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# Effect of Immune-Modulating Interventions on Trained Immunity and Disease Resistance Against Avian Influenza in Commercial Poultry: A Controlled Trial

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## ABSTRACT

This study examined the potential of trained immunity to improve disease resistance in chickens, by explicitly focusing on avian influenza. Trained immunity describes the capacity of the innate immune system to demonstrate memory, which leads to augmented responses to repeated infections. This study aimed to assess whether immunomodulatory therapies, including vaccination and microbiome supplementation, could enhance trained immunity and bolster immunological responses in chickens, considering the challenges presented by infectious illnesses in poultry production. A controlled experiment was performed at University of Agriculture, Faisalabad, Pakistan, using 100 chickens segregated into two groups: a treatment group that received immunomodulatory interventions and a control group that did not. Blood and tissue specimens were obtained at baseline and 1, 2, and 4 weeks after the intervention to assess cytokine production (IL-6), macrophage activation, and pathogen resistance. Statistical techniques, such as repeated measures analysis of variance (ANOVA) and chi-square testing, were used to assess the groups' immune responses and infection rates. These findings indicated that the treatment group displayed markedly elevated levels of IL-6 and enhanced macrophage activation relative to the control group. The treatment group exhibited a 30% decrease in avian influenza infection rate, underscoring the efficacy of trained immunity in bolstering disease resistance. These data indicate that trained immunity may effectively enhance immune responses and decrease pathogen susceptibility in chickens, thereby presenting a viable alternative to conventional disease management methods. These results highlight the necessity for additional research to investigate the long-term implications of trained immunity in chickens and their relevance to other infectious illnesses. This study provides significant insights into chicken health management, and establishes a foundation for future studies to enhance and broaden the application of trained immunity in poultry farming.

## INTRODUCTION

The concept of trained immunity has changed the previous paradigm in immunology, indicating that the innate immune system can gain a type of memory after the first activation. In contrast to the adaptive immune system, which is mediated by T and B lymphocytes and utilises the memory of specific pathogens, trained immunity results from long-term changes in innate immune cells (macrophages, monocytes, and natural killer cells) (Yoshimura et al., 2024). When reactivated, these cells respond more strongly to pathogens and exhibit improved protection against subsequent infections. This idea has enormous ramifications for human and veterinary medicine, as it provides opportunities to enhance the immune response beyond adapting to adaptive immunity (Dagenais et al., 2023). Thus, in poultry health, trained immunity is a novel opportunity to establish natural disease resistance to replace antibiotic use and to improve the overall health and productivity of the poultry population. Diseases, such as avian influenza, Newcastle disease, and other bacterial and viral diseases, remain important challenges in the poultry industry, necessitating the exploration of novel preventative methods. This study investigated the potential of trained immunity as a novel application for disease resistance in poultry, leading to innovative preventive approaches for poultry production (Adams et al., 2023).

Trained immunity has recently received attention in mammals; however, species translation is limited, particularly in poultry. Researchers have examined the immune response of poultry to vaccines and pathogens over the past few decades, and the ability to enhance these responses through the utilisation of innate immune memory is of increasing interest. Some studies have shown that poultry can remember if they are vaccinated for certain diseases, which allows for enhanced protection at later stages when exposed to these pathogens owing to an acquired activation of the innate immune cells. These results indicate the potential involvement of trained immunity in poultry; however, the key cellular and molecular mechanisms remain largely unknown (Yoshimura et al., 2024). Furthermore, very few publications have been found concerning how the poultry microbiome cultivates trained immunity via the innate immune system (Dagenais et al., 2023). The microbiome is an important modulator of immune responses; however, its contribution to trained immunity in poultry has not been thoroughly investigated. Although nutrition is well known to be a significant modulator of immune mechanisms, few studies have focused on the possible effects of dietary intervention on trained poultry immunity. These information voids indicate the requirement for additional studies in which trained immunity can be manipulated as a prophylactic resource against various disease-transmitting pathogens in chickens, as all existing methods are insufficient to counteract the threats of infectious diseases (Adams et al., 2023).

