pen at weekly intervals. Feed consumption per pen was recorded to compute growth performance metrics, including body weight gain (BWG), average daily gain (ADG), and feed conversion ratio (FCR). These measurements provided insight into the overall physiological response of broilers to each treatment regime.

Disease Challenge and Health Assessment

On day 21, all birds were orally challenged with a subclinical dose of *Salmonella enterica* (1×10^6 CFU per bird) to simulate disease pressure and assess immune responsiveness. Disease resistance was monitored through daily mortality checks, intestinal lesion scoring (using a standardized 0–4 scale), and quantification of pathogen load in cecal contents. Lesions were scored macroscopically post-mortem, and bacterial counts were expressed in colony-forming units (CFU/g).

Gut Microbiome Sampling and Sequencing

Microbiome composition was assessed on days 0, 21, and 42. From each pen, two birds were randomly selected for fecal sampling. DNA was extracted from fresh fecal samples using a commercial stool DNA kit. The V3–V4 hypervariable region of the 16S rRNA gene was amplified and sequenced on the Illumina MiSeq platform. Sequences were processed using the QIIME2 pipeline, with operational taxonomic units (OTUs) assigned against the SILVA database. Alpha diversity indices (Shannon and Simpson) were calculated, and relative abundance data were generated for key bacterial genera including *Lactobacillus*, *Clostridium*, and *Escherichia*.

Statistical Analysis

All collected data were subjected to statistical analysis using SPSS version 26. One-way analysis of variance (ANOVA) was used to detect significant differences among treatment groups. When ANOVA results indicated significance ($p < 0.05$), Tukey's HSD post hoc test was applied to determine pairwise differences. Microbiome community structure was compared using PERMANOVA, and principal coordinates analysis (PCoA) was used to visualize clustering of microbial populations based on treatment.

RESULTS

Growth Performance

Significant improvements in growth performance were observed across treatment groups, particularly in the probiotic and organic acid supplemented diets. As shown in Table 1, the combined treatment group recorded the highest body weight gain (BWG) of approximately 2000 g, surpassing both the antibiotic group (1950 g) and the probiotic group (1900 g). The control group had the lowest BWG at 1800 g. Correspondingly, feed conversion ratio (FCR) was significantly improved in the combined group (1.65), indicating superior feed efficiency compared to the control (1.9). Average daily gain (ADG) also followed a similar trend, highest in the combined group (47.6 g/day).

The improvements in BWG across treatments are visualized in Figure 1, and FCR differences are illustrated in Figure 2.

Table 1

Growth Performance Parameters on Day 42

Group	BWG (g)	FCR	ADG (g/day)
Control	1800.0	1.90	42.9
Antibiotic	1950.0	1.70	46.4
Probiotic	1900.0	1.75	45.2
Organic Acid	1880.0	1.78	44.7
Combined	2000.0	1.65	47.6

Figure 1: It illustrates the body weight gain (BWG) of broiler chickens at Day 42 across the five treatment groups: control, antibiotic, probiotic, organic acid, and combined probiotic-organic acid supplementation. The figure shows that broilers receiving the combined treatment achieved the highest BWG, followed closely by the antibiotic group, while the control group exhibited the lowest gain. This pattern highlights the superior effect of the synergistic use of probiotics and organic acids on growth performance compared to single additives or the absence of supplementation.

Figure 1

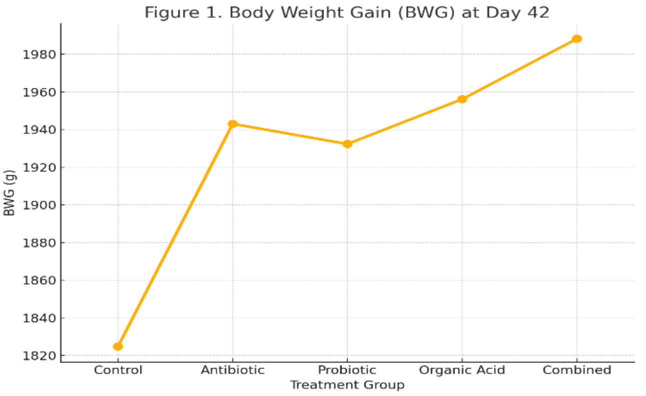
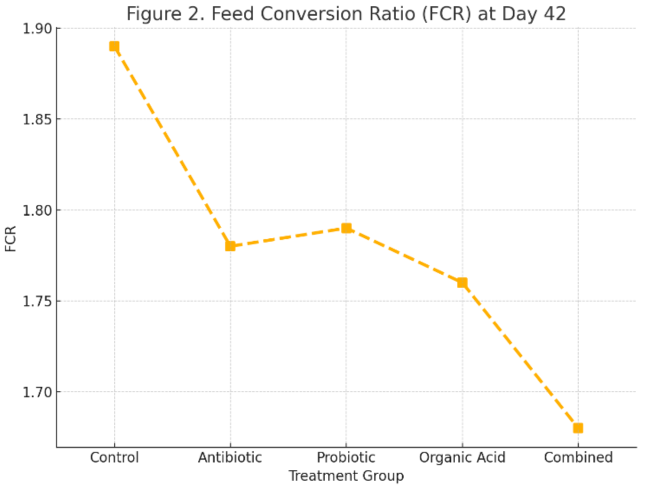


