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Combined *Salmonella* and *Pasteurella multocida* Antigens Single-Dose and Adsorbed in Aluminum Hydroxide–Chitosan Protect Swiss Mice Against Lethal Challenge

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Abstract

Background/Objectives: Avian cholera and typhoid are major bacterial diseases in the poultry industry, causing reduced productivity and economic losses. Here, we developed a single-dose, bivalent, inactivated vaccine. Methods: Different concentrations of the experimental vaccine containing *Pasteurella multocida* (*P. multocida*) and *Salmonella* Gallinarum (*S.* Gallinarum) antigens adsorbed to an aqueous matrix based on chitosan, aluminum hydroxide, and saponin were evaluated in mice to determine the optimal single-dose concentration. Results: Vaccine efficacy was assessed by survival rates; the experimental vaccine provided complete protection, whereas 67% of animals immunized with a commercial vaccine survived a challenge with 1×10^{10} CFU/mL (2×10^{9} CFU/mice) of *P. multocida* and 5×10^{10} CFU/mL (1×10^{10} CFU/mice) of *S.* Gallinarum. Subsequently, experiments were conducted to determine the cytokine profiles of animals immunized with the experimental vaccine (single-dose vs. double-dose regimens) followed by a challenge. Pre-challenge cytokine analysis revealed similar profiles for interleukin (IL)-2, IL-4, IL-10, IL-17, interferon gamma (IFN-γ), and Tumor necrosis factor (TNF-α) across groups; however, IL-6 levels were significantly higher in the single-dose experimental vaccine group (G1) than in the commercial vaccine group (G2). In the post-challenge comparative study, the cytokine

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Academic Editors: Haibo Li and Hongwu Sun

Received: 11 September 2026
Revised: 19 October 2026
Accepted: 23 October 2026
Published: 27 October 2026

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response induced by the single-dose vaccine was robust and stable, showing no negative interference following the *S*. Gallinarum challenge, although the double-dose regimen did boost cellular and regulatory cytokines (IFN-γ, TNF-α, and IL-10) specifically against *S*. Gallinarum. Conversely, no significant differences in cytokine profiles were observed among the treatments for groups challenged with *P. multocida* or for unchallenged groups. Conclusions: The single-dose regimen proved sufficient to confer complete protective immunity against both pathogens, suggesting it is a promising strategy for simultaneously preventing avian typhoid and cholera.

Keywords: adjuvants; bivalent vaccine; *Pasteurella multocida*; poultry farming; *Salmonella* Gallinarum; single-dose

1. Introduction

Fowl cholera and fowl typhoid are bacterial diseases with major economic impact, causing significant losses to poultry production in various regions of the world. Fowl cholera is caused by *Pasteurella multocida*, a pathogen capable of infecting different animal species; it causes hepatic lesions and sudden death in infected chickens, posing a serious threat to the poultry industry. It is classified into five capsular serogroups (A, B, D, E, and F), with serogroups A and F (and more rarely D) primarily associated with disease cases [1]. In contrast, *Salmonella enterica* serovar Gallinarum biovar Gallinarum (*S*. Gallinarum) infections remain a persistent challenge for poultry production. Live attenuated vaccines, one of the control strategies employed against this pathogen, carry an inherent risk of secondary mutation and virulence reversion, as well as the potential to cause severe reactions in immunocompromised animals [2,3]; inactivated vaccines, such as the one developed in this study, avoid this risk by eliminating replicative bacterial capacity prior to administration. The persistence and dissemination of these agents within poultry production systems present additional control challenges. In fowl cholera, wild birds act as important reservoirs for *P. multocida*, facilitating the spread of the agent to commercial flocks [4]. Furthermore, once introduced into a farm, the bacterium can establish persistent infections and become endemic, hindering eradication efforts and fostering recurrent outbreaks [5]. Similarly, fowl typhoid remains endemic in various regions of Central America, South America, Africa, and Southeast Asia, posing an obstacle to the sustainability of poultry production and to the international trade of poultry and poultry products [2]. Inactivated vaccines are generally well-tolerated by host organisms and serve as key strategies for controlling infectious diseases in poultry farming, contributing to reduced mortality, improved production metrics, and decreased economic losses associated with infectious outbreaks [3]. However, commercially available inactivated vaccines still face limitations regarding the breadth and duration of the induced immune response [6]. Strategies involving antigen selection and adsorption methods utilizing novel immunomodulatory adjuvants show promise for enhancing vaccine efficacy. Adjuvants can boost antigen immunogenicity, prolong the immune response, stimulate cellular and humoral immunity mechanisms, and reduce antigenic competition in combination vaccines [7–9]. Research into novel adjuvants highlights the use of chitosan, valued for its mucoadhesive properties and ability to facilitate interactions between antigens and immune system cells. Saponins enhance the simultaneous stimulation of cellular and humoral responses, contributing to mechanisms associated with both Th1 and Th2 profiles when combined with aluminum hydroxide matrices; the latter are predominantly used in commercial vaccines due to their role in inducing Th2-type responses [10–15].

Therefore, this study proposes an experimental vaccine utilizing inactivated *S*. Gallinarum antigens and *P. multocida* adsorbed onto a matrix composed of chitosan, aluminum

hydroxide, and saponin, aiming to enhance the immune response and extend the protection conferred by immunization in murine models. The proposal seeks to contribute to the development of more efficient vaccination strategies for the poultry industry, with the potential to reduce the handling associated with repeated administrations and optimize immunization programs for poultry.

2. Materials and Methods

2.1. Bacterial Strains and LD_{50} Determination

The *P. multocida* type A strain was provided by the Center for Diagnosis and Research in Avian Pathology at the Federal University of Rio Grande do Sul (UFRGS), and its identification was performed as described by Furian et al. (2014) [16]. The *Salmonella enterica* serovar Gallinarum strain used in this study was provided by the Avian Medicine Laboratory at the State University of Londrina (UEL), Brazil, and its identification was performed as described by Batista et al. (2016) [17]. The isolates were cultured in Brain Heart Infusion (BHI) broth (Merck® New Jersey, NJ, USA) in an orbital shaking incubator for 12 h at 37 °C and 180 rpm. Cells were harvested using a benchtop centrifuge (model 80-2B) at 3000 rpm for 10 min; the cells were collected and resuspended in phosphate-buffered saline (PBS, pH 7.2), a process repeated three times. The median lethal dose (LD_{50}) of *P. multocida* and *S.* Gallinarum was determined according to the Reed and Muench method [18,19]. To determine the LD_{50} of *P. multocida*, mice (n = 6 per group) were inoculated intraperitoneally with 200 µL of active bacterial suspension at concentrations of 7.50×10^{13}; 8.37×10^{12}; 2.17×10^{11}; 1.16×10^{10}; 1.30×10^{9}; and 6.26×10^{8} CFU/mL. For the LD_{50} determination of *S.* Gallinarum, animals were distributed into five groups (n = 6 per group) and inoculated intraperitoneally with the same volume (200 µL) of active suspension at concentrations of 1.37×10^{15}; 4.90×10^{12}; 5.41×10^{10}; 1.84×10^{9}; and 5.97×10^{8} CFU/mL. The animals were monitored for 48 h, with events and clinical signs recorded, to determine the LD_{50}.