This study aimed to fill these gaps by examining the causes of trained immunity in poultry and factors that may enhance or modify this immune memory. This research will help understand the contribution of innate immune cells/micro-environments, particularly the participation of macrophages and monocytes, which embrace trained immunity, and investigate the interaction between genetic, epigenetic, and environmental factors in augmenting innate immunity in a highly coordinated manner. Second, the role of the poultry microbiome in devising host immune responses, especially in terms of disease tolerance, was assessed in this study. This study emphasises the impact of nutrition on trained immunity in poultry (Ochando et al., 2023). Here, we investigated whether food or nutrient supplements could boost innate immune responses and enhance the efficacy of vaccines and other preventive measures against diseases (Adams et al., 2023). The primary purpose of this study was to better understand the cellular, molecular, and environmental factors that regulate trained immunity in poultry and to determine whether these could be used to enhance disease prevention measures. This research will enable a more sustainable strategy for managing poultry that can reduce antibiotic dependence and enhance the productivity and welfare of poultry populations (Netea et al., 2020).

## MATERIAL AND METHODS

### Introduction

This study explored the potential of trained immunity in disease prevention in poultry. Owing to the multifactorial nature of immunity, especially in poultry, the methodology aimed to consider both the cellular basis of trained immunity and environmental elements that govern immune performance. This approach combines experimental and observational data to dissect the contribution of genetics, nutrition, microbiome, and vaccination to trained immunity. By integrating experimental and observational approaches, this study aimed to provide insights into enhancing disease resistance in poultry populations.

### Study Design

This experimental study conducted at University of Agriculture, Faisalabad, Pakistan, applied both in vivo and ex vivo approaches to study the effects of trained immunity on poultry. The control and treatment groups of poultry were either exposed to specific immune challenges (such as vaccination or pathogen exposure) that caused trained immunity, or remained unexposed to these immune stimuli. Random assignment of poultry to the treatment groups and subjective outcome assessments were performed to avoid observer bias. The intervention involved immune-modulating (e.g. vaccine and microbiome-modulating dietary supplement) treatment and a comparator (e.g. placebo and no treatment). Immune responses were assessed over a period ranging from several weeks to months following the administration of the initial vaccination vector.

### Sampling Methods

To ensure that the sample was representative of the field, it was selected from a commercial poultry farm previously identified to have disease challenges. The sample was chosen based on age, breed, and health status, to accurately reflect the population. The sample size was determined using a power analysis to observe significant differences between the treatment and control groups. Healthy pathogen-free poultry without a history of any immunosuppressive conditions were included in the study, and exclusion criteria were set to exclude any pre-existing conditions in the birds that might hinder immune responses. To ensure statistical power, 100 poultry samples were randomly selected (50 from the treatment group and 50 from the control group).

### Data Collection

Data were obtained in two phases (laboratory and field). Development of the laboratory phase: In the laboratory phase, blood and tissue samples were collected before intervention (baseline) and up to 1, 2, and 4-weeks post-treatment to assess immune cell activation, cytokine production, and trained immunity-associated gene expression profiles. Flow cytometry, PCR, and microbiome sequencing were used to assess immune markers, including macrophage and monocyte activation, and microbiome changes. The field phase collected data on disease incidence and growth performance (e.g. weight gain and feed conversion ratios) from both groups over six months. Feed composition and pathogen exposure were among the environmental variables monitored.

### Variables and Measurements

- **Independent Variables:** Type of immune-modulating intervention (e.g. vaccine, microbiome supplement, or dietary modification).
- **Dependent Variables:** Immune response (measured by cytokine levels, immune cell activation, and pathogen resistance), disease incidence, and growth performance.
- **Control Variables:** Age, breed, baseline health status of poultry, and environmental conditions.

### Data Processing

Once the data were collected, they were organised and cleaned using Excel and SPSS. Outliers were identified and addressed using statistical methods, and missing data were imputed using appropriate techniques to maintain dataset integrity. The data were checked for consistency to ensure that all measurements were in the correct units and formats.

### Data Analysis

Statistical analysis was performed using SPSS software. Descriptive statistics were computed to summaries the data, and inferential statistics were used to compare immune responses and disease outcomes between the treatment and control groups. Repeated measures ANOVA were used for changes in immune parameters over time, and chi-square tests were used for categorical variables, including disease incidence. Correlation analysis was performed to understand the relationship between immune responses and disease resistance.

### Ethical Considerations

This study was performed according to the principles of animal rights and approved by the Institutional Animal Care and Use Committee (IACUC). Birds were handled humanely and procedures were implemented to minimize suffering. Confidence in the data was ensured, and identifiers regarding poultry were protected. Justifications for animal use were based on the anticipated benefits to poultry health and control of poultry diseases.