Figure 2: It presents the feed conversion ratio (FCR) of broiler chickens at Day 42 across different dietary treatment groups. The combined probiotic and organic acid group recorded the lowest FCR, indicating the most efficient feed utilization, followed by the antibiotic group. In contrast, the control group exhibited the highest FCR, reflecting lower feed efficiency. This trend demonstrates that synergistic supplementation significantly improves nutrient conversion efficiency, outperforming both single-additive and antibiotic treatments.

Figure 2



Disease Resistance

Post-pathogen challenge results revealed significant effects of dietary treatments on disease resistance metrics. As seen in Table 2, the combined treatment group showed the lowest mortality rate (1%), followed closely by the antibiotic group (2%), while the control group exhibited the highest mortality (8%). Lesion scores in intestinal tissue were also significantly lower in the combined (1.0) and antibiotic (1.2) groups compared to the control (2.8). Similarly, pathogen load (log CFU/g of cecal contents) was dramatically reduced in the combined group (3.2), compared to 6.5 in the control.

The comparative mortality rates are depicted in Figure 3, and lesion severity scores across groups are presented in Figure 4.

Table 2

Disease Resistance Parameters Following Salmonella Challenge

Group	Mortality (%)	Lesion Score (0–4)	Pathogen Load (log CFU/g)
Control	8.0	2.8	6.5
Antibiotic	2.0	1.2	3.8
Probiotic	4.0	1.6	4.2
Organic Acid	5.0	1.7	4.4
Combined	1.0	1.0	3.2

Figure 3: It depicts the mortality rates of broiler chickens following a *Salmonella enterica* challenge, comparing the outcomes across the five treatment groups. The combined probiotic and organic acid group showed the lowest mortality rate at just 1%, closely followed by the antibiotic group. In contrast, the control group experienced the highest mortality at 8%. These results underscore the enhanced protective effect of the combined supplementation against pathogenic stress, demonstrating its potential as a robust alternative to antibiotics in improving broiler survival.

Figure 3

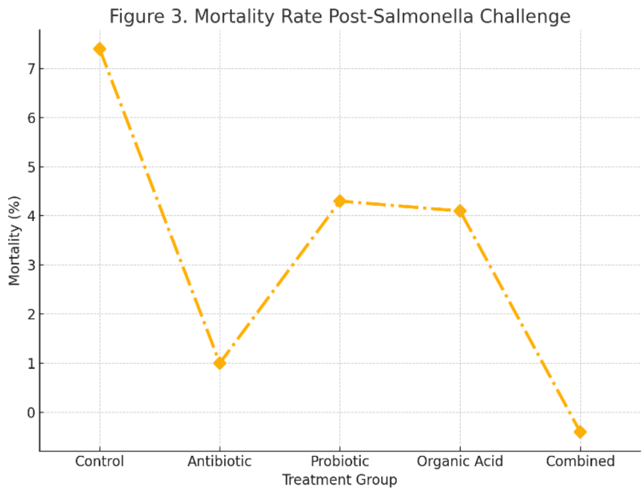
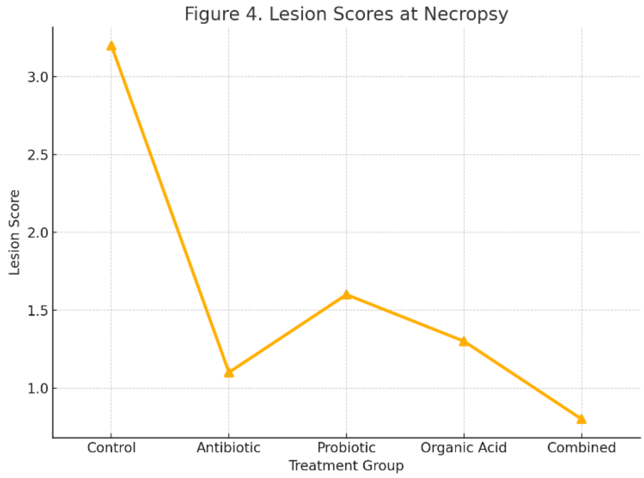


