Immunomodulatory Effects Of *Lactobacillus Plantarum* NC8 In Poultry: Enhancing Immune Response Against Salmonellosis

Junaid Nazir[1,2], Shazia Tabassum[3], Saima Tabassum[4], Areeba Tabassum[1], Priyanka Jha[2*], Syed Mudasir Ahmad[1*]

[1]*Division of Animal Biotechnology, Faculty of Veterinary Sciences & Animal Husbandry, SKUAST Kashmir, Shuhama, J&K, India -190006. junaidnaxir@gmail.com, areebamir25@gmail.com, mudasirbio@gmail.com*
[2*]*Department of Clinical Biochemistry, Lovely Professional University, Phagwara Punjab, India - 144411. priyanka.27775@lpu.co.in*
[3]*Department of physiology, Government medical College, Nagpur, Maharashtra, India-440003. shaziamir16@gmail.com*
[4]*Department of Otorhinolaryngology - Head and Neck Surgery, Government Medical College, Srinagar-190010. saima_t2007@yahoo.co.in*

***Corresponding Author:** Syed Mudasir Ahmad*
**Professor, Animal Biotechnology, Faculty of Veterinary Sciences and Animal Husbandry, Shuhama, SKUAST-Kashmir, 190006 mudasirbio@gmail.com*

***Co- Corresponding Author:** Priyanka Jha
*Associate professor, Department of Clinical Biochemistry, Lovely Professional University, Phagwara Punjab, India, 144411 priyanka.27775@lpu.co.in

Received - 17/02/2025 Acceptance - 18/02/2025

KEYWORDS
Salmonella Gallinarum, fowl typhoid, *Lactobacillus plantarum* NC8, immunomodulation, cytokine expression, probiotic intervention, poultry immunity

ABSTRACT
Salmonella Gallinarum is a major pathogen in poultry, causing fowl typhoid and significant economic losses. This study investigates the immunomodulatory effects of *Lactobacillus plantarum* NC8 in enhancing resistance against *S.* Gallinarum infection. A total of 45 chicks were divided into experimental groups and administered different doses of *L. plantarum* NC8. Birds that received a double-dose regimen (Day 1 and Day 10) revealed reduced tissue damage in the vaccinated groups compared to controls in histopathological analysis. The expression of key cytokines (IL-4, IL-10, and IL-17) was significantly upregulated, suggesting a balanced immune response that enhances both protection and immune regulation. These findings highlight the potential of *Lactobacillus plantarum* NC8 as a probiotic intervention to improve poultry immunity and reduce *Salmonella* infections, offering a natural and sustainable alternative to antibiotics in disease control.

Introduction

Salmonella infections, particularly those caused by *Salmonella* Gallinarum, pose a significant challenge to poultry health and the poultry industry worldwide. *Salmonella* Gallinarum is the causative agent of fowl typhoid, a severe systemic disease that leads to high mortality rates in chickens and substantial economic losses (Berhanu & Fulasa, 2020; Shivaprasad, 2000). Unlike zoonotic *Salmonella* species, which can be transmitted to humans through contaminated poultry products, *S.* Gallinarum is host-adapted to birds and does not commonly cause human infections (Nazir et al., 2024). Controlling fowl typhoid remains a priority for poultry production, yet effective strategies are limited.(Berhanu & Fulasa, 2020)

Current approaches for managing *Salmonella* infections in poultry include vaccination, antibiotic treatment, and improved biosecurity measures (Hohmann, 2001). However, the widespread use of antibiotics has contributed to the emergence of antimicrobial-resistant strains, raising concerns about the sustainability of antibiotic-based disease control (Nazir et al., 2025). The search for alternative methods has led to increasing interest in probiotics, particularly *Lactobacillus* species, for their ability to enhance disease resistance and modulate the immune system in poultry (Ahmad et al., 2023).

Lactobacillus plantarum NC8, a probiotic commonly found in the gut microbiota and fermented foods, has been shown to support gut health and immune function. Probiotics contribute to disease resistance by promoting a balanced gut microbiome, preventing colonization by harmful pathogens, and stimulating the host immune system.(Bruno et al., 2009; Hurley et al., 2014) In poultry, the immune response involves both innate and adaptive mechanisms, with the gut-associated lymphoid tissue (GALT) playing a critical role in defence against intestinal pathogens like *Salmonella* (Gut et al., 2018; Yuan et al., 2022).