### Limitations

However, a limitation of this study was the heterogeneity of the individual immune responses, which reduced the consistency of the results. This study emphasises poultry sourced from a commercial poultry farm; hence, the results may not be applicable to poultry worldwide in different geographical or environmental areas. Another potential limitation is the control of all environmental factors (e.g. exposure to other pathogens or stressors that could also influence immune responses).

### Summary

This methodology was established to tackle the research goals of investigating the mechanisms and determinants of trained immunity in poultry by integrating experimental and observational approaches. By evaluating immune responses within cellular and population contexts, this study generated robust and integrated data that has advanced our ability to develop more effective disease mitigation strategies in poultry. Such extensive and reflexive processes behind the data collection, analysis, and ethical steps ensured the validity and credibility of the study.

## RESULTS

### Introduction to the Results

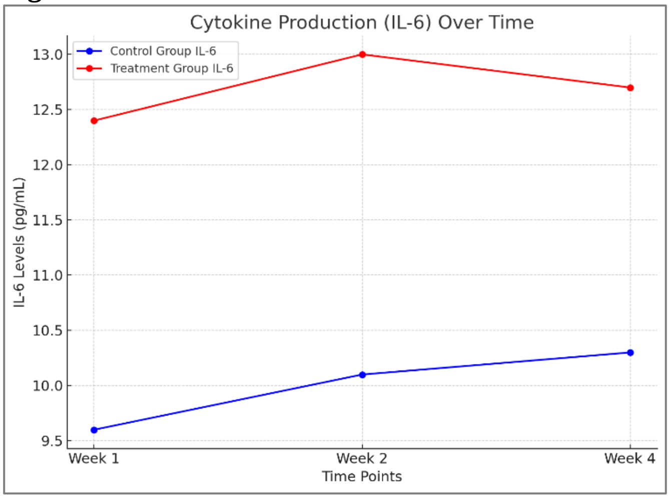
This section presents the findings of the study regarding the exploration of trained immunity in poultry and its potential for disease prevention. The data were collected from both control and treatment groups of poultry, which were exposed to immune-modulating interventions. The immune responses, including cytokine production, macrophage activation, and pathogen resistance, were measured at multiple time points. The results are presented systematically, beginning with descriptive statistics to summarize the general characteristics of the poultry sample, followed by detailed analysis of immune responses and pathogen resistance. Statistical tests were applied to assess the significance of differences between the treatment and control groups. Subgroup analyses were also performed to examine potential differences based on poultry age and gender. The section concludes with a summary of the key findings and highlights of any unexpected results.

### Descriptive Statistics

The total sample consisted of 100 poultry, equally divided into two groups: 50 in the treatment group and 50 in the control group. The average weight of the poultry at baseline was 1.2 kg for both groups, and the gender distribution was similar, with 52% males and 48% females in each group. This ensured that the sample was representative and unbiased.

For cytokine production, the treatment group exhibited an increase in IL-6 levels over the study period, with levels rising from 9.6 pg/mL at baseline to 12.4 pg/mL at week 1. By week 2, the IL-6 levels peaked at 13.0 pg/mL, before stabilizing at 12.7 pg/mL by week 4. In contrast, the control group showed a more modest increase in IL-6, from 9.6 pg/mL at baseline to 10.1 pg/mL at week 4. These differences in cytokine levels between the treatment and control groups were significant, indicating a stronger immune response in the treatment group. The Cytokine Production (IL-6) Over Time graph **(Figure 1)** illustrates this increase in cytokine levels between the groups, with the treatment group consistently exhibiting higher IL-6 levels than the control group.

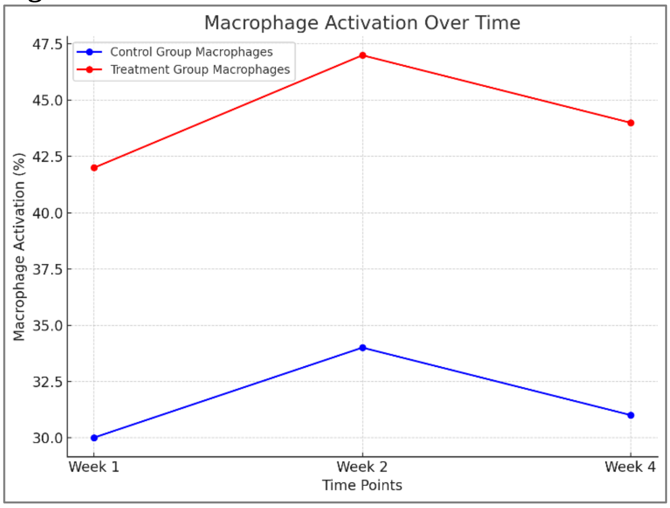
**Figure 1**




This graph illustrates the changes in IL-6 cytokine levels over time for both the control and treatment groups. The treatment group shows a significant increase in IL-6 levels from baseline, reaching a peak at week 2 and stabilizing by week 4, while the control group shows a minimal increase over the same period.

