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Research Paper

Investigating Immunogenicity and Protectivity of Subcutaneous Administration of *Salmonella* Dublin Bacterin in Mice

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ABSTRACT

Background: Given the significant zoonotic threat posed by *Salmonella enterica* serovar Dublin (*S.* Dublin) and its substantial impact on animal populations and public health, the objective of the present study was to assess the immunogenicity and protectivity of subcutaneous administration of *Salmonella* Dublin bacterin in a murine model.

Materials and Methods: Specific pathogen-free female BALB/c mice were tested for *Salmonella*-free status, and housed in controlled conditions. A formalin-killed bacterin was prepared from a local isolate of *S.* Dublin using a well-established protocol, ensuring bacterial inactivation and safety. Groups 1 through 3 of mice were received, respectively, either phosphate buffered saline plus alum or a single dose of inactivated bacterins with and without alum adjuvant via subcutaneous route. Immune responses were evaluated through microagglutination, enzyme-linked immunosorbent assay, delayed-type hypersensitivity, interferon-gamma assays, and challenge with viable *S.* Dublin.

Results: Microagglutination and enzyme-linked immunosorbent assay tests revealed alum-adjuvanted injection as the best method for stimulation of anti-*S.* Dublin antibodies production. The gamma interferon production and delayed hypersensitivity tests, crucial for cellular immunity, were also most elevated in mice injected with alum-adjuvanted *S. Dublin* bacterin. After the challenge with the live bacteria, the isolation rate of *S.* Dublin was significantly different (P=0.03) among the different groups but only mice injected with alum-adjuvanted showed a significant difference (P≤0.05) compared to the control group.

Conclusion: This study emphasizes the efficacy of alum as an adjuvant in inactivated *S.* Dublin vaccines. Insights gained from both humoral and cellular immune responses, provide valuable knowledge for the development of *S.* Dublin vaccination strategies.

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Introduction

Salmonella enterica serovar Dublin (*S.* Dublin) poses a significant threat as an animal pathogen, primarily affecting cattle and presenting risks of transmission to other species, including humans [1]. Renowned for its high invasiveness and severe clinical manifestations, *S.* Dublin can lead to substantial mortality rates in infected animals [2]. Given the potential for the economic impact on the livestock industry and zoonotic transmission, there is considerable interest in veterinary medicine and public health in controlling and preventing *S.* Dublin infections [2, 3]. Immunization strategies against *S.* Dublin encompass both live attenuated and inactivated vaccines, with mice serving as a reliable model organism for assessing vaccine efficacy [4, 5]. A comprehensive investigation into this matter, utilizing a murine model infected with *Salmonella Typhimurium*, was conducted by Cameron and Fuls in 1974, which their study revealed that a highly effective immune response could be elicited through a formalin-inactivated alum-precipitated vaccine [6]. Live vaccines, including those derived from avirulent rough mutants of *S.* Dublin, exhibit variable degrees of protection in mice and calves [7]. The live vaccine strain HB 1/17 has demonstrated substantial protection in mice against *S.* Dublin and even displayed cross-protection against *S. Typhimurium* [8]. Inactivated vaccines, confer lasting immunity, with durations extending up to at least 12 weeks post-immunization; while the immunogenicity of these vaccines can decline over time, administering multiple injections and using adjuvants has been shown to ease this effect [9, 10]. The development of effective vaccines against *S.* Dublin is crucial for reducing the pathogen's impact on animal populations and minimizing the risk of zoonotic transmission. In this context, the ongoing debate surrounding the comparative efficacy of live versus inactivated vaccines has prompted focused research efforts [5, 11]. Accordingly, this study compares the immunogenicity and protectivity of subcutaneous administration of locally prepared *S.* Dublin bacterin in a murine model.

Materials and Methods

Bacterial strains and growth conditions

Three clinical samples (liver, spleen, and lung) from 4 cases of cows with signs of septicemia and sudden death were subjected in September 2022 to the veterinary bacteriology laboratory of Shahrekord University, Shahrekord Province, Iran. In the laboratory, all these samples underwent cultivation on various culture media, including blood agar medium, xylose lysine deoxycholate agar, and, MacConkey agar. The incubation temperature for the cultivation process was maintained at 37 ˚C. Differential diagnosis of isolated bacteria revealed the same gram-negative *Salmonella* spp. from all specimens of all 4 cases [12]. Polymerase chain reaction and serotyping of the isolate revealed it as *S.* Dublin [13].