Recent studies have demonstrated that *Lactobacillus plantarum* NC8 can regulate cytokine production, influencing immune responses without causing excessive inflammation (Maldonado et al., 2004; Wang et al., 2019). Key cytokines such: IL-4 supports B-cell activation and antibody production, IL-10 regulates inflammation, and IL-17 helps protect against bacterial infections by promoting mucosal defence. The balance between these cytokines is crucial for an effective immune response (Choi & Reiser, 1998; Ng et al., 2013; Ruiz de Morales et al., 2020).

Despite growing evidence supporting the immunomodulatory effects of probiotics, there is still limited research on how *Lactobacillus plantarum* NC8 influences immune responses against *Salmonella Gallinarum*.(LeCureux & Dean, 2018; Maldonado et al., 2004) Most probiotic studies focus on gut microbiota composition rather than systemic immunity and disease resistance. Therefore, this study aims to evaluate the role of *Lactobacillus plantarum* NC8 in modulating immune responses in poultry and its potential to enhance protection against *Salmonella* Gallinarum.(Chadfield et al., 2003) By investigating its effects on immunoglobulin levels and cytokine expression, this research could contribute to the development of sustainable, antibiotic-free strategies for controlling fowl typhoid and improving poultry health.

This research could provide new insights into how probiotics like *Lactobacillus plantarum* NC8 can be used as a tool to enhance the immune system in poultry, potentially leading to more effective and sustainable methods for controlling *Salmonella* infections. The findings of this study may contribute to the development of natural, non-antibiotic strategies to improve poultry health, reduce the spread of *Salmonella*, and minimize the risk of foodborne illnesses in humans.

Materials and Methods

Animals and Experimental Design

A total of 45 one-day-old broiler chicks were obtained from the Poultry Department at SKUAST-Kashmir and housed under controlled and hygienic conditions in individual cages. The chicks were provided with standard commercial feed and water ad libitum throughout the experiment. Before the initiation of the study, all chicks were screened for *Salmonella* infection using an ELISA-based assay and confirmed to be *Salmonella*-free.

The chicks were randomly divided into nine experimental groups (n = 5 per group). The groups were designed to evaluate the effect of Lactobacillus plantarum NC8 on immune responses in poultry, with variations in dose and administration schedule.

Preparation of *Lactobacillus plantarum* NC8 Suspension and Oral Dosage

Lactobacillus plantarum NC8 was cultured overnight at 37°C in de Man, Rogosa, and Sharpe (MRS) broth, harvested by centrifugation (4,000 × g, 10 min at 4°C), and washed twice with sterile PBS. The bacterial suspension was adjusted to a final concentration of 1×10^8 CFU/mL in PBS to ensure consistent dosing across all treatment groups. The birds were orally administered *Lactobacillus plantarum* NC8 using a sterile syringe, following a structured experimental setup: Groups 1, 2, and 3 received two doses of *Lactobacillus plantarum* NC8 (1×10^8 CFU in PBS) on days 1 and 10, while Groups 4, 5, and 6 received one dose (1×10^8 CFU in PBS) on day 1, followed by PBS on day 10. Groups 7, 8, and 9 served as control groups, receiving PBS alone on both days. Throughout the study, all birds were closely monitored for behavioural changes, feed intake, and general health status.

Salmonella Gallinarum Challenge

At 20 days of age, all chicks were challenged intraperitoneally with *Salmonella* Gallinarum at an ID_{50} dose of 6.5×10^8 CFU per bird (Nazir et al., 2024). The bacterial inoculum was prepared by culturing *S.* Gallinarum overnight in Brain Heart Infusion (BHI) broth at 37°C with shaking (200 rpm). The suspension was then adjusted to an optical density (OD_{600}) of 0.8, corresponding to 6.5×10^8 CFU/mL, which was confirmed through serial dilution and plating. Each bird was injected intraperitoneally with 0.2 mL of the bacterial suspension. Post-challenge, the birds were monitored daily for 10 days for clinical signs of infection, including lethargy, loss of appetite, body weight changes, and mortality.

Sample Collection: Blood Samples: Blood was collected from the wing vein on days 10 and 20 using a sterile 25G needle and vacutainer tubes. The collected blood was allowed to clot at room temperature for 30 min before being centrifuged at 3,000 × g for 10 min to obtain serum for antibody analysis.

On day 30, all birds were humanely euthanized by cervical dislocation following Institutional Animal Ethics Committee (IAEC) guidelines. The intestinal and spleen tissues were harvested for histopathological analysis and cytokine expression profiling respectively.