2.2. Ethical/Legal Requirements and Use of Animals in Experiments

Female Swiss mice, approximately 6 weeks of age and weighing 20–24 g, were obtained from the Federal University of Goiás (UFG) and housed in the preclinical testing room of the Biomolecules and Vaccines Laboratory (LaBVac) at the Federal University of Tocantins (UFT), Brazil. Animals were randomly allocated to experimental groups by laboratory personnel. The animals were kept in pathogen-free primary enclosures with ad libitum access to food and water, maintained at a controlled temperature (24 °C), with odor and lighting controls adjusted to a 12 h light/dark cycle. During the immunization phase, animals from all groups were housed together in the same room; after bacterial challenge, animals were separated into distinct, clearly identified cages/shelves by treatment group. All animals used in this experiment were previously dewormed via access to an Ivermectin® solution (1:20 dilution) for 7 days. The experiments were conducted in accordance with ethical recommendations established by the law governing procedures for the scientific use of animals. They were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Tocantins (protocol no. 23.101.001.307/22-31). Every effort was made to avoid undue suffering or pain; the animals were monitored for clinical status, changes in body weight, and clinical signs such as lethargy, hypothermia, and/or respiratory distress. The onset of such clinical signs was treated as a death event and recorded to determine the cumulative mortality for each experimental group and subsequently calculate the LD_{50}. At the end of the observation period, the animals were euthanized using ketamine (300 mg/kg) (Vetbrands®, Perdões/MG, Brazil) and xylazine hydrochloride (22.5 mg/kg) (Syntec®, São Paulo/SP, Brazil).

2.3. Antigen Production, Inactivation, and Vaccine Formulation

The vaccine antigen was obtained from inactivated *P. multocida* and *S.* Gallinarum bacterial cells derived from pathogen cultures in BHI broth. Inactivation was achieved by adding a 0.6% (*v*/*v*) formalin solution under agitation at 150 rpm for 48 h at 37 °C. We verified inactivation efficacy by plating 1 mL of each inactivated sample onto BHI agar and incubating for 24 h at 37 °C. After confirming inactivation, residual formalin was removed by washing the bacterial suspension three times. For each wash, the cells were centrifuged at 3000 rpm for 10 min, the supernatant was discarded, and the pellet was resuspended in PBS. The adjuvant used was prepared by first solubilizing chitosan powder (Polymar Ltda., Fortaleza/CE, Brazil, 81% degree of deacetylation) in 0.8% (*v*/*v*) acetic acid and 0.9% (*w*/*v*) saline solution, using a 2:1 ratio of chitosan (12.5 mg/mL of final concentration) to aluminum hydroxide (4.5 mg/mL of final concentration) [15], supplemented with saponin at a final concentration of 500 µg/mL. The experimental vaccine was formulated with both antigens at a concentration of 10^9 cells/mL (2.5×10^8 cells/mice), using a ratio of 25% (*v*/*v*) adjuvant and 10% (*v*/*v*) saponin.

2.4. Definition of Vaccine Dose

To determine the dose of the experimental vaccine, mice were assigned to four experimental groups of 12 animals each, designated GC1, GC2, GC3, and PBS. Groups GC1, GC2, and GC3 received a single intramuscular dose of 250 µL, 125 µL, and 50 µL of the experimental vaccine, respectively. The PBS group received a single 250 µL dose of PBS and served as the negative control. Animals were monitored daily from day 1 to day 21 post-immunization and evaluated for clinical signs including changes in feed intake, lethargy, prostration, and diarrhea as well as variations in body weight (Figure 1A). Following this analysis period, the groups were subdivided and challenged intraperitoneally with 200 µL of a suspension containing the respective viable bacteria at a concentration corresponding to the LD_{50} previously determined for each pathogen. A second study was designed based on data obtained from the standardization of the experimental vaccine dose. Thus, the vaccine dose defined in the previous study was compared with the vaccination protocol used by the manufacturer of a commercial poultry vaccine against both pathogens, licensed for avian use and administered intramuscularly to mice in this study to allow direct comparison with the experimental vaccine under the same murine challenge model (Figure 1B). Mice were assigned to four experimental groups of 12 animals each: experimental vaccine (single 250 µL dose); commercial vaccine (two 250 µL doses with a 14-day interval); adjuvant (single 250 µL dose of the formulation without bacterial antigen); and negative control (250 µL of PBS). Twenty-one days after immunization, the animals were challenged intraperitoneally with 200 µL of a virulent bacterial suspension of *P. multocida* and *S.* Gallinarum. Following the challenge, the animals were monitored daily for seven days, with clinical signs and mortality recorded.

2.5. Cytokine Determination Assay

Cytokine analysis of animals was carried out and distributed into experimental groups, considering three animals per group: G1 and G2, composed of animals immunized, respectively, with a single dose of the experimental vaccine and two doses of the commercial vaccine, without subsequent challenge; G3 and G4, immunized with a single dose of the experimental vaccine and challenged with *P. multocida* and *S.* Gallinarum, respectively; and G5 and G6, immunized with two doses of the experimental vaccine and subsequently challenged with *P. multocida* and *S.* Gallinarum, respectively. We collected blood plasma samples from the unchallenged groups 21 days after the first immunization. In contrast, in the groups subjected to the experimental challenge, we collected samples three days

after pathogen inoculation, consistent with previously described time points for assessing the systemic cytokine response following *Salmonella* and *Pasteurella* challenge in murine models [20,21]. Plasma was obtained by centrifuging whole blood in a microcentrifuge (Microcentrifuge G-L, IKA® São Paulo/SP, Brazil) at 15,000 rpm for 30 min. After separation, the samples were stored at 2 °C until flow cytometry analysis. The levels of interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-17, tumor necrosis factor alpha (TNF-α), and interferon-gamma (IFN-γ) were determined using Cytometric Bead Array (CBA) technology. For this analysis, the BD™ CBA Mouse Th1/Th2/Th17 kit was used, following the manufacturer's instructions. Data were acquired on a flow cytometer (FACSCanto™ II, San Diego, CA, USA), and results were expressed as mean fluorescence intensity (MFI).