Study animals

Male BALB/c mice, aged 6 weeks and certified as specific pathogen-free (SPF), were prepared. A serology (microagglutination) test was conducted to confirm the *Salmonella*-free status of purchased mice. These mice were individually accommodated in rearing isolators and provided with *Salmonella*-negative commercial feed and drinking water. The research activities accurately adhered to the guidelines established by both the institutional Administrative Committee and the Ethics Committee for laboratory animals.

Preparation of experimental inactivated bacterin

The inactivation of *S.* Dublin was according to the method of Hashizume-Takizawa and Germanier, with some modifications [14, 15]. In a brief 200 mL sterile nutrient broth was prepared and inoculated with above mentioned *S.* Dublin local isolate, followed by incubation at 37 ˚C for 48 h. The bacterial population in the suspension was harvested by centrifugation at 4000 g for 5 min and the supernatant was carefully discarded. The bacterial pellet was suspended in phosphate-buffered saline (PBS) in a falcon tube and treated with 0.5% formaldehyde, followed by incubation at room temperature for 48 h. After formaldehyde treatment, centrifugation was repeated, and all residual formaldehyde was removed by triple washing with PBS. To confirm bacterial inactivation, 10 µL of *S.* Dublin bacterin was cultured on nutrient agar medium in triplicate and incubated at 37 ˚C for 48 h. All experimental steps, including the inoculation, incubation, and confirmation of bacterial inactivation, were conducted under sterile conditions. McFarland turbidity standards were used to standardize the approximate number of *S.* Dublin bacterin in resulting suspensions and stored at 4 ˚C for further use.

Determination of infectious dose of 50% (ID_{50}) of *S.* Dublin for mice

To assess the ID_{50} of *S.* Dublin local isolates, 40 mice were randomly divided into 8 equal groups and subjected to various doses of the bacterium. Each mouse of group 1 through group 7 received a subcutaneous injec-

tion of 100 µL of various serial 10-fold dilutions (10^8, 10^7, …. and 10^2 CFU/mL) of *S.* Dublin fresh culture, and the controls (group 8) received PBS via the subcutaneous route. All subcutaneous injections were performed on loose skin on the neck. Two days after the disease induction, the mice were euthanized and their spleens were streaked on XLD and blood agar media for isolation of *S.* Dublin by quadrant technique. The ID_{50} was calculated using the method of Reed and Muench [16].

Immunization of mice

A total of 45 mice were randomly classified into 3 equal groups. The experimental groups were as follows:

Group 1 (control group): Group 1, consisting of mice received 0.5 mL of PBS containing 10% alum via a subcutaneous route. Its purpose was to provide a reference point for evaluating the effects of interventions in the other groups.

Group 2 (formalin-inactivated bacterin group): Mice in group 2 were immunized with 0.6×10^8 bacterial cells of formalin-inactivated *S.* Dublin in 0.5 mL of PBS via a subcutaneous injection. The aim was to assess the efficacy of this inactivated bacterin in stimulating an immune response against *S.* Dublin and to observe its impact on virulence.

Group 3 (alum-adjuvanted formalin killed bacterin group): Group 4 received subcutaneous immunization with 0.6×10^8 bacterial cells of formalin-inactivated *S.* Dublin in 0.5 mL of PBS containing 10% alum as an adjuvant.

Evaluation of immune response: Microagglutination test (MAT)

The MAT was carried out as described elsewhere [17]. Briefly, the serum samples were meticulously collected from 5 immunized mice of each group at day 21 post-immunization through cardiac puncture under anesthesia. Following blood clotting at room temperature and subsequent centrifugation, the serum was carefully separated and stored at -20 ˚C. Tenfold dilutions of the collected serum were prepared using sterile PBS. The bacterial suspension of inactivated *S.* Dublin was adjusted to a 0.5 McFarland standard and an equal volume of it was added to duplicate serially diluted serum specimens in 96-well u-bottom microtiter plates. The plates were then incubated at 37 ˚C for 24 h. Following incubation, agglutination patterns were scrutinized by the naked eye, and the highest dilution at which agglutination occurred was meticulously recorded for each serum sample. Titers were determined according to standard criteria as the highest serum dilutions that agglutinated at least 50% of the cells for each group used.