Cytokine Expression Analysis (IL-4, IL-10, IL-17) using RT-PCR

Total RNA was extracted from spleens using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. RNA purity and concentration were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). For cDNA synthesis, 1 µg of RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA). The expression levels of IL-4, IL-10, and IL-17 were quantified using a SYBR Green-based real-time PCR assay (Applied Biosystems, USA), with GAPDH serving as the housekeeping gene. Relative cytokine expression levels were analysed using the $2^{-\Delta\Delta Ct}$ method, allowing for a comparative assessment across different treatment groups.

Histopathological Examination

The spleen and intestinal tissues were fixed in 10% neutral buffered formalin for 48 hours, embedded in paraffin, and sectioned at 5 µm thickness using a microtome (Leica RM2235, Germany). The sections were stained with haematoxylin and eosin (H&E) and examined under a light microscope (Olympus BX53, Japan). A blinded pathologist assessed tissue sections for inflammation, necrosis, and bacterial lesions.

Statistical Analysis

Data were analysed using GraphPad Prism. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test for multiple group comparisons and unpaired t-test for two-group comparisons. A p-value < 0.05 was considered statistically significant. Results are presented as mean ± standard error of the mean (SEM).

Ethical Approval

All experimental procedures involving animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of SKUAST-Kashmir. The study was conducted in strict compliance with CPCSEA guidelines for the care and use of laboratory animals in research.

Results

Mortality and Clinical Observations

Following the *Salmonella* Gallinarum challenge, mortality rates varied across the experimental groups. The double-dosed *Lactobacillus plantarum* NC8 groups (Groups 1, 2, and 3) exhibited the lowest mortality. Specifically, Group 1 recorded 2 deaths, Group 2 had 3 deaths, and Group 3 reported 1 death. Groups receiving a single dose of *Lactobacillus plantarum* NC8 (Groups 4, 5, and 6) showed a higher mortality rate, with each group experiencing 3 deaths. The PBS control groups (Groups 7, 8, and 9) exhibited the highest mortality rates: Group 7 had 4 deaths, Group 8 had 5 deaths, and Group 9 reported 3 deaths (Figure 1).

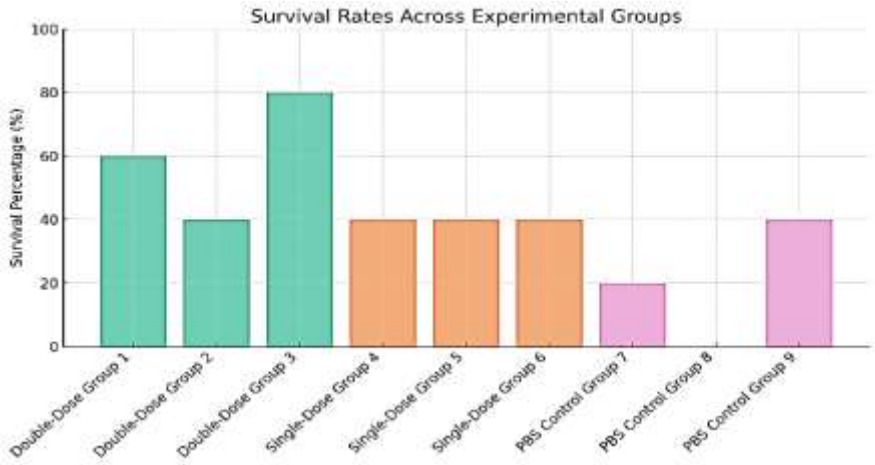

Figure1: Survival curve of experimental groups following *Salmonella* Gallinarum challenge.

Cytokine Expression (IL-4, IL-10, IL-17)

Cytokine profiling of serum samples taken on day 20 post-challenge showed a significant upregulation of IL-4, IL-10, and IL-17 in the double-dosed groups. All Ct values were normalized using GAPDH as the housekeeping gene (Table 1).

Table 1: Mean Ct values (± SD) for IL-4, IL-10, and IL-17.

Group Type	IL-4	IL-10	IL-17
Double Dose	24.5 ± 0.5	24.7 ± 0.4	25.8 ± 0.6
Single Dose	26.0 ± 0.6	26.5 ± 0.5	26.2 ± 0.5
PBS Control	27.5 ± 0.4	27.0 ± 0.3	28.8 ± 0.4

IL-4 levels were significantly elevated in the double-dosed groups compared to the single-dosed and PBS control groups, suggesting a shift towards a Th2-type immune response. IL-10, an anti-inflammatory cytokine, was also significantly elevated, indicating enhanced immune regulation. IL-17 levels were notably higher, indicating potential involvement in mucosal immunity and enhanced protection against *Salmonella* (Figure 2).