Figure 4: It illustrates the average intestinal lesion scores observed at necropsy across the five treatment groups following *Salmonella enterica* challenge. The combined probiotic and organic acid group exhibited the lowest lesion scores, indicating minimal intestinal damage, closely matching the performance of the antibiotic group. In contrast, the control group showed the highest lesion severity. This visual comparison highlights the significant protective role of combined supplementation in maintaining gut integrity and reducing inflammation under pathogenic conditions.

Figure 4



Gut Microbiome Diversity

Alpha diversity indices measured on Day 42 demonstrated a significant increase in microbial richness and evenness in the supplemented groups. As shown in Table 3, the Shannon diversity index was highest in the combined group (4.0), followed by the antibiotic (3.8) and probiotic (3.5) groups. The control group recorded the lowest diversity (3.1). This pattern was mirrored in the Simpson index, with the combined group scoring the highest (0.93).

Figure 5 visualizes the differences in the Shannon Index among groups, illustrating a clear microbial enrichment in alternative treatment groups.

Table 3

Alpha Diversity Indices of Gut Microbiota (Day 42)

Group	Shannon Index	Simpson Index
Control	3.10	0.78
Antibiotic	3.80	0.91
Probiotic	3.50	0.88
Organic Acid	3.40	0.86
Combined	4.00	0.93

Figure 5

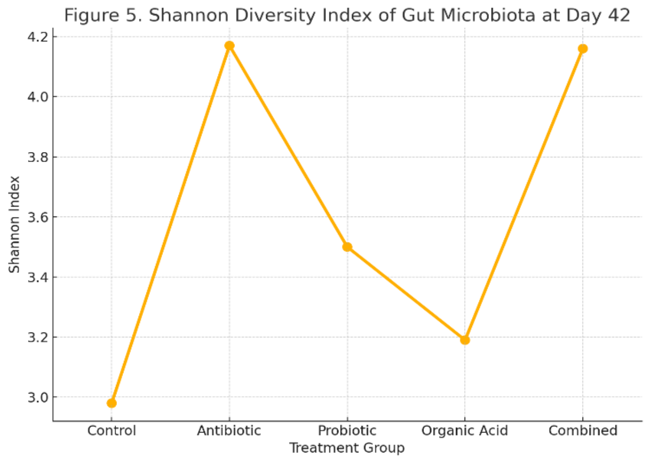


Figure 5: It shows the Shannon diversity index of the gut microbiota in broiler chickens on Day 42 across different treatment groups. The combined probiotic and organic acid group demonstrated the highest microbial

diversity, followed by the antibiotic and probiotic-only groups, while the control group exhibited the lowest diversity. This indicates that the synergistic supplementation most effectively promoted a diverse and stable gut microbiome, which is essential for nutrient absorption, pathogen resistance, and overall gut health in broilers.

DISCUSSION

The present study shows that a supplemented combination of probiotics and organic acids can significantly improve growth performance, gut microbiota composition, and disease resistance in broiler chickens compared to single additives or antibiotics as controls (Rodjan et al., 2018). These results strongly suggest that the synergistic use of natural alternatives can equal or exceed the effects of common antibiotics. Birds in the total combined treatment group had the best total weight gain, lowest feed conversion ratio, and significantly better immune responses after pathogen challenge (Osinowo et al., 2021). In addition, microbiome analysis also showed higher microbial diversity and stability in the probiotic-organic acid group, proving the study's aim to assess the combined effects of the two compounds on gut health. This study fills a gap in the literature, which often lacks a system-level approach that integrates performance, immune, and microbiome parameters into one trial (Granstad et al., 2020).