Enzyme-linked immunosorbent assay test

On the above-mentioned days post-immunization, a critical assessment of the humoral immune response was conducted through the quantification of specific immunoglobulin G titers. This analysis was performed utilizing an in-house indirect enzyme-linked immunosorbent assay (ELISA) [17]. In summary, the optimum concentration of sonicated *S.* Dublin antigen (0.5 µg/mL) in carbonate-bicarbonate buffer was coated in an ELISA plate (Nunc, Denmark) and the plate was incubated at 4 ˚C overnight and after 3 washes with PBS, blocking was done with 5% skimmed milk (HiMedia, India) for 2 h at room temperature. Optimum dilution of serum samples (1:200) was added to duplicate wells after washing and the plate was incubated at room temperature for 1 h. After 3 washes as above, the secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (Sigma-Aldrich, USA) was used at a dilution of 1:10,000. Finally, the plate was washed again and TMB/H_2O_2 chromogen/substrate (Rahazistpadtan, Iran) was added to each well. After 15 min the reaction was stopped by the addition of H_2S0_4 one molar and the optical density at 450 nm (OD450) was measured using an ELISA reader (Bio-Rad 680, USA).

Assessment of delayed-type hypersensitivity

On day 28 after the immunization, 1×10^6 killed *S.* Dublin in a volume of 0.1 mL were injected subcutaneously into the left foot pad of 3 mice from each group. The same volume of PBS was injected into the sole of each mouse's right foot as a negative control. After 48 h, the thickness of the foot pad was checked by a digital caliper (Mitutoyo, Japan) and these mice were excluded from the experiment. The difference between left and right foot pad thickness was considered as the delayed-type hypersensitivity (DTH) response to injected bacterin [18].

Study challenges

Four of the mice of each group were challenged with 1.5×10^7 CFU (3 ID_{50}) of *S.* Dublin, 8 weeks after immunization. Two days after the challenge, the mice were euthanized and their spleens were cultured for isolation of *S.* Dublin.

Gamma interferon test

On day 28 post-immunization, blood was collected from 3 mice of each group for the performance of the gamma interferon test. Peripheral blood cells were collected from anesthetized mice via cardiac puncture into EDTA-coated tubes. Following the isolation of peripheral blood mononuclear cells through density gradient centrifugation, these cells were plated at 2×10^5 cells/well in RPMI-1640 medium (BioIdea, Iran), which consists of 10% FBS (BioIdea, Iran). The cells were subjected to heat-inactivated *S.* Dublin (10^8 CFU/0.1 mL per well) and incubated at 37 ˚C in 5% CO_2 for 48 h. After incubation, supernatant from cell cultures was collected, and IFN-γ concentrations were measured using the Mouse IFN-γ ELISA kit (Becton Dickinson, USA). Duplicate measurements were performed for more accuracy.

Statistical analysis

The data were presented as Mean±SEM. Statistical analysis was conducted using GraphPad Prism software, version 5 (GraphPad Software Inc., CA, USA), using a one-way analysis of variance with Tukey's multiple comparison test. Meanwhile, P≤0.05 was considered statistically significant.

Results

Inefctious dose 50% of *S.* Dublin for mice

None of the mice in the control group or in groups 1 to 5, which received 10^1 to 10^5 CFU of *S.* Dublin, were infected with the bacterium. In contrast, in groups 6 to 8, which received 10^6 to 10^8 CFU, *S.* Dublin was isolated from the spleens of 1, 4, and 5 mice, respectively. Using the method of Reed and Muench, ID_{50} of *S.* Dublin for mice was determined to be 0.5×10^7 CFU.

Humoral immune response to *S.* Dublin

The antibody responses to *S.* Dublin bacterin in the sera of immunized mice are presented in Figure 1. According to the MAT, at day 21 post-immunization, the Mean±SEM of serum anti-*S.* Dublin antibody titers of groups group 1 through group 3 were 16±4, 188±8, and 604±32. Based on the ELISA results (OD), at day 21 post-immunization, the Mean±SEM of serum anti-*S.* Dublin immunoglobulin G of groups group 1 through group 3 were 0.115±0.014, 0.451±0.024 and 1.429±0.048. In both tests serum anti-*S.* Dublin antibody titers were significantly (P≤0.05) increased in both groups receiving antigen, with the group receiving alum-adjuvanted antigen through injection showing the highest increase. Statistical analysis by one-way analysis of variance revealed a very significant difference (P=0.0001) among different groups in terms of serum anti-*S.* Dublin antibody titers.