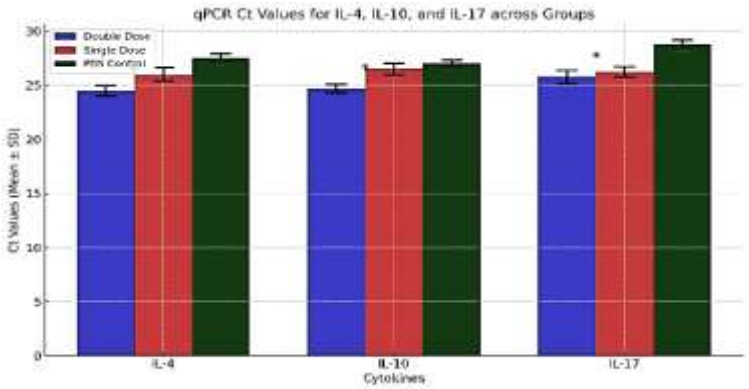

Figure2: qPCR Ct Values for IL-4, IL-10, and IL-17 Across Groups

Histopathological Analysis

Histopathological examination of intestinal tissues revealed notable variations in tissue damage across the treatment groups. The double-dosed Lactobacillus plantarum NC8 groups exhibited minimal lesions, with preserved mucosal architecture and no significant signs of inflammation or necrosis. In contrast, the single-dosed and PBS control groups demonstrated moderate to severe histopathological changes, including marked infiltration of inflammatory cells, necrosis, and haemorrhage as shown in figure 3. These findings suggest that the double-dose regimen of *Lactobacillus plantarum* NC8 effectively reduced both mortality and the pathological impact of *Salmonella* Gallinarum infection.

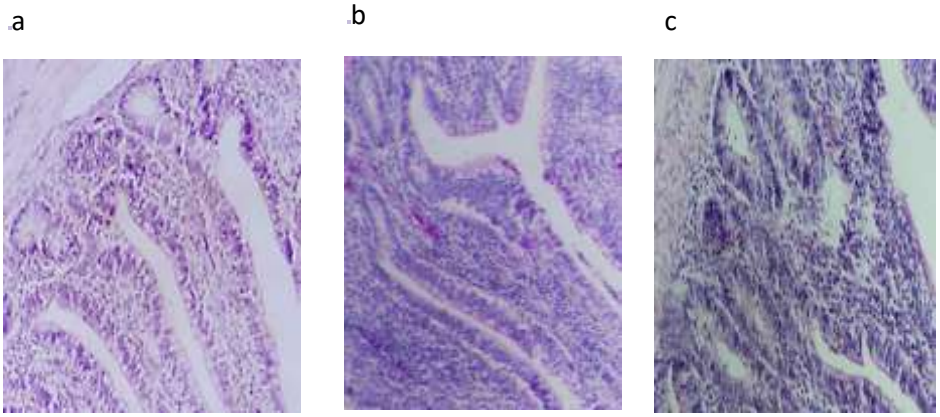

Figure 3: Histopathological examination of intestinal tissues. (a) Double-dose *Lactobacillus plantarum* group: minimal lesions. (b) Single-dose group: moderate inflammation and necrosis. (c) PBS control group: severe tissue damage with extensive inflammation and heterophil infiltration.

Discussion

This study demonstrates the immunomodulatory potential of *Lactobacillus plantarum* NC8 in protecting poultry against *Salmonella* Gallinarum infection. The findings indicate significant improvements in cytokine expression, histopathological outcomes, and mortality rates with a double-dose regimen of *L. plantarum* NC8. The upregulation of IL-4, IL-10, and IL-17 in the double-dosed groups suggests enhanced immune regulation and protection. IL-4 elevation reflects a shift towards a Th2-type immune response, promoting antibody-mediated immunity—a critical defence mechanism against extracellular pathogens like *Salmonella* (Choi & Reiser, 1998). The increased IL-10 expression indicates strong anti-inflammatory effects, helping to mitigate excessive immune responses that could lead to tissue damage (Ng et al., 2013). Notably, IL-17 upregulation suggests enhanced mucosal immunity, crucial for preventing *Salmonella* colonization and systemic dissemination.(Ruiz de Morales et al., 2020)

