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Antibody Responses in Pigs Induced by Inactivated Vaccine Against *Streptococcus suis* Formulated with Montanide ISA 201 and Montanide Gel 01 Adjuvants

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ABSTRACT

Streptococcus suis (S. suis) is a zoonotic pathogen responsible for streptococcosis, causing substantial economic losses in swine production worldwide. The present study evaluated the humoral immune response in 8-week-old Landrace pigs immunized with an inactivated autogenous *S. suis* vaccine formulated with either Montanide™ ISA 201 VG or Gel 01 adjuvants. A completely randomized experimental design assigned 12 male, 8-week-old Landrace male pigs to three groups. The control group received Montanide™ ISA 201 VG adjuvant without antigen (P1), the second group received antigen and Montanide™ ISA 201 VG (P2), and the third group received antigen and Montanide™ Gel 01 (P3). Vaccination was performed by intramuscular injection into the neck muscle using 4 mL of vaccine suspension. Serum samples from all groups were collected weekly for nine weeks post-vaccination, and antibody titers were quantified using an indirect enzyme-linked immunosorbent assay. Clinical parameters, including body temperature, behavioral changes, and weight gain, were monitored weekly for nine weeks. Both adjuvanted vaccine groups (P2 and P3) demonstrated significantly higher antibody titers compared to the control group, with no significant difference between adjuvant types. A progressive increase in antibody levels was observed from week one to week nine in Groups 2 and 3. No vaccine-associated adverse effects were noted. The *S.suis* vaccine formulated with either Montanide™ ISA 201 VG or Montanide™ Gel 01 adjuvants demonstrated a proper safety profile, with no adverse effects on health or growth performance, and was effective in stimulating strong antibody responses in Landrace pigs. The average antibody titer produced by the vaccine using Montanide™ ISA 201 VG was 0.404 ± 0.201, whereas the vaccine with Montanide™ Gel 01 achieved a titer of 0.404 ± 0.199. The adjuvants elicited comparable immune responses in pigs with no statistically significant difference in antibody titers. The present findings indicated that Montanide™ ISA 201 VG and Montanide™ Gel 01 adjuvants effectively enhanced the immunogenicity of inactivated *S. suis* vaccines in Landrace pigs.

Keywords: Antibody response, Oil-based adjuvant, *Streptococcus suis*, Swine vaccine, Vaccine efficacy

INTRODUCTION

Streptococcus suis (*S. suis*) is a significant pathogenic bacterium in pigs, responsible for streptococcosis, a disease that causes considerable economic losses in the global swine industry (Li et al., 2024). *Streptococcus suis* infection in pigs in Bali, Indonesia, characterized by high morbidity and mortality rates, poses a significant zoonotic threat, particularly to individuals with direct exposure to pigs or their derived products (Winaya et al., 2023). In Indonesia, the incidence of streptococcosis cases in both humans (Tarini et al., 2022) and pigs (Besung et al., 2019) has risen markedly over the past decade, with serotypes 2 and 9 reported as the most common causative agents.

The challenge of controlling *S. suis* infections is further exacerbated by the emergence of antimicrobial resistance among field isolates (Lunha et al., 2022). While antibiotics remain a primary tool for treatment, their overuse contributes to the development of resistant strains, reducing treatment efficacy and posing long-term public health risks (Muteeb et al., 2023). To mitigate the public health risks associated with the widespread infection of *S. suis*, it is crucial to develop alternative prevention methods. In particular, creating effective vaccines that match the antigenic characteristics of local strains found in the specific geographic area is essential (Yao et al., 2015). Vaccines developed from local isolates provide improved protective efficacy by closely matching the antigenic profiles of circulating strains, thus eliciting more specific and robust immune responses compared to traditional vaccines (Choy et al., 2022).