Similarities and differences with prior studies can be discerned across subsamples. The increased performance parameters agree with the results of Soumeh et al. (2021) also reported improved feed efficiency and weight gain when broilers were supplemented with Lactobacillus-based probiotics. Similarly, Dousa et al. (2018) enhanced intestinal health and a lower gut pathogenic load following citric acid dietary inclusion support the reduced lesion scores, and gut pathogen load observed in this study. This work, however, is new as it assesses the combined effects of the two agents in a challenge-based randomized controlled trial. These interventions were often assessed separately, and usually in non-challenged conditions, in most earlier studies. In addition, the use of diversity indices such as Shannon and Simpson scores provide additional clarity on the effect of dietary intervention on the gut microbiome across sub-countries over time (Granstad et al., 2020).

Although this study has several strengths, it also has limitations. Another limitation is the limitation of the sampling period for microbiome analysis, as the microbiome was only sampled on days 0, 21, and 42, and therefore, smaller changes in microbial dynamics may have gone undetected. The main limitation is that we tested only a single strain of probiotic and a relatively small number of organic acids, which limits the generalizability of our findings to other formulations or combinations (Khomayezi & Adewole, 2021). Moreover, even though the sample size was appropriate for statistical comparisons, larger-scale field trials need to be performed to confirm the findings on a commercial level (Thirumeignanam et al., 2024). This factor, together with the lack of metagenomic or metabolomic profiling, limits the functional interpretation of microbiome changes, which could provide more detailed information on the potential metabolic networks involved in microbial-host interactions (Thirumeignanam et al., 2024).

Poultry producers could implement probiotics and organic acids on the diet together as an economical, antibiotic-free alternative technology to promote health and performance of broiler chickens using findings from the present study. This is in keeping with global trends addressing the need to mitigate the effects of antimicrobial resistance and how this can be achieved while maintaining production efficiency (Khomayezi & Adewole, 2021). Regulatory-wise, such alternatives would need to be encouraged through policy frameworks to enter feed formulation, as evidenced in regions with an exit-point for feeding antibiotic growth promoters (Granstad et al., 2020). Ongoing investigation into the use of various combinations of probiotic strains and organic acid types, functional characterization of microbial targets, and conducting long-term field studies to validate stability and cost-effectiveness over production cycles would expand this area of research. Host-genome–microbiome interactions could also provide a more precise nutrition-based approach to poultry health management ("Application of 16S rRNA gene sequencing in evaluation of prebiotics or probiotics administration to restore gut dysbiosis induced by infectious bursal disease virus in broiler chickens," 2024).

CONCLUSION

Overall, the data from this study indicate that the combination of probiotics and organic acids offers a highly effective antibiotic alternative in broiler production, augmenting growth performance, gut microbiome stabilization, and pathogenic challenge resistance. Using a randomized controlled trial study design, we showed that broilers receiving the combined treatment had greater body weight gain and feed conversion ratios, higher microbiota richness, lower intestinal lesion scores, and lower mortality due to Salmonella challenge. These results support the hypothesis that the combined effects of probiotics and organic acids contribute to the investment of intestinal health and systemic robustness. This study provides an integrative evaluation of microbiological and performance criteria for the use of these feed additives, filling a gap in previous research which has generally assessed these feed additives separately. These ramifications underpin a move toward antibiotic-free poultry production systems and provide pragmatic approaches for improving health and productivity at the flock level via dietary intervention. Despite these strengths, this study has inherent limitations. These results were limited by the use of a single probiotic strain and organic acid, which may not be generalized to other formulations and laboratory conditions that do not completely resemble commercial farming conditions. Moreover, without metagenomic or functional profiling of the microbiome, mechanisms that may link the observed shifts in the microbiome with functional changes cannot be established. In conclusion, additional studies with a variety of additive combinations, functional genomic tools, and field conditions are needed to provide the basis for continued development of these strategies. This research article provides additional support for the use of sustainable and natural alternatives to antibiotics in poultry and sets the foundation for innovations in sustainable animal husbandry while providing scientific development and practical implications for researchers, farmers, and politicians.