Histopathological analysis revealed minimal tissue damage in the spleen and intestines of the double-dosed groups, highlighting the protective effects of L. plantarum NC8. In contrast, control and single-dose groups exhibited severe lesions, including inflammation, necrosis, and haemorrhaging. These results underscore the dose-dependent role of *L. plantarum* NC8 in mitigating *Salmonella*-induced pathology and align with the cytokine expression data, supporting a balanced immune response that limits both infection severity and collateral tissue damage

The reduced mortality rates in the double-dosed groups further demonstrate the effectiveness of this probiotic regimen(Kazemifard et al., 2022). By enhancing systemic immunity and reducing tissue injury, *L. plantarum* NC8 may limit bacterial dissemination and improve overall survival in *Salmonella*-challenged birds. Given concerns over antimicrobial resistance due to the overuse of antibiotics, probiotics such as *L. plantarum* NC8 present a promising alternative for disease control in poultry. The observed immunomodulatory effects suggest that *L. plantarum* NC8 could be integrated into poultry management programs to reduce antibiotic reliance, improve flock health, and lower the risk of *Salmonella* outbreaks.

This study provides strong evidence for the protective effects of *L. plantarum* NC8, but certain limitations must be acknowledged. The sample size was relatively small, and only one breed of poultry was tested. Long-term effects of continuous *L. plantarum* NC8 administration were not evaluated. Future studies should explore its effects across different poultry breeds, age groups, and environmental conditions. Additionally, investigating the molecular mechanisms underlying its immunomodulatory effects could further clarify its role in immune regulation. Research into its potential interactions with other probiotics or antibiotics may also provide insight into more comprehensive disease control strategies.

Lactobacillus plantarum NC8 demonstrates significant potential as an immunomodulator for protecting poultry against *Salmonella* Gallinarum. By reducing the reliance on antibiotics, this approach offers a sustainable and effective solution for managing poultry infections. Beyond *Salmonella* protection, these benefits may extend to promoting overall gut health and immune function in poultry. Further research is needed to explore broader applications and fully elucidate the mechanisms driving these effects.

Data availability

All data generated or analysed during this study are included in this manuscript. All relevant data are available from the authors.

Acknowledgements

We extend our gratitude to the Division of Livestock Production and Management, Faculty of Veterinary Sciences and Animal Husbandry (FVSc & AH), SKUAST-K, which played a crucial role in the successful execution of our experimental trials.

Author contributions

J.N. and S.M.A. designed the project. J.N., and S.T. wrote the paper. J.N., designed and carried out the experiments relating to the infection of chicks. S.T., S.T., A.T., S.M.A. and P.J. contributed to experiments and data interpretation analysed the re-isolated *Salmonella* from infected birds. All authors critically reviewed the manuscript.

Declaration of Interests

All authors declare no competing interests.

Ethics approval and consent to participate

The experimental protocol, including animal acquisition, vaccination, and challenge trials, was thoroughly reviewed and approved by the Animal Ethics Committee of SKUAST-K. This committee operates in compliance with the national guidelines on animal care and welfare as set by the Government of India.

Funding Statement

We acknowledge the financial support provided by the Department of Science and Technology, Science and Engineering Research Board (DST-SERB), under the grant CRG/2021/000677 for the project on the "Development of Recombinant Lactic Acid Bacteria-Based Vaccine for Salmonellosis in Poultry."