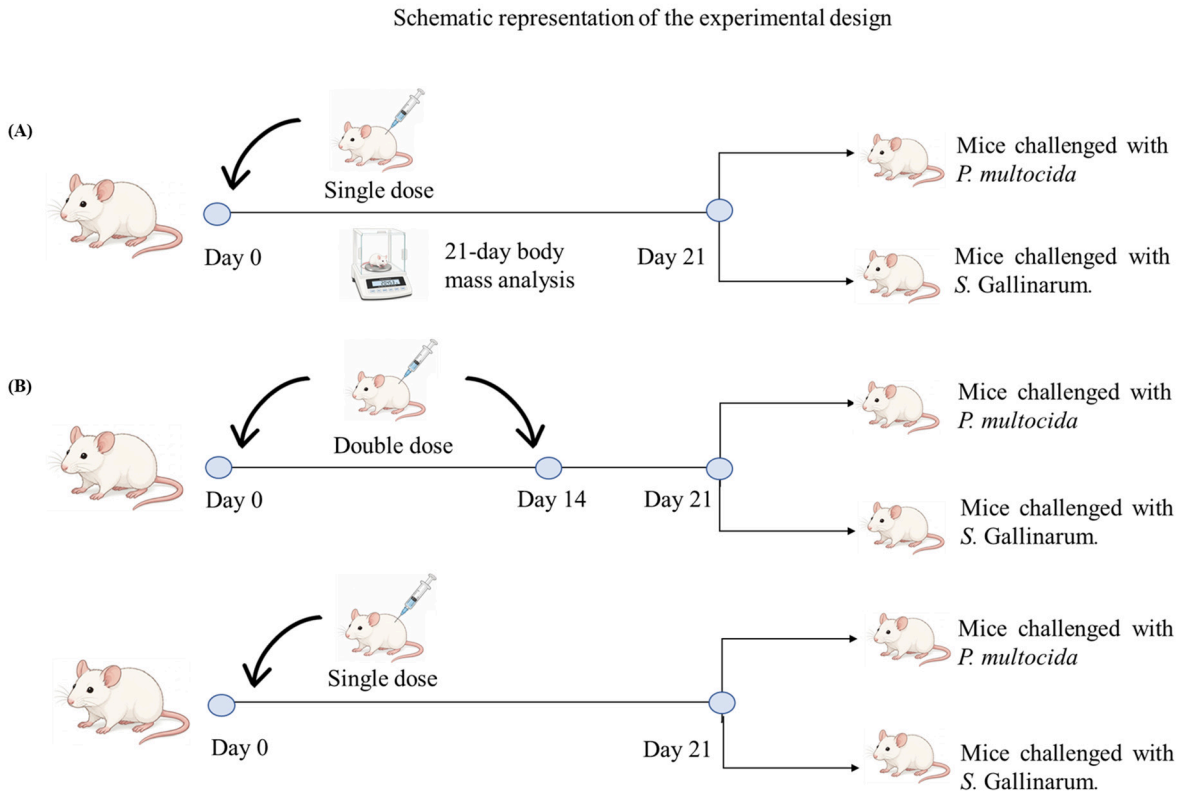


Figure 1. Schematic representation of the experimental design. (**A**) Protocol used to determine the optimal dose of the experimental vaccine, including single-dose immunization, monitoring of mice body weight over a 21-day period, and assessment of protective efficacy following experimental challenge with *P. multocida* or *S*. Gallinarum. (**B**) Comparison of the immunization protocols used for the experimental vaccine (single dose) and the commercial vaccine (two doses administered on days 0 and 14), followed by assessment of protective efficacy after experimental challenge with *P. multocida* or *S*. Gallinarum.

2.6. Statistical Analysis

Statistical tests were performed using GraphPad Prism 8.0 software. The log-rank test was used to compare two or more groups with censored data, defined as individual death, evaluated via Kaplan–Meier analysis. We assessed body weight and plasma cytokine levels using two-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Normality of the body weight and cytokine data was confirmed using the Shapiro–Wilk test prior to ANOVA. Results are presented as mean values ± SD. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Determination of the Lethal Dose of S. Gallinarum and P. multocida in Murine Models

The virulence of *P. multocida* strains in Swiss mice resulted in clinical signs consistent with avian cholera at concentrations ranging from 7.5×10^{13} to 2.17×10^{11}, leading to the most acute form of the disease and 100% mortality in the group (Figure 2A). Conversely, animals inoculated with concentrations equal to or lower than 1.16×10^{10} included survivors throughout the experimental period (Figure 2A), indicating a lethal infection dose corresponding to 95 LD_{50} (1×10^{10} CFU/mL; 2×10^{9} CFU/mice). Regarding the LD_{50} of *S.* Gallinarum, all animals inoculated with a concentration of 1.37×10^{15} died within 12 h of observation; lower doses showed progressively higher survival rates as the bacterial infection concentration was reduced (Figure 2B), indicating a lethal infection dose corresponding to 40 LD_{50} (5×10^{10} CFU/mL; 1×10^{10} CFU/mice).