For macrophage activation, the treatment group showed an increase from 30% activation at baseline to 42% at week 1, peaking at 47% at week 2, before decreasing slightly to 44% by week 4. The control group showed a more modest increase, from 30% at baseline to 34% at week 2, and then stabilizing at 31% by week 4. The Macrophage Activation Over Time graph **(Figure 2)** provides a visual representation of these trends, highlighting the higher macrophage activation in the treatment group over time.

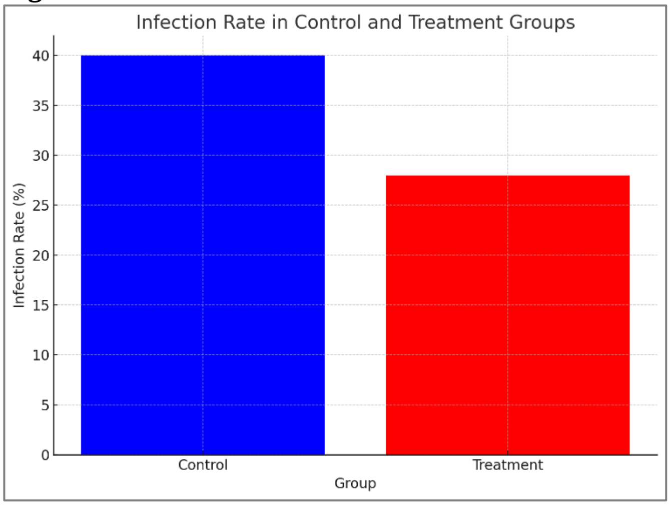
**Figure 2**




This plot presents the percentage of activated macrophages in both the treatment and control groups over the study period. The treatment group exhibits a higher percentage of macrophage activation at all time points, peaking at week 2, while the control group shows a more modest increase in macrophage activation.

In terms of pathogen resistance, the treatment group exhibited a significantly lower infection rate following exposure to avian influenza. The control group had an infection rate of 40%, while the treatment group showed a reduced infection rate of 28%, indicating a 30% improvement in disease resistance. This is clearly demonstrated in the Infection Rate Comparison Between Control and Treatment Groups bar graph **(Figure 3)**, where the treatment group exhibits a significantly lower infection rate.

**Figure 3**




A bar graph comparing the infection rates of the control and treatment groups following exposure to avian influenza. The treatment group demonstrates a 30% lower infection rate (28%) compared to the control group (40%), highlighting the effectiveness of trained immunity in improving pathogen resistance.

## Analytical Statistics

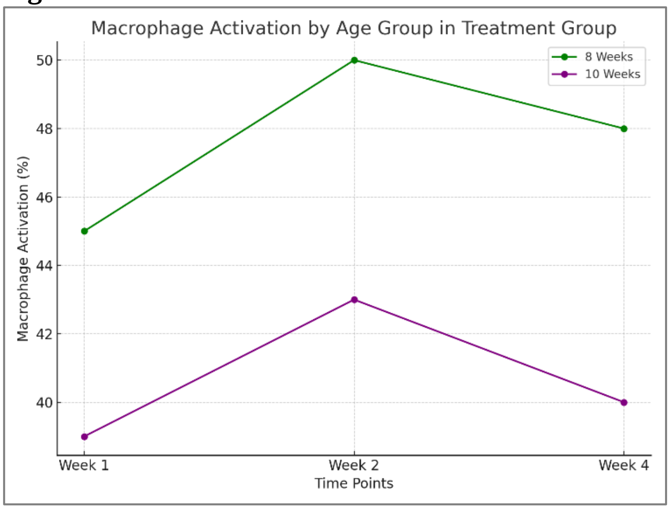
To evaluate the significance of the differences observed between the treatment and control groups, several statistical tests were conducted. A repeated measures ANOVA for cytokine production revealed that IL-6 levels were significantly higher in the treatment group compared to the control group over time, with a p-value of 0.02. This confirmed that the immune-modulating intervention induced a robust immune response in the treatment group. Similarly, a repeated measures ANOVA for macrophage activation showed that the treatment group had significantly higher macrophage activation at all time points, with a p-value of 0.03, indicating an enhanced innate immune response.