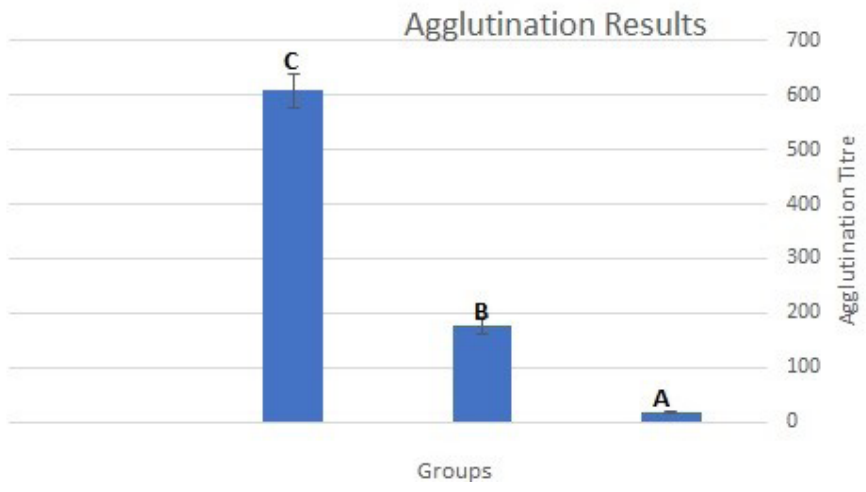

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Figure 1. Serum anti-*Salmonella* Dublin immunoglobulin G (Mean±SE) of mice received either phosphate buffered saline plus alum (group 1), *S.* Dublin bacterin (group 2), or *S.* Dublin bacterin plus alum (group 3) via subcutaneous route

Notes: Different letters on bars indicate significant differences (P≤0.05) between groups.

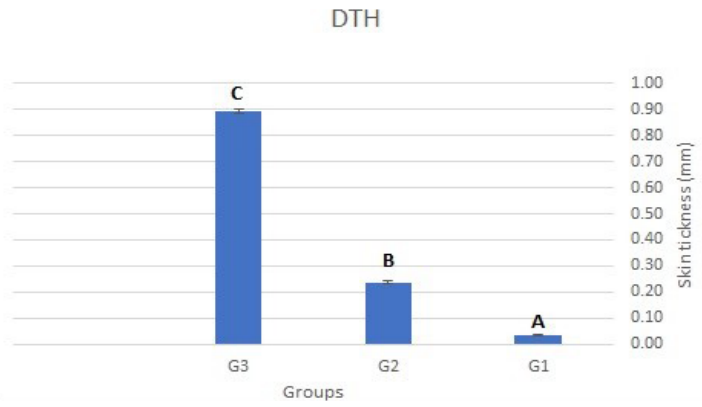

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Figure 2. The DTH response (thickness of food pad skin after intradermal injection of *S.* Dublin bacterin) of mice groups group 1 through group 3 which primed either phosphate buffered saline plus alum (group 1), *S.* Dublin bacterin (group 2), or *S.* Dublin bacterin plus alum (group 3) via subcutaneous route

Notes: Different letters on bars indicate significant differences (P≤0.05) between groups.

DTH result

According to the DTH test, at day 28 post-immunization, the Mean±SEM of the thickness of the foot pad of groups group 1 through group 4 of mice were 0.03±0.004 mm, 0.24±0.007 mm, and 0.89±0.007 mm. Figure 2 illustrates that groups receiving the antigen with or without adjuvants exhibit an augmented foot sole thickness (P≤0.05) compared to the control group when exposed to *S.* Dublin bacterin. The group group 3 which received bacterin with alum adjuvant compared to the other groups displays the highest increase (P=0.0001) in sole thickness.

Gamma interferon test result

The Mean±SEM of IFN-γ production after stimulation of isolated peripheral blood cells with *S.* Dublin bacterin at day 28 post-immunization in groups group 1 through group 3 of mice were 0.358±0.011, 0.673±0.014 and 0.728±0.021 pg/mL (Figure 3). Statistical analysis by one-way analysis of variance showed a very significant difference (P=0.0001) among different groups in terms of IFN-γ production after stimulation by *S.* Dublin bacterin. As depicted in Figure 4, the highest IFN-γ production (P=0.0001) compared to the control group was evident in the group group 3 which received bacterin plus alum adjuvant.