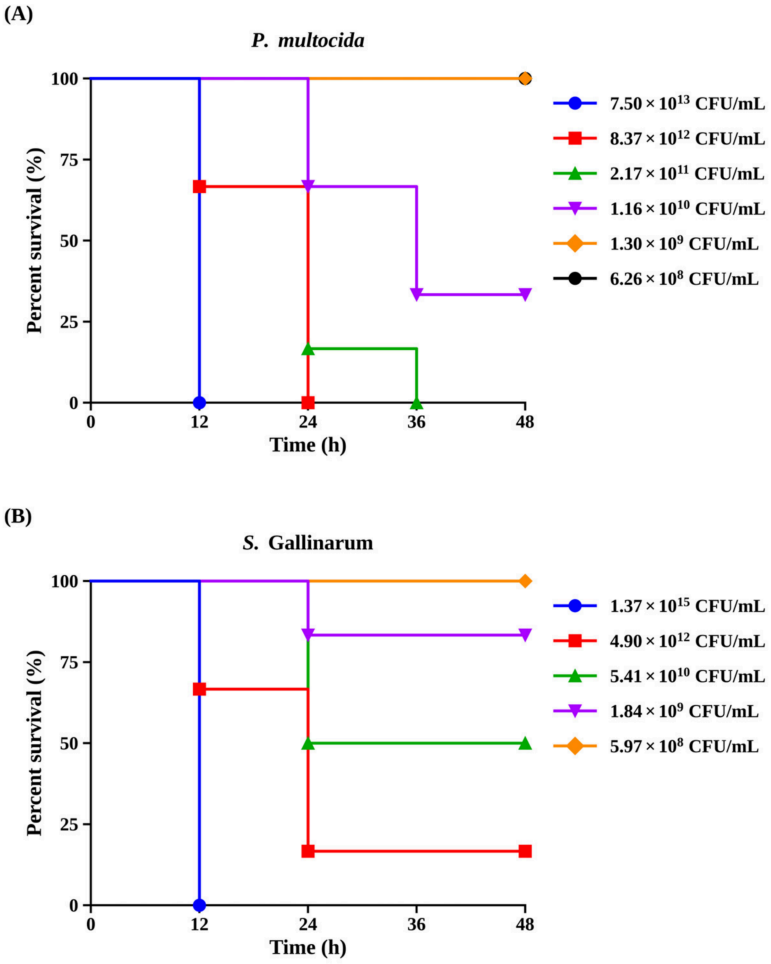


Figure 2. Standardization of the LD_{50} of *P. multocida* and *S.* Gallinarum in mice. Kaplan–Meier survival curves following intraperitoneal inoculation with increasing concentrations (10^{8} to 10^{13} CFU/mL) of (**A**) *P. multocida* and (10^{8} to 10^{15} CFU/mL) (**B**) *S.* Gallinarum. The x-axis represents time in hours post-inoculation (0–48 h). Mortality was monitored every 12 h for two days post-inoculation. LD_{50} values were calculated using the Reed–Muench method to determine the doses used in subsequent challenge assays.

3.2. Evaluation of Vaccine Dosage Using Murine Models

In the preliminary study of vaccine dosages GC1 (250 µL), GC2 (125 µL), and GC3 (50 µL), the evaluation of the experimental animals' mean body mass revealed no significant changes in weight gain throughout the 21-day post-immunization period. At the end of

this period, a statistically significant difference ($p < 0.05$) was observed only between the GC2 group and the control group. In contrast, the GC1 and GC3 groups showed no differences, thereby demonstrating the formulation's safety and the maintenance of the animals' zootechnical performance (Figure 3A). As this isolated difference was not dose-dependent, occurring only in the intermediate-dose group (GC2) and not in the higher-dose (GC1) or lower-dose (GC3) groups receiving the same formulation, it is attributed to normal biological variation among individual animals rather than to a formulation-related effect. For animals immunized and challenged with *P. multocida* LD_{50}, the GC1 group showed 100% survival, the highest observed rate. In contrast, animals in the GC2 and GC3 groups showed survival rates of 83% and 67%, respectively ($p < 0.05$) (Figure 3B). Meanwhile, the survival rate of animals immunized and challenged with *S*. Gallinarum showed a progressive decline over the monitoring period, varying according to the administered dose of the experimental vaccine; the GC1 group demonstrated the highest survival rate (83%), whereas the GC2 and GC3 groups showed a more marked reduction over time, resulting in 33% survival in both groups ($p < 0.05$) (Figure 3C).