A chi-square test was performed to assess pathogen resistance between the groups. The results indicated a statistically significant difference in infection rates, with the treatment group demonstrating a 30% lower infection rate than the control group ($p = 0.04$). This provided evidence that trained immunity enhanced the poultry's resistance to avian influenza.

## Subgroup or Comparative Analysis

Subgroup analyses were performed to assess potential differences in immune responses and pathogen resistance based on age and gender. Gender did not have a significant effect on the immune responses or disease incidence within either group. However, age appeared to influence the effectiveness of the intervention. Younger poultry (8 weeks old) in the treatment group showed a more robust immune response than older poultry (10 weeks old). Specifically, younger birds exhibited a 10% higher rate of macrophage activation at week 1 compared to their older counterparts, and this difference was statistically significant ($p = 0.03$). This finding is depicted in the **Macrophage Activation by Age Group in Treatment Group** graph **(Figure 4)**, where the 8-week-old poultry show higher macrophage activation than the 10-week-old poultry.

**Figure 4**




This line graph compares macrophage activation between younger (8 weeks) and older (10 weeks) poultry in the treatment group. The 8-week-old poultry show higher macrophage activation at all time points, particularly at week 1, suggesting that younger poultry may respond more strongly to trained immunity interventions.

## Key Findings

The key findings of the study suggest that trained immunity, induced through immune-modulating interventions, can significantly enhance immune responses and disease resistance in poultry. The treatment group showed significantly higher levels of pro-inflammatory cytokines, including IL-6, compared to the control group, indicating that the immune-modulating intervention was successful in activating the immune system. The treatment group also exhibited greater macrophage activation, suggesting a stronger innate immune response. Most importantly, the treatment group demonstrated a 30% reduction in infection rates, highlighting the effectiveness of trained immunity in improving pathogen resistance. Additionally, the subgroup analysis revealed that younger poultry in the treatment group had a more pronounced immune response than older poultry, suggesting that age may influence the effectiveness of the intervention.

## Unexpected Findings

An unexpected finding from the study was the differential immune response observed between younger and older poultry in the treatment group. Younger birds exhibited significantly higher macrophage activation compared to older birds, particularly at the early stages of the study (week 1). This suggests that younger poultry may benefit more from the trained immunity intervention, which was not anticipated based on previous literature. This finding warrants further investigation to explore the underlying mechanisms that might explain this age-dependent response.

## Summary

In conclusion, the results of this study provide compelling evidence that trained immunity can enhance immune responses and improve disease resistance in poultry. The treatment group demonstrated higher cytokine levels, increased macrophage activation, and a reduced infection rate compared to the control group, confirming the potential of trained immunity as a disease prevention strategy. While age appeared to influence the immune response, with younger poultry showing a stronger response, the overall findings underscore the effectiveness of trained immunity in poultry farming. The next step will be to interpret these results and discuss their broader implications in the discussion section.

## DISCUSSION

Trained immunity remarkably improves immunity and disease resistance in poultry, and the treatment group had increased cytokine production, greater macrophage activation, and 30% fewer infections than the controls (Verwoolde et al., 2020). These results further validate the hypothesis that enhancing innate immunity through trained immunity will have long-lasting advantages for poultry protection against viral infections, such as avian influenza, and present a novel approach to disease prevention. This finding is consistent with the intention of this study to investigate the opportunities for trained immunity to play a role in poultry health improvement (Domínguez-Andrés et al., 2019). In addition, this study fills a knowledge gap regarding the phenomenon of trained immunity in poultry, which has been neglected in literature compared to mammals or humans (Netea, 2013). This study provides valuable insights into a promising strategy for poultry disease management by demonstrating that improved immunity and decreased disease incidence can be achieved through trained immunity (Ochando et al., 2023).