Commercially available vaccines often exhibit suboptimal efficacy against local strains due to the antigenic diversity of *S. suis* isolates circulating in specific regions (Segura et al., 2020). In contrast, autogenous vaccines developed from local isolates have shown promising results in inducing strain-specific immunity (Miryala and Swain, 2025). Nevertheless, inactivated vaccines generally require adjuvants to enhance immunogenicity (Nooraei et al., 2023). Among

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different types of adjuvants, oil-based adjuvants, such as Montanide™ ISA 201 and ISA 01, have demonstrated the ability to improve the magnitude and duration of immune responses in veterinary vaccines (Mohamed, 2022). However, data on the effectiveness of adjuvants in generating protective immunity and antibody responses with *S. suis* vaccines derived from local field isolates is limited. The present study aimed to evaluate the antibody responses in Landrace pigs induced by an inactivated *S. suis* vaccine prepared from a local Indonesian isolate, which was derived from clinical cases and formulated using either Montanide™ ISA 201 or ISA 01.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Indonesia, under approval letter number B/181/UN14.2.9/PT.01.04/2024. The authors considered the farmers' ethical concerns and obtained their consent before conducting the present study.

Study design

The present study employed a completely randomized design with repeated measures, comprising three treatment groups based on the type of adjuvant, including Montanide™ ISA 201 VG without antigen (P1) as the control group, *S. suis* vaccine formulated with Montanide™ ISA 201 VG adjuvant (P2), and *S. suis* vaccine formulated with Montanide™ Gel 01 adjuvant (P3). Observations were conducted weekly for 9 weeks. Each treatment group consisted of four replicates, with four piglets per replicate.

A total of 12 eight-week-old male Landrace piglets were used in the present study, which was conducted at a pig farm in Bangbang Village, Bangli Regency, Bali Province, Indonesia. All piglets were clinically healthy, with no prior history of illness, and had not received any vaccinations or medications before the commencement of the study. All piglets were housed in an open, ventilated, concrete pen with a tiled roof, measuring 6 x 12 meters. Ambient temperatures during the day and night ranged from 21°C to 28°C, with relative humidity levels ranging from 75% to 98%. The piglets were fed twice daily with a balanced ration ingredient (BRI) feed (PT. Japfa Comfeed Indonesia Tbk) and had access to drinking water *ad libitum*. After a 14-day acclimatization period under established husbandry conditions (Maxwell et al., 2024), the piglets were randomly assigned to three treatment groups (P1, P2, and P3), with four piglets per group.

Three treatment groups (P1, P2, and P3) were administered during the first and fifth weeks, following the vaccination schedule outlined in the study conducted by Rumapea et al. (2022). During the nine-week observation period following vaccination, weekly clinical monitoring was conducted, which included evaluations of body weight, behavioral changes, and rectal temperature. Weekly blood samples were drawn from the jugular vein of each pig to isolate serum, which was then utilized for antibody titer measurement using the indirect enzyme-linked immunosorbent assay (ELISA) technique. A total of 108 serum samples were obtained from three treatment groups during the entire nine-week monitoring period.

Bacterial culture

An isolate of *S. suis*, previously identified as strain IIA3 through polymerase chain reaction, was obtained from the culture collection of the Biomedical Laboratory at the Faculty of Veterinary Medicine, Udayana University, Indonesia. To initiate culture, the isolate was grown on sheep blood agar (SBA, Catalog Number: 212750, Difco™, USA) and incubated overnight at 27°C. Subsequently, five colonies of *S. suis* grown on sheep blood agar were transferred into 500 mL of tryptone soy broth (TSB, Catalog Number: 211825, Difco™, USA) and incubated at 37°C for 48 hours with continuous shaking. After incubation, the bacterial concentration was adjusted to match the McFarland 0.5 turbidity standard. To confirm the purity and morphology of the isolate, subculturing was performed on SBA, and Gram staining was conducted (Besung et al., 2019).

Bacterial inactivation

The cultured suspension was divided into two 50 mL aliquots and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded, and the bacterial pellets were resuspended in physiological saline 0.9% (NaCl) to a final volume of 50 mL. Inactivation was carried out in two phases. Initially, the suspension underwent ultrasonication at 70% amplitude for 20 minutes, repeated in three cycles using an ultrasonic processor (Newton, CT, USA). The suspension was subsequently subjected to heat treatment at 80°C for two hours in a water bath incubator. To verify complete inactivation, the treated suspension was streaked on Mueller-Hinton agar (MHA, Catalog Number: 22520, Difco™, USA) and incubated at 37°C for 18 hours to assess for any residual bacterial growth (Besung et al., 2019).