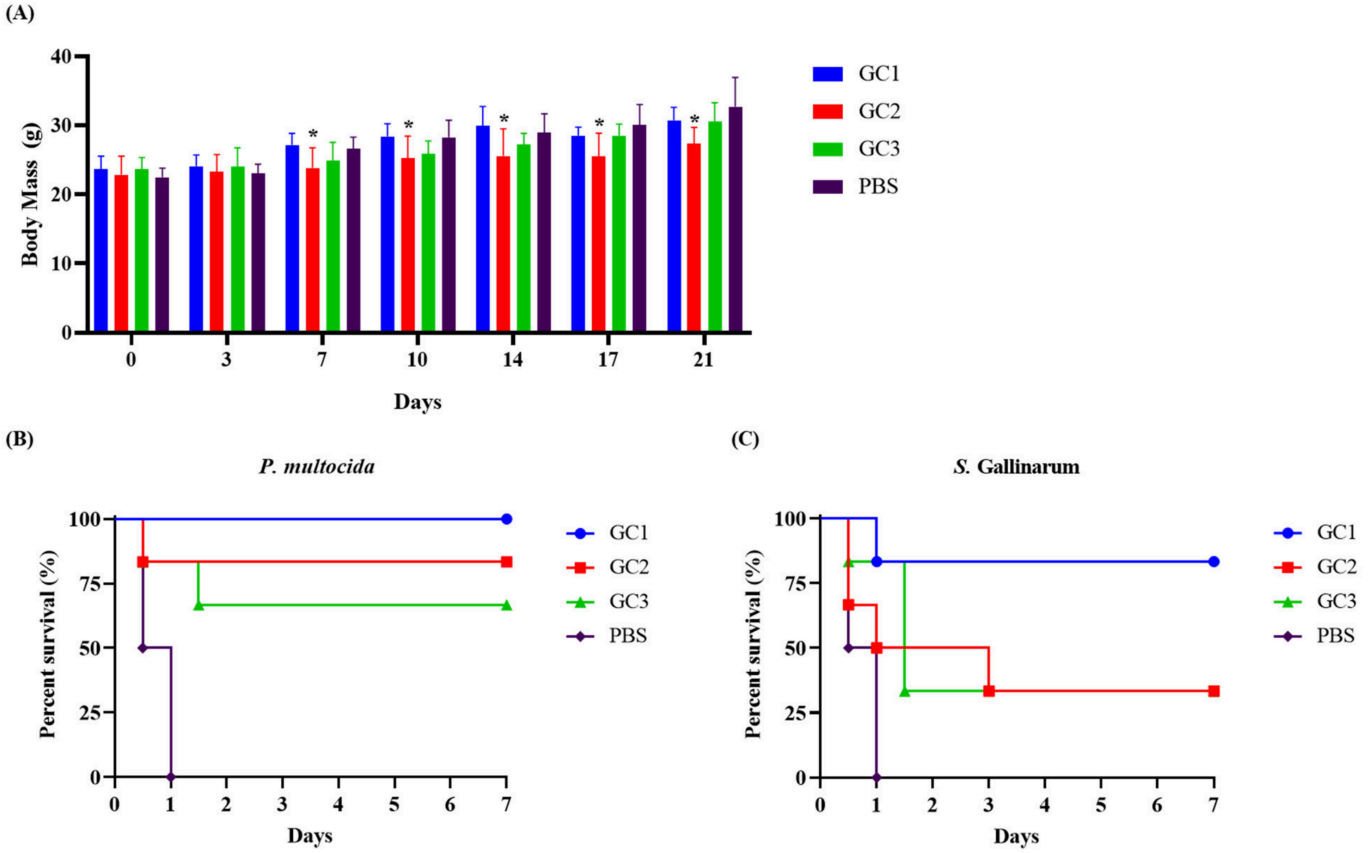


Figure 3. Groups GC1, GC2, GC3, and PBS were immunized and monitored for body weight over 21 days, then subdivided for bacterial challenges. (**A**) Variation in mouse body weight during the 21 days following immunization with the experimental vaccine. GC1: 250 µL; GC2: 125 µL; GC3: 50 µL; PBS: 250 µL of PBS. (**B**) Survival analysis of groups GC1, GC2, GC3, and PBS following challenge with *P. multocida*, and (**C**) survival analysis of groups GC1, GC2, GC3, and PBS following challenge with *S*. Gallinarum. Body weight data were analyzed by two-way ANOVA followed by Tukey's post hoc test; survival curves were compared using the log-rank test. * = Statistically significant difference ($p < 0.05$).

With the vaccine dosage established, the experimental vaccine provided 100% survival for animals challenged with the LD_{50} of *P. multocida* and *S*. Gallinarum during the seven-day post-challenge observation period. In contrast, animals immunized with the commercial

vaccine showed a survival rate of 67%, whereas those that received only the adjuvant formulation showed a survival rate of 17%. No surviving animals were observed in the control group inoculated with PBS ($p < 0.05$) (Figure 4A,B).

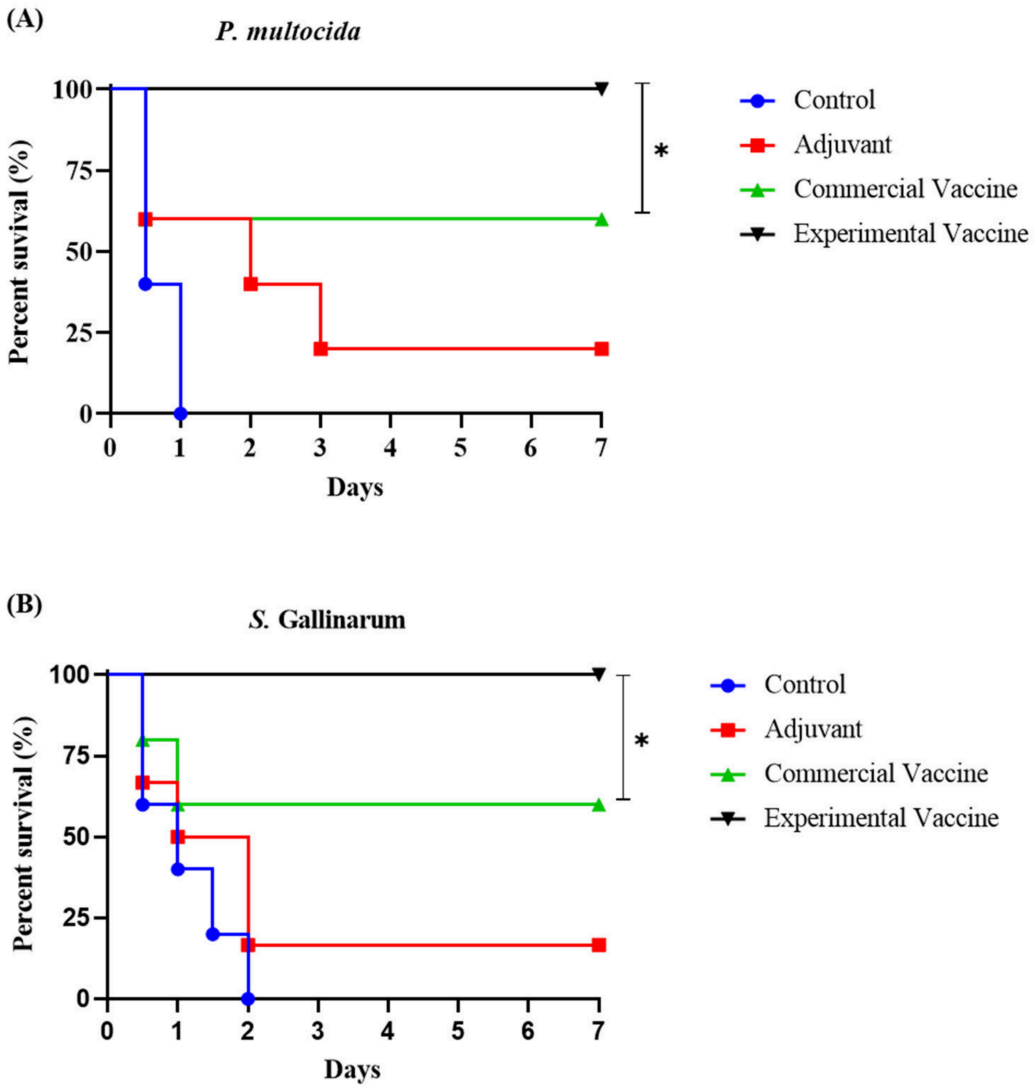


Figure 4. Protective efficacy of the experimental vaccine against bacterial challenges. Kaplan–Meier survival curves following challenge with the LD_{50} of (**A**) *P. multocida* and (**B**) *S.* Gallinarum. The experimental groups consisted of unvaccinated animals (control, blue line —●—), animals immunized only with the adjuvant solution (red line —■—), animals immunized with the commercial vaccine using a two-dose regimen (green line —▲—), and animals immunized with the experimental vaccine using a single-dose regimen (black line —▼—). The animals (n = 12/group initially; n = 6/group per pathogen) were challenged 21 days after immunization in the single-dose protocol or seven days after the second immunization in the two-dose protocol. Survival was monitored daily for seven days following the challenge. * = indicates a statistically significant difference by log-rank test ($p < 0.05$).