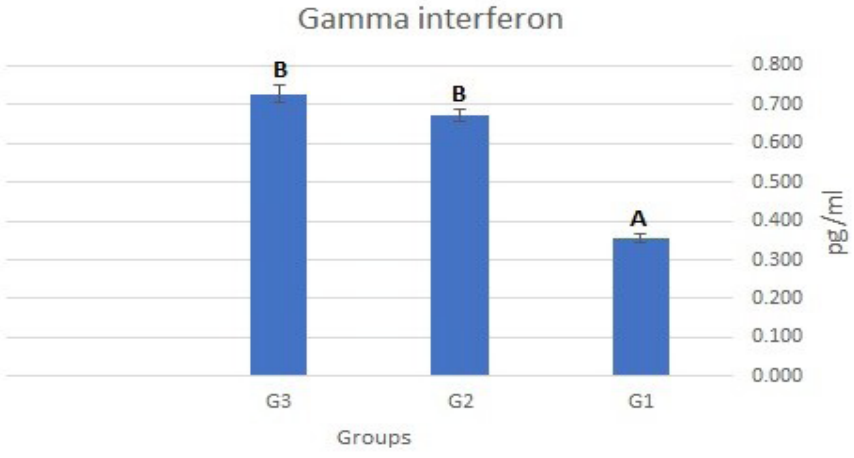

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Figure 3. The cell-mediated immune response (production of gamma interferon after stimulation with *S.* Dublin bacterin) of mice group 1 through group 3 which primed either phosphate buffered saline plus alum (group 1), *S.* Dublin bacterin (group 2), or *S.* Dublin bacterin plus alum (group 3) via subcutaneous route

Notes: Different letters on bars indicate significant differences (P≤0.05) between groups.

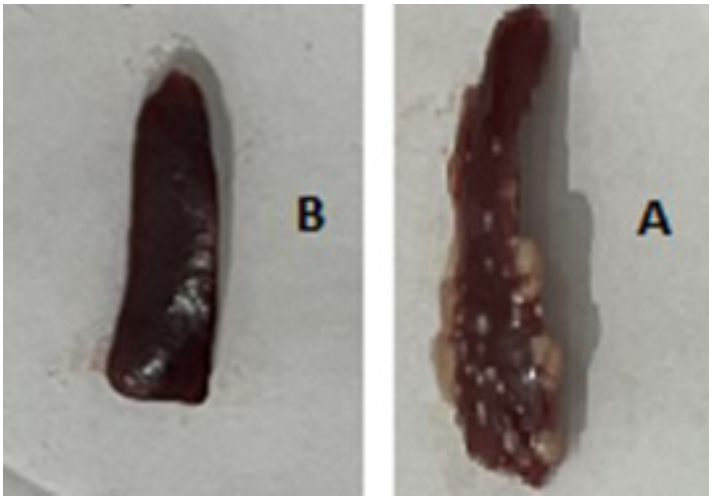

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Figure 4. The spleens of two mice from the control group (A) and *Salmonella* Dublin bacterin plus alum injected group (B) 2 days post-challenge

The challenge with live *S.* Dublin

S. Dublin was isolated from the spleen of all 4 mice of the control group (Figure 4). It isolated from 2 and none of the spleens of group 2 through group 3 of test groups, respectively. The *S.* Dublin isolation rates were significantly different (P=0.03) among different groups but only group 3 showed a significant difference (P≤0.05) compared to the control group (group 1).

Discussion

The present study investigated the immunogenicity and protectivity of different *S.* Dublin bacterin in mice, focusing on antibody titers, delayed-type hypersensitivity, gamma interferon production, and the challenge with live *S.* Dublin. The findings offer valuable insights into the effectiveness of different vaccination approaches and provide a basis for comparison with other studies in the field.