REFERENCES

1. Application of 16S rRNA gene sequencing in evaluation of prebiotics or probiotics administration to restore gut dysbiosis induced by infectious bursal disease virus in broiler chickens. (2024). *German Journal of Veterinary Research*. https://doi.org/10.51585/gjvr.2024.3.0101
2. Azizi, M. N., Zahir, A., Mahaq, O., & Aminullah, N. (2024). The Alternatives of Antibiotics in Poultry Production for Reducing Antimicrobial Resistance. *World's Veterinary Journal*.
3. Chachaj, R., Sembratowicz, I., Krauze, M., & Ognik, K. (2019). The effect of partial replacement of soybean meal with fermented soybean meal on chicken performance and immune status. *Journal of Animal and Feed Sciences*. https://doi.org/10.22358/jafs/110777/2019
4. Dousa, B. M., Adam, M. S., Malik, H. E. E., Ali, O. H. A., ElhassanM.M., O., Elamin, K. M., & Elagib, H. A. A. (2018). Impact of Probiotics and Acidifiers on Growth Performance and Blood Chemistry of Broiler Chickens.
5. Eid, K., Radwan, A. A., & Gebriel, G. M. (2010). The Interaction Effect Of Strain , Sex And Live Body Weight On Antibody Response To Srbc S In Broiler Chickens.
6. Granstad, S., Kristoffersen, A. B., Benestad, S. L., Sjurseth, S. K., David, B., Sørensen, L., Fjermedal, A., Edvardsen, D. H., Sanson, G., & Løvland, A. (2020). Effect of feed additives as alternatives to in-feed antimicrobials on production performance and intestinal Clostridium perfringens counts in broiler chickens. *Animals*, *10*(2), 240. https://doi.org/10.3390/ani10020240
7. Hu, Z., Liu, L., Guo, F., Huang, J., Qiao, J., Bi, R., Huang, J., Zhang, K., Guo, Y., & Wang, Z. (2023). Dietary supplemental coated essential oils and organic acids mixture improves growth performance and gut health along with reduces Salmonella load of broiler chickens infected with Salmonella Enteritidis. *Journal of Animal Science and Biotechnology*, *14*(1), 95. https://doi.org/10.1186/s40104-023-00889-2
8. Khomayezi, R., & Adewole, D. I. (2021). Probiotics, prebiotics, and synbiotics: an overview of their delivery routes and effects on growth and health of broiler chickens. *World's Poultry Science Journal*, *78*, 57 - 81. https://doi.org/10.1080/00439339.2022.1988804
9. Nataraja, D., Malathi, V., Naik, J., Indresh, H. C., & Sreedhara, J. N. (2020). Effect of Butyric Acid Supplementation on Growth Performance and Immune Response in Broilers. *International Journal of Current Microbiology and Applied Sciences*, *9*, 2422-2430. https://doi.org/10.20546/ijcmas.2020.902.276
10. Niyonshuti, E., Ng'ang'a, Z. W., Bayraktar, Ö. H., & Kırkpınar, F. (2022). Antibiotic Free Poultry Production-Focus on Antimicrobial Resistance, Challenges, and Alternatives. *6th International Students Science Congress Proceedings Book*. https://doi.org/10.52460/issc.2022.008
11. Omidiwura, B. R. O., & Agboola, A. F. (2024). Performance, gut microbial population and histology of broiler chickens fed probiotics and organic acid supplemented diets. *Nigerian Journal of Animal Production*. https://doi.org/10.51791/njap.vi.7888
12. Osinowo, O. A., Ogbonna, C. C., & Omoare, A. M. (2021). Growth Performance and Gut Morphological Responses of Broiler Chickens to Fermented Diets. *Journal of Experimental Agriculture International*. https://doi.org/10.9734/jeai/2021/v43i930733
13. Rodjan, P., Soisuwan, K., Thongprajukaew, K., Theapparat, Y., Khongthong, S.,

Jeenkeawpieam, J., & Salaeharae, T. (2018). Effect of organic acids or probiotics alone or in combination on growth performance, nutrient digestibility, enzyme activities, intestinal morphology and gut microflora in broiler chickens. *Journal of animal physiology and animal nutrition*, *102*(2), e931-e940. https://doi.org/10.1111/jpn.12858
14. Soumeh, E. A., Cedeno, A. D. R. C., Niknafs, S., Bromfield, J., & Hoffman, L. C. (2021). The efficiency of probiotics administrated via different routes and doses in enhancing production performance, meat quality, gut morphology, and microbial profile of broiler chickens. *Animals*, *11*(12), 3607. https://doi.org/10.3390/ani11123607
15. Thirumeignanam, D., Chellapandian, M., Arulnathan, N., Parthiban, S., Kumar, V., Vijayakumar, M. P., & Chauhan, S. (2024). Evaluation of natural antimicrobial substances blend as a replacement for antibiotic growth promoters in broiler chickens: enhancing growth and managing intestinal bacterial diseases. *Current microbiology*, *81*(2), 55. https://doi.org/10.1007/s00284-023-03573-w