3.3. Determination of the Cytokine Profile of Animals Immunized with Experimental and Commercial Vaccines, With and Without Challenge, in Murine Models

Evaluation of the cytokine profile post-immunization and before experimental challenge (Figure 5A) revealed slight differences between the single-dose immunization group (G1) and the commercial double-dose vaccine group (G2) (Figure 5B); however, among the cytokines evaluated, IL-6 showed a significant difference ($p < 0.05$), with higher levels in the single-dose group (G1). We also investigated the effect of bacterial challenge with either

P. multocida (G3) or *S.* Gallinarum (G4) on the immune response induced by immunization. Data showed no significant differences for most of the cytokines evaluated (Figure 5C). However, it was again observed that IL-6 levels were significantly higher in immunized, unchallenged animals compared to the group immunized and subsequently challenged with *P. multocida* ($p < 0.05$), whereas *S.* Gallinarum (G4) did not interfere with cytokine stimulation before or after experimental challenge; this indicates that the primary stimulus was driven by immunization, without further stimulation interference following the experimental challenge (Figure 5C). We also conducted a comparative study of immunization protocols and cytokine stimulation data using single-dose (G3 and G4) and double-dose (G5 and G6) experimental vaccines, assessing the period post-immunization and following the respective bacterial challenges to contribute to the understanding of the protective immune response against both pathogens (Figure 5D). Here, we observed that animals immunized with either the single-dose or double-dose experimental vaccine protocols were protected following the challenge test, with 100% survival; this demonstrates that the stimulated cytokine levels conferred a protective response even when the animals were challenged with either pathogen. Following challenge, animals that received two vaccine doses exhibited enhanced cytokine stimulation. Of note were the groups challenged with *S.* Gallinarum, where the two-dose regimen resulted in significantly higher ($p < 0.05$) levels of IFN-γ, TNF-α, and IL-10 compared to the single-dose group; this indicates greater activation of cellular and regulatory immunological mechanisms associated with the response to intracellular pathogens. In contrast, no significant differences were observed between the evaluated vaccination protocols in the groups challenged with *P. multocida*.