Adjuvant formulation

The inactivated antigen was formulated into two vaccine candidates using distinct oil-based adjuvants from SEPPIC (Fairfield, NJ, USA). The Montanide™ Gel 01 formulation consisted of 7.5% adjuvant, 42.5% NaCl, and 50% antigen. Meanwhile, the Montanide™ ISA 201 VG formulation consisted of equal parts of adjuvant and insoluble lysate from *S. suis* culture, serving as an antigen (50% each). To enhance emulsification, 4 µL of polysorbate (Seppic, France) was added to 40 mL of vaccine preparation, and the mixture was homogenized using a magnetic stirrer at 1500 rpm for 25 minutes (Obradovic et al., 2021).

Vaccination and sample collection

Each pig in groups P2 (Vaccine with 50% v/v, Montanide™ ISA 201 VG) and P3 (Vaccine with 7.5% v/v Montanide™ Gel 01) received a 4 mL intramuscular injection of the assigned vaccine formulation. Pigs in Group P1 received 4 mL of Montanide™ ISA 201 VG without antigen at a final concentration of 50%. Vaccination was administered twice, with a 28-day interval between the prime and booster doses (Obradovic et al., 2021). Weekly, blood samples (5 mL) were collected from the jugular vein, allowed to clot, and centrifuged at 5000 × g for 10 minutes to separate the serum. The serum was aliquoted and stored at -20°C pending immunological analysis (Kralova et al., 2022).

Enzyme-linked immunosorbent assay

Antibody levels in the serum of pigs from all treatment groups were evaluated from week one to week nine and assessed using an indirect ELISA, as described by Obradovic et al. (2021). The ELISA plates were coated with 50 µL of a 1:10 diluted *S. suis* insoluble lysate antigen and incubated overnight at 4°C. Plates were washed three times with PBS-Tween (Difco PBS-Tween 20, Catalog Number: 28378-020, USA), followed by blocking with 100 µL of 10% skim milk at room temperature for 1 hour. After three washes, serum samples diluted 1:100 were added (1 µL per well) and incubated at 37°C for one hour. Plates were rewashed, and then 50 µL of anti-swine Immunoglobulin G (IgG, H+L) conjugated to alkaline phosphatase (Sigma-Aldrich, USA) was diluted 1:1000 and added. The plates were incubated at 37°C for one hour. After final washes, 50 µL of p-nitrophenyl phosphate (Sigma-Aldrich, USA) substrate was added to each well and incubated at room temperature for 15 minutes. The colorimetric reaction was measured using an ELISA plate reader (Bio-Rad, USA) at the appropriate wavelength of 405 nm (Jeffery et al., 2024).

Statistical analysis

The analysis of body weight gain, feed intake, body temperature, and clinically observed signs post-vaccination was conducted descriptively. To assess differences in antibody titers among the adjuvant groups (P1, P2, and P3) and across the observation periods from week one to nine, repeated measures analysis of variance (ANOVA) was utilized, followed by the least significant difference (LSD) post hoc test, which was performed using IBM SPSS Statistics version 29. A P-value of less than 0.05 was deemed statistically significant ($p < 0.05$).

RESULTS

During the observation period, the control pigs (P1) and those vaccinated with inactivated *S. suis*, either formulated with Montanide™ ISA 201 VG adjuvant (P2) or Montanide™ Gel 01 adjuvant (P3), did not exhibit any signs of health disturbances. No clinical signs indicative of illness, such as high fever, lethargy, or behavioral changes, were observed. The pigs maintained a consistent appetite and normal activity levels, and the average recorded body temperature was approximately 39°C, which falls within the normal physiological range for swine (Teixeria et al., 2020). Piglets exhibiting regular activity following vaccination indicated that the experimental animals tolerated the administered vaccine well. Furthermore, body weight progressively increased ($p < 0.05$) from week one to week nine (Figure 1), suggesting that the growth process was not adversely affected during the experimental period.

Figure 1 demonstrates that both the control group (P1) and groups P2 and P3 exhibited a statistically significant ($p < 0.05$) and consistent weekly increase in body weight from week one to week nine. Analysis of variance revealed no significant differences ($p > 0.05$) in body weight among groups P1, P2, and P3. However, a statistically significant increase in body weight ($p < 0.05$) was observed from week one to week nine. The body weight recorded in the second week was significantly higher ($p < 0.05$) than that in the first week, and this upward trend continued progressively until week 9. The antibody titer profile in groups P1, P2, and P3 from week one to week nine is presented in Figure 2.