The ELISA and microagglutination results revealed distinct patterns in antibody responses among the experimental groups. Mice receiving *S.* Dublin bacterin through injection with alum adjuvant exhibited significantly higher anti-*S.* Dublin antibody levels compared to those receiving bacterin alone. This suggests that alum adjuvant, particularly when administered through injection, enhances the humoral immune response, resulting in elevated antibody production. The enhanced antibody production observed in the alum-adjuvanted groups is consistent with the known immunostimulatory effects of alum, which is a widely used adjuvant in vaccine formulations [9, 19]. Alum has been shown to promote the activation of antigen-presenting cells, leading to an increased immune response [19, 20]. The results indicate that the combination of *Salmonella* and alum adjuvant, particularly through injection, synergistically amplifies the production of *S.* Dublin specific antibodies. Consistent with our results, several studies have reported alum's potent ability to boost antibody responses [21, 22]. In a study by Buonsanti et al. (2016), alum-adjuvanted vaccines elicited higher antibody titers compared to non-adjuvanted formulations [22]. This aligns with our findings where mice receiving *S.* Dublin bacterin through injection with alum exhibited significantly elevated *Salmonella*-specific antibody levels. In a study conducted by O'Hagan et al. (2021), alum-adjuvanted influenza vaccines demonstrated a marked increase in antibody titers compared to non-adjuvanted formulations [23]. Moni et al. (2023), investigated the impact of alum adjuvant on hepatitis B vaccines. Their findings revealed a substantial elevation in specific antibody titers in individuals who received the alum-adjuvanted hepatitis B vaccine compared to those who received the non-adjuvanted version [9]. Krauss et al. (2022), conducted a clinical trial assessing the efficacy of an alum-adjuvanted human papillomavirus (HPV) vaccine. The results demonstrated a significant increase in HPV-specific antibody levels in the group receiving the alum-adjuvanted vaccine compared to the control group [24]. The results of these studies align with our study and exemplify alum's consistent adjuvant effect in promoting antibody responses across different pathogens.

The DTH results further emphasized the impact of adjuvants on the cellular immune response. The foot sole skin thickness significantly increased in mice in-

jected with *S.* Dublin bacterin along with alum adjuvant, in contrast to groups receiving bacterin alone. This indicates that alum, as an adjuvant, not only enhances humoral immunity but also contributes to a robust cell-mediated immune response. This aligns with the work of Mutiso et al. (2010), where alum-adjuvanted vaccines induced a robust cell-mediated response, characterized by increased skin thickness in a DTH assay [25]. Similarly, our findings show that mice injected with *S.* Dublin bacterin along with alum exhibited a significant increase in foot sole skin thickness, indicating enhanced cellular immune reactions. In a study led by Ebensen et al. (2019), alum-adjuvanted tetanus toxoid vaccines were investigated for their impact on cellular immune responses. Their findings demonstrated a significant increase in footpad swelling in mice receiving the alum-adjuvanted vaccine compared to the control group in DTH experiments [26]. Osuala et al. (2009), conducted a study evaluating the impact of an alum-adjuvanted tuberculosis (TB) vaccine on cellular immunity. The DTH results showed an enhanced skin induration in individuals who received the alum-adjuvanted TB vaccine compared to the non-adjuvanted group [27]. These studies highlight alum's ability to promote a heightened cell-mediated immune response, as evidenced by increased thickness in response to antigen exposure, in line with our study.

The present research showed that immunization of mice with injectable *S.* Dublin bacterin with or without adjuvant has an increasing effect on IFN-γ production by peripheral blood lymphocytes following stimulation with *S.* Dublin antigen. This cytokine is produced by innate (NK) and specific (Th_1) immune cells and strengthens both cellular and humoral immune systems [28]. Among the disadvantages of the alum adjuvant is its incapability to provoke Th_1 cell responses that are an essential part of cell-mediated immune response to combat most obligative and facultative intracellular pathogens [29], which the present study also confirms this because no significant difference was observed between the groups receiving bacterin with or without alum. In several studies [30-32], an increase in interferon-gamma following vaccination with inactivated *salmonella* spp. has been observed, which is a confirmation of the results of the present research.

Conclusion

The administration of formalin killed *S.* Dublin and alum as an adjuvant can stimulate both cellular and humoral immunity in BALB/c mice. The distinct patterns observed in antibody titers, DTH responses, and IFN-γ production provide a comprehensive understanding of the multifaceted effects of alum on the immune system.

This study lays the groundwork for future research in optimizing vaccination strategies against *Salmonella* spp. and other pathogens. Further work is planned to assess alternative routes of administration and the effect of dose.

Ethical Considerations

Compliance with ethical guidelines

All procedures related to the experimentation and management of animals were approved by the Animal Welfare and Ethics Committees of Shahrekord University, Shahrekord, Iran (Code: IR.SKU.REC.1402.030.

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Authors' contributions

Conceptualization and Supervision: Masoud Ghorbanpoor; Investigation and writing: All authors.

Conflict of interest

The authors declared no conflict of interests.

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