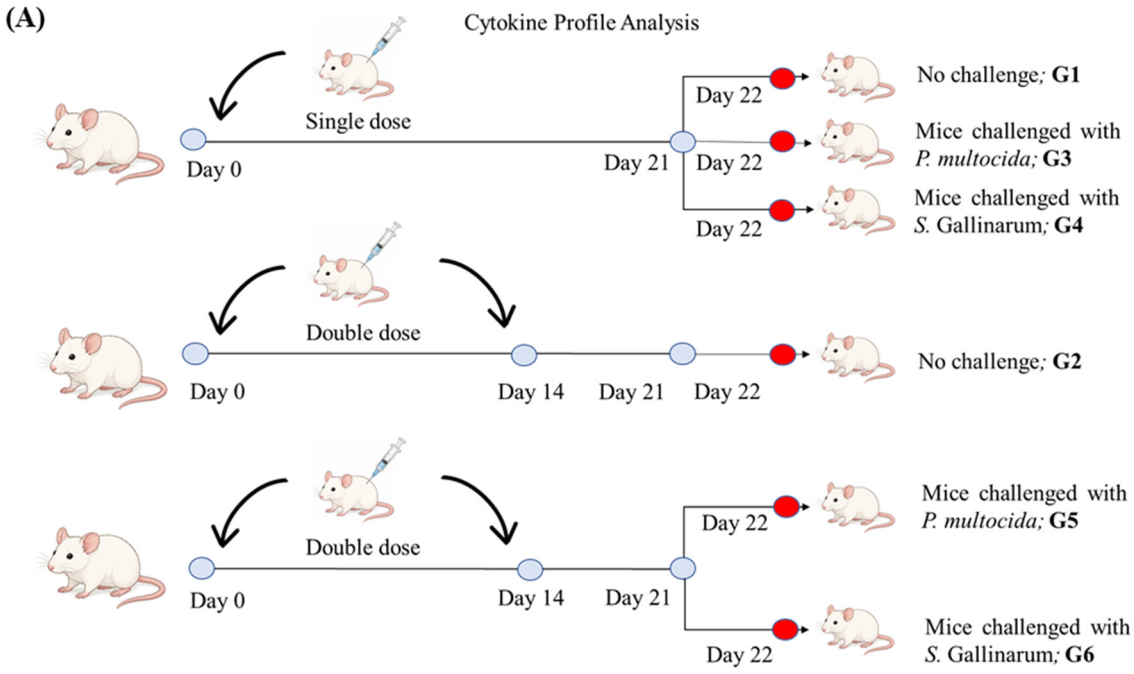


Figure 5. *Cont.*

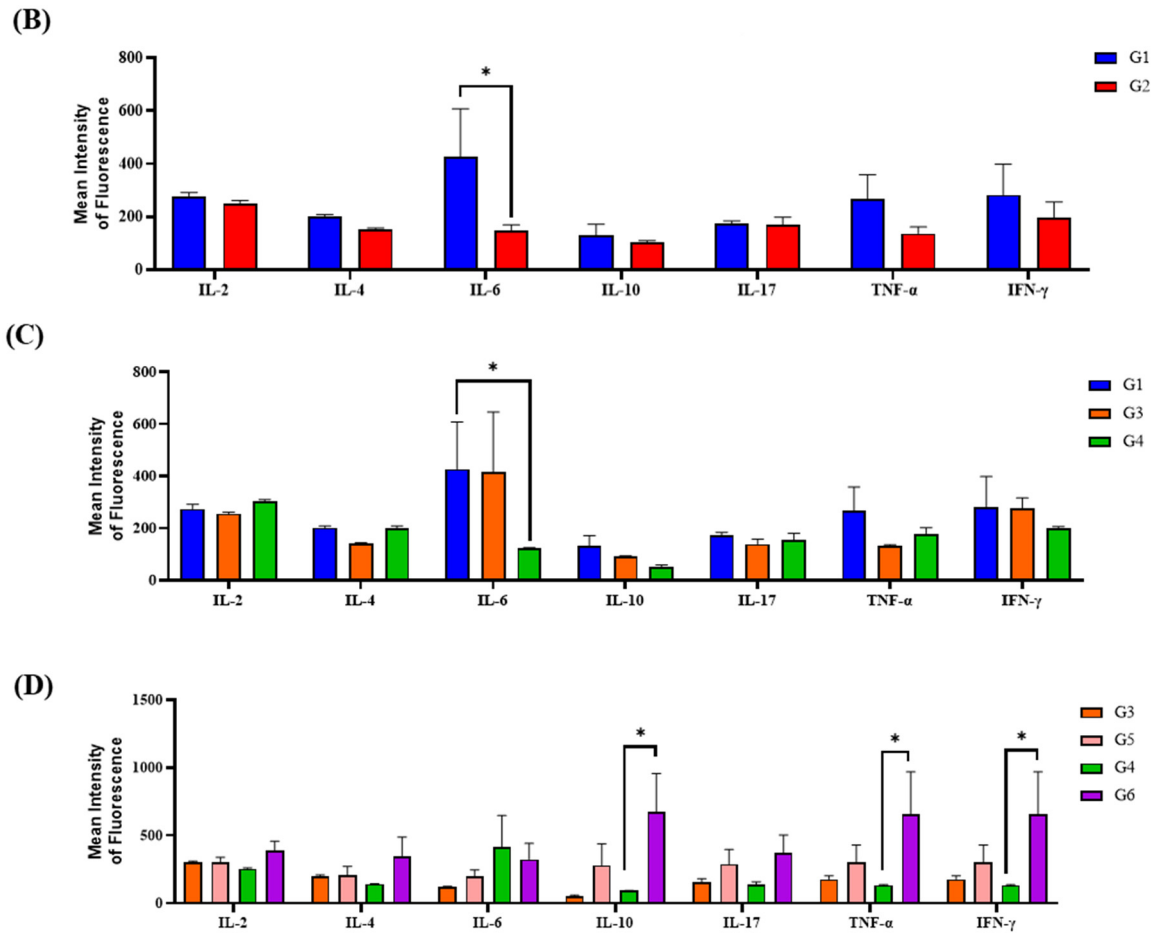


Figure 5. Plasma cytokine profile in mice under different vaccination regimens and bacterial challenges. (**A**) Experimental group scheme: G1, experimental vaccine (single-dose, no challenge); G2, commercial vaccine (double-dose, no challenge); G3 and G4, experimental vaccine (single-dose) challenged with *P. multocida* or *S*. Gallinarum, respectively; G5 and G6, experimental vaccine (double-dose) challenged with *P. multocida* or *S*. Gallinarum, respectively. (**B**) Plasma levels of IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ (G1 vs. G2), expressed as mean fluorescence intensity (MFI), during the pre-challenge period. (**C**) Effect of bacterial challenge on the immune response induced by the single dose of the experimental vaccine (G1 vs. G3 and G4). (**D**) Comparison of the post-challenge cytokine profile between single-dose (G3, G4) and double-dose (G5, G6) regimens. Data presented as mean ± SD. * $p < 0.05$ indicates a statistically significant difference (two-way ANOVA followed by Tukey's test).

4. Discussion

Inactivated vaccines serve as an alternative that enables broader coverage against infectious agents through simultaneous antigen exposure. They stimulate the herd's immune system, offer logistical efficiency and reduced transport costs, and improve animal management. However, in some cases, they may induce weaker immune responses; therefore, it is important to devise strategies, such as using adjuvants to enhance immunogenicity [22]. Commonly used adjuvants include saponin [23], chitosan [24], and aluminum hydroxide [25]. In this study, we developed a single-dose bivalent inactivated vaccine targeting *S*. Gallinarum and *P. multocida*, pathogens that cause significant economic losses in the global poultry industry. Data from a murine model demonstrated the high efficacy of the experimental vaccine, ensuring complete protection following challenge with virulent strains of *P. multocida* (Figure 4A) and *S*. Gallinarum (Figure 4B), and showing superior performance compared to the reference commercial vaccine, which requires a two-dose regimen.

Given the sanitary and economic significance of these diseases, developing effective vaccination strategies is crucial for controlling fowl typhoid and fowl cholera in poultry production systems. Various approaches, such as vaccines using *S.* Gallinarum strains attenuated via *SptP* gene deletion, radiation-inactivated vaccines, inactivated vaccines combined with L-proline supplementation, and the use of adjuvants derived from bacterial DNA have been reported to enhance humoral and cellular immune responses in murine models [26–29]. Consequently, data from numerous studies reinforce the potential of inactivated vaccines as a cost-effective disease control strategy, corroborating the findings of the present study.

In this context, the route of administration can also inform the immune response. The intramuscular immunization route used in this study may facilitate local antigen retention at the injection site, promoting recruitment of antigen-presenting cells and activation of the systemic immune response in regional lymph nodes [30]. Conversely, the intraperitoneal challenge widely used in experimental models of bacterial infection mimics systemic pathogen dissemination, making the systemic immunity induced by intramuscular vaccination biologically relevant and capable of conferring protection in this experimental model [31]. Regarding the cytokine profile, IFN-γ levels increased significantly in the group immunized with a double dose of the experimental vaccine and subsequently challenged with *S.* Gallinarum (G6) (Figure 5D); this pattern was also observed in the TNF-α levels of the immunized animals in group G6 and may be compatible with the involvement of Th1-associated immune mechanisms. However, this interpretation should be made with caution, as antigen-specific antibody titers and cellular immune phenotyping were not assessed in this study. Such mechanisms are crucial for containing intracellular pathogens, as they promote macrophage activation and enhance cell-mediated immunity [32]. Activation of this immunological pathway is a key mechanism in eliminating intracellular pathogens, contributing to control of bacterial replication and the establishment of a more efficient protective response against *S.* Gallinarum infection [33].

IL-17 levels in group G6 do not allow us to confirm Th17 involvement under the conditions evaluated ($p > 0.05$). Given the limited sample size (n = 3 per group), this result should be interpreted with caution and does not rule out a possible role for this immunological mechanism, which should be reevaluated in future studies with larger cohorts. This response has been linked to the recruitment of inflammatory cells to sites of infection and the amplification of immunity against invasive bacteria [30]. The higher IL-10 observed in group G6 than in G4 suggests that administering double doses of the experimental vaccine promoted a more pronounced modulation of the immune response following the *S.* Gallinarum challenge. IL-10 plays a key regulatory role, helping control inflammation and limit tissue damage from the immune response [34].