Pigs in groups P1, P2, and P3 exhibited antibody formation, indicated by the antibody titer value (Figure 2). From week one to nine, Group P1 demonstrated a very low and stable increase in antibody titer every week ($p > 0.05$). Meanwhile, the vaccinated groups (P2 and P3) exhibited an increase in antibody titer at week two, one week after the first vaccination, with a more significant rise ($p < 0.05$) noted after the second vaccination at week five. Antibody titers continued to increase until they peaked at week seven, then remained high with slight fluctuation until week nine ($p <$

0.05). The results of groups P2 and P3 suggested that the antigen in the vaccine was capable of eliciting an immune response, leading to antibody production. However, the magnitude of the response varied depending on the adjuvant used. Further analysis of antibody titer levels using an LSD test revealed statistically significant differences ($p < 0.05$) among the adjuvant treatments (Table 1).

Antibody titers were significantly higher ($p < 0.05$) in pigs from the vaccinated groups that received antigen combined with different adjuvants (P2 and P3) compared to those in the control group that did not receive the antigen (Table 1). However, no significant differences ($p > 0.05$) were observed in the antibody titers between groups P2 and P3, suggesting that both adjuvants elicited comparable immune responses in terms of antibody production. Antibody titers in response to the different adjuvants over the weekly observation period are presented in Table 2.

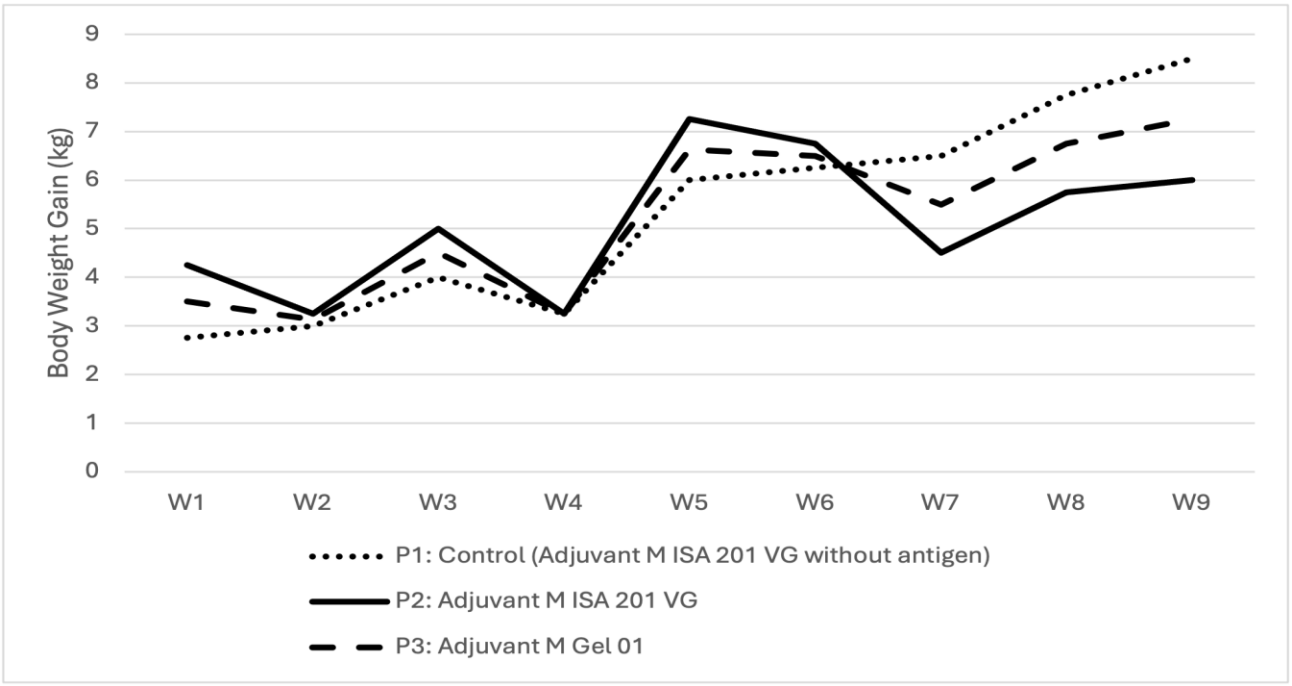

Figure 1. Body weight gain in 8-week-old male Landrace pigs. P1: Control group, P2: Vaccinated group with Montanide™ ISA 201 VG adjuvant, and P3: Vaccinated group with Montanide™ Gel 01 adjuvant.