References

1. Ahmad, S. M., Bhat, S. S., Shafi, S., Dar, M. A., Saleem, A., Haq, Z., Farooq, N., Nazir, J., & Bhat, B. (2023). Identification of key transcription factors and their functional role involved in Salmonella typhimurium infection in chicken using integrated transcriptome analysis and bioinformatics approach. *BMC Genomics*, *24*(1). https://doi.org/10.1186/s12864-023-09315-3
2. Berhanu, G., & Fulasa, A. (2020). Pullorum Disease and Fowl Typhoid in Poultry: A Review. *British Journal of Poultry Sciences*, *9*(3).
3. Bruno, V. M., Hannemann, S., Lara-Tejero, M., Flavell, R. A., Kleinstein, S. H., & Galán, J. E. (2009). Salmonella typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells. *PLoS Pathogens*, *5*(8). https://doi.org/10.1371/journal.ppat.1000538
4. Chadfield, M. S., Brown, D. J., Aabo, S., Christensen, J. P., & Olsen, J. E. (2003). Comparison of intestinal invasion and macrophage response of Salmonella Gallinarum and other host-adapted Salmonella enterica serovars in the avian host. *Veterinary Microbiology*, *92*(1–2). https://doi.org/10.1016/S0378-1135(02)00290-0
5. Choi, P., & Reiser, H. (1998). IL-4: Role in disease and regulation of production. In *Clinical and Experimental Immunology* (Vol. 113, Issue 3). https://doi.org/10.1046/j.1365-2249.1998.00690.x
6. Gut, A. M., Vasiljevic, T., Yeager, T., & Donkor, O. N. (2018). Salmonella infection – Prevention and treatment by antibiotics and probiotic yeasts: A review. In *Microbiology (United Kingdom)* (Vol. 164, Issue 11). https://doi.org/10.1099/mic.0.000709
7. Hohmann, E. L. (2001). Nontyphoidal salmonellosis. *Clinical Infectious Diseases*, *32*(2). https://doi.org/10.1086/318457
8. Hurley, D., McCusker, M. P., Fanning, S., & Martins, M. (2014). Salmonella-host interactions - modulation of the host innate immune system. In *Frontiers in Immunology* (Vol. 5, Issue OCT). https://doi.org/10.3389/fimmu.2014.00481
9. Kazemifard, N., Dehkohneh, A., & Baradaran Ghavami, S. (2022). Probiotics and probiotic-based vaccines: A novel approach for improving vaccine efficacy. In *Frontiers in Medicine* (Vol. 9). https://doi.org/10.3389/fmed.2022.940454
10. LeCureux, J. S., & Dean, G. A. (2018). Lactobacillus Mucosal Vaccine Vectors: Immune Responses against Bacterial and Viral Antigens . *MSphere*, *3*(3). https://doi.org/10.1128/msphere.00061-18
11. Maldonado, A., Ruiz-Barba, J. L., & Jiménez-Díaz, R. (2004). Production of plantaricin NC8 by Lactobacillus plantarum NC8 NC8 is induced in the presence of different types of gram-positive bacteria. *Archives of Microbiology*, *181*(1). https://doi.org/10.1007/s00203-003-0606-8
12. Nazir, J., Manzoor, T., Saleem, A., Gani, U., Bhat, S. S., Khan, S., Haq, Z., Jha, P., & Ahmad, S. M. (2025). Combatting Salmonella: a focus on antimicrobial resistance and the need for effective vaccination. *BMC Infectious Diseases*, *25*(1), 84.
13. Nazir, J., Tabassum, S., Manzoor, T., Jha, P., & Mudasir Ahmad, S. (2024). Estimation Of Infectious Dose 50 (ID50) And Immune Response to Salmonella Gallinarum in Vanaraja Layers. *African Journal of Biomedical Research*, *27*(4S), 12994–13001. https://doi.org/10.53555/AJBR.v27i4S.6542
14. Ng, T. H. S., Britton, G. J., Hill, E. V., Verhagen, J., Burton, B. R., & Wraith, D. C. (2013). Regulation of adaptive immunity; the role of interleukin-10. In *Frontiers in Immunology* (Vol. 4, Issue MAY). https://doi.org/10.3389/fimmu.2013.00129
15. Ruiz de Morales, J. M. G., Puig, L., Daudén, E., Cañete, J. D., Pablos, J. L., Martín, A. O., Juanatey, C. G., Adán, A., Montalbán, X., Borruel, N., Ortí, G., Holgado-Martín, E., García-Vidal, C., Vizcaya-Morales, C., Martín-Vázquez, V., & González-Gay, M. Á. (2020). Critical role of interleukin (IL)-17 in inflammatory

and immune disorders: An updated review of the evidence focusing in controversies. In *Autoimmunity Reviews* (Vol. 19, Issue 1). https://doi.org/10.1016/j.autrev.2019.102429

16. Shivaprasad, H. L. (2000). Fowl typhoid and pullorum disease. *OIE Revue Scientifique et Technique*, *19*(2). https://doi.org/10.20506/rst.19.2.1222
17. Wang, S., Geng, N., Zhou, D., Qu, Y., Shi, M., Xu, Y., Liu, K., Liu, Y., & Liu, J. (2019). Oral Immunization of Chickens With Recombinant Lactobacillus plantarum NC8 Vaccine Against Early ALV-J Infection. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02299
18. Yuan, F., Sharma, J., Nanjappa, S. G., Gaulke, C. A., & Fang, Y. (2022). Effect of Killed PRRSV Vaccine on Gut Microbiota Diversity in Pigs. *Viruses*, *14*(5). https://doi.org/10.3390/v14051081