Although this profile indicates active engagement of the adaptive immune response, it is worth noting that the group immunized with a single dose (G4), despite showing no statistically significant changes in cytokine levels after the challenge ($p > 0.05$) (Figure 5C), achieved a survival rate equivalent to that of the double-dose group, with both groups showing 100% protection. This finding suggests that the single dose was capable of establishing sufficient protective immunity to control the *S.* Gallinarum infection without requiring intense systemic inflammatory activation; this pattern may be compatible with a more efficiently modulated immune response, in which the immunity established by vaccination could have obviated the need for extensive recruitment of pro-inflammatory mediators following the challenge, as previously reported by Vinod et al. (2017) [35]. From this perspective, cytokine stability in the single-dose group does not indicate an absence of immune response but rather evidence of effective infection control with a lower inflammatory cost—a desirable characteristic for vaccine formulations, especially considering

that single-dose regimens reduce animal stress, operational costs, and vaccination coverage failures associated with multidose schedules in the field [28]. In the group challenged with *S*. Gallinarum, levels of IL-6 and the other cytokines evaluated (IL-2, IL-4, IL-10, IL-17, TNF-α, and IFN-γ) remained stable relative to pre-challenge levels ($p > 0.05$); this is biologically consistent with an immune response efficiently modulated by vaccination, wherein previously established immunological memory obviated the need for secondary systemic inflammatory activation, as described in murine models of vaccine-mediated protection against *Salmonella* [35,36]. The stability of plasma cytokines post-challenge observed in vaccinated animals aligns with studies demonstrating distinct immunological profiles between immunized and non-immunized animals following an LD_{50} challenge, often characterized by the absence of exacerbated systemic inflammatory amplification in protected groups [37].

Immunization against *P. multocida* and the resulting cytokine profile are consistent with a response to a predominantly extracellular pathogen, which is typically associated with humoral immunity, as previously reported by Homayoon et al. (2020) [38]. Further assessment of this interpretation would benefit from measuring antigen-specific antibody titers, which were not evaluated in the present study. In non-challenged animals, the group receiving the experimental vaccine (G1) exhibited basal plasma IL-6 levels significantly higher than those of the group receiving the commercial vaccine (G2) ($p < 0.05$) (Figure 5B). This effect is attributed to the immunostimulatory potential of the adjuvant—composed of chitosan, aluminum hydroxide, and saponin—corroborating findings by Zhao et al. (2009) [39], who observed elevated IL-6 levels in mice immunized with a bivalent pasteurellosis vaccine delivered via chitosan nanoparticles.

The data show that the evaluated bivalent vaccine induced an immune response and provided effective protection against *S*. Gallinarum and *P. multocida* in the study model; this protection is associated with modulation of immunological mechanisms linked to cellular and regulatory responses. However, the cytokine profile alone does not allow direct confirmation of the proposed protective mechanisms, including a potential Th1-associated response or effective modulation of the immune response, as antigen-specific antibody titers and cellular immune phenotyping were not assessed in this study. Such findings reinforce the potential of this experimental vaccine as a promising strategy for developing bivalent vaccines aimed at minimizing the impact of poultry diseases. Future studies in the target species are needed to expand knowledge of the efficacy and safety of the vaccine developed in this pilot study. This is particularly relevant because avian cytokine biology differs from that of mammals, including distinct gene organization and receptor systems, which may limit the direct translation of the cytokine-based mechanisms observed in this murine model to chickens. Additionally, further characterization of the vaccine-induced immune response, potentially including comparative studies of specific antibodies in murine and avian models, is warranted to assess its direct application for disease control in the poultry industry.

5. Conclusions

The results indicate that the single-dose bivalent inactivated vaccine developed in this study conferred high protective efficacy against *S*. Gallinarum and *P. multocida* challenges in a murine model, conferring 100% survival to immunized animals. Although the two-dose regimen increased cellular and regulatory cytokines after *S*. Gallinarum challenge, the single-dose immunization was sufficient to activate the immunological mechanisms required for complete protection. Among the formulations evaluated, the 250 µL dose exhibited the best protective performance and an excellent safety profile, without affecting animal weight gain. Taken together, the data highlight the potential of combining chitosan, aluminum hydroxide, and saponin as a promising adjuvant platform, providing a basis

for future studies in the target avian species, an essential step to bridge the immunological differences between the models.

Author Contributions: H.S.B.: Writing—original draft, data curation, investigation, formal analysis, validation, methodology, and conceptualization; M.O.F.: Writing—review and editing, investigation, and methodology; G.M.M.: Writing—review and editing, investigation, and methodology; T.T.D.: Writing—review and editing and methodology; W.S.M.: Writing—review and editing and methodology; F.E.: Writing—review and editing and methodology; E.W.S.C.: Writing—review and editing and data curation; B.A.: Methodology and validation; G.R.d.S.: Writing—review and editing and methodology; M.G.S.: Methodology, validation, and writing—review and editing; A.A.S.B.: Resources and methodology; V.P.d.N.: Resources and methodology; I.V.B.: Writing—review and editing, formal analysis, and validation; L.A.M.M.: Writing—review and editing, formal analysis, and validation; K.F.V.: Writing—review and editing, formal analysis, and validation; L.S.S.d.S.: Writing—review and editing, and methodology; V.M.C.: Writing—review and editing and methodology; R.O.A.: Writing—review and editing, investigation, and methodology; P.A.N.: Conceptualization and writing—review and editing; K.D.P.C.: Resources and writing—review and editing; Y.O.C.: Methodology, validation, and resources; A.S.R.C.: Writing—review and editing, project administration, methodology, resources, supervision, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Council of Scientific and Technological Development (CNPq, Brazil), Funding Authority for Studies and Projects (FINEP, Brazil) and ANTIGEN LTDA, projects: CNPq RHAE 424435/2021 and CNPq RHAE 441791/2024; FINEP 03.22.0448.00/2025. ASRC (CNPq 427304/2018-0, CNPq 305589/2021-0, CNPq 309054/2025-7), VPN (CNPq 318754/2025-8). The Graduate Program for Biotechnology and the Graduate Program for Biodiversity and Biotechnology of the Legal Amazon from the Federal University of Tocantins.

Institutional Review Board Statement: In this study, all procedures involving animals were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Tocantins, Brazil (Approval Date: 10 September 2022, No. 23.101.001.307/22-31). All experiments were conducted in strict accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA), and all animal care and experimental protocols strictly adhered to relevant national and institutional standards regarding the use of laboratory animals.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: Authors A.S.R.C. and K.D.P.C. are shareholders of Antigen Ltda., a company dedicated to the research, development, and innovation of veterinary products. The authors declare that the research was conducted independently and that this relationship did not influence the study design, data collection, analysis, interpretation, or the conclusions presented in this manuscript. All other authors declare no conflicts of interest.

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