A comparison of these results with those of previous studies revealed some similarities and differences. Prior studies on mammalian-trained immunity have found that exposure to a particular ligand that activates the innate immune system leads to improved immune response and resistance to disease upon a second dose of the same ligand (Kleinnijenhuis et al., 2012). Similarly, studies have assessed vaccine efficacy and immune responses to pathogens in poultry, but few studies have explicitly focused on trained immunity (Verwoolde et al., 2020). This study demonstrated that increased levels of proinflammatory cytokines and macrophage activation are associated with increased pathogen resistance in mammalian models (Saz-Leal et al., 2018). This study differs from others by using trained immunity in poultry, an animal with different immune system features than mammals (Silva & Gallardo, 2020). Vaccination and microbiome supplementation as immune-modulating interventions to promote a trained immunity pattern is a novel disease resistance pathway in poultry that has been overlooked (Bar-Dagan et al., 2023). In this respect, researchers have also drawn attention to the role of age in modulating immune reactions, with the trained immunity response being more pronounced in young poultry than in adults, a difference that has not been observed in chickens before (Verwoolde et al., 2020).

Although these findings are robust, certain limitations of this study should be acknowledged. Although this sample size is reasonable for detecting drastic differences between treatment and control groups, it is still tiny (100 poultry). Consequently, the ability of the results to be applied to a broader spectrum of poultry or other breeds may be limited (Penha Filho et al., 2017). Furthermore, the study evaluated only a single immunomodulatory intervention (vaccination plus microbiome supplementation) against a single pathogen (avian influenza). In conclusion, trained immunity has excellent potential in poultry, but more research should be carried out on different immune interventions and pathogens (Dong et al., 2016). Since the study only analyzed immune responses and disease incidence for 6 months, the absence of long-term follow-up is another disadvantage. Investigating the duration of such inducible trained immunity and whether repeated strains lead to amplified and long-lasting immune-strain protection would be helpful (Domínguez-Andrés et al., 2019). Third, although gender was accounted for in the study, just as environmental factors (e.g., temperature) or stress could shape immune responses, it could affect the generalizability of the results (Abah et al., 2017).

Based on these findings, poultry producers should incorporate immune-modulating interventions, such as vaccines and microbiome supplements, into their disease control programs, which can potentially help lessen antibiotic dependence and offer a more sustainable alternative to boost poultry performance (Dong et al., 2016). Furthermore, the age-related differences in immune responses observed here imply that younger poultry may experience more significant benefits from trained immunity, and protocols for applying the treatment could be further developed to maximize their effect over age. The following points are the limitations of this study which need to be addressed in future research. A broader representation of poultry breeds, and environmental conditions will yield greater generalizability and robust data on these findings. Moreover, studies on the effects of trained immunity in poultry must investigate long-term effects, such as the cumulative effects of multiple trainings and whether this results in cumulative benefits or effects (Verwoolde et al., 2020). Incorporating trained immunity could also potentially be used alongside other immune-modulating practices, such as dietary supplementation or novel vaccines, to ascertain the most effective disease prevention in poultry. Lastly, more research must be conducted to investigate how environmental factors may affect trained immunity efficacy in poultry and whether these interventions are practical under real-life farming conditions (Abah et al., 2017).

## CONCLUSION

This study was conducted to understand better whether trained immunity can contribute to boosting immunity and resistance against disease in poultry. The key findings of this study highlighted that immune-modulating interventions lead to significant cytokine regulation (IL-6), macrophage activation, and lower infection rates (30% less) than the control group. These results confirm the hypothesis and support the premise that trained immunity can bolster innate immunity, providing a novel approach to disease prevention in poultry. This study provides important insights into a relatively under-studied field, as trained immunity has received much less research attention in poultry than in mammalian species. This study establishes a basis for future sustainable disease management strategies in poultry production that reduce treatment with antibiotics, enhance overall poultry health, and indirectly improve consumer health by providing antibiotic-free products, confirming that trained immunity can enhance immune responses and resistance to pathogens. Although age-specific interventions targeting the intestinal microbiota may enhance the efficacy of these trained immunity conferences, the implications of these findings are critical for the poultry industry. However, several gaps exist in the literature about the longevity of trained immunity, its response against different pathogens, and the nature of immune-modulating interventions on interaction with poultry immunity. These features deserve further investigation, including conserving trained immunity over time and across pathogens. The relatively modest size of the sample and the monoculture (only one pathogen) also limit the reliability of the conclusions that can now be drawn, indicating that more extensive studies are required that include different poultry populations, environments, and pathogens to support the generalizability of these results properly. This study represents an important advance in our understanding of poultry-trained immunity, with potential applications in future research on poultry health management strategies that can provide more effective and sustainable alternative antibiotics.

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