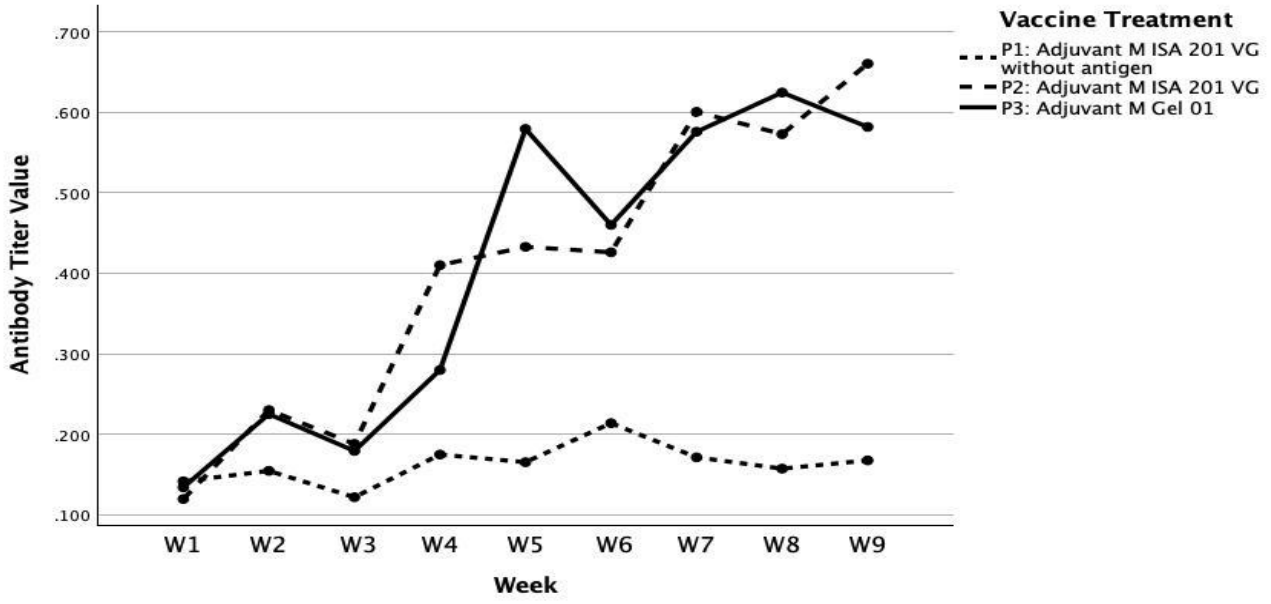

Figure 2. Antibody titers in 8-week-old Landrace male pigs from week one to nine. P1: Control group, P2: Vaccinated group with Montanide™ ISA 201 VG adjuvant, and P3: Vaccinated group with Montanide™ Gel 01.

Table 1. Antibody titers in 8-week-old male landrace pigs treated with Montanide ISA 201 VG without antigen, Montanide ISA 201 VG with antigen, and Montanide Gel 01 with antigen

Groups	Mean antibody titer
Adjuvant Montanide™ ISA 201 VG without antigen (P1)	0.163 ± 0.056^{a}
Adjuvant Montanide™ ISA 201 VG (P2)	0.404 ± 0.201^{b}
Adjuvant Montanide ™ Gel 01 (P3)	0.404 ± 0.199^{b}

[ab] Means that different superscript letters in a column indicate significant differences ($p < 0.05$).

Table 2. Antibody titers in 8-week-old male landrace pigs vaccinated with *Streptococcus. suis* from week one to nine

Observation period	Mean antibody titer
Week 1	0.132 ± 0.035^{a}
Week 2	0.203 ± 0.114^{b}
Week 3	0.163 ± 0.078^{b}
Week 4	0.288 ± 0.148^{c}
Week 5	0.392 ± 0.186^{d}
Week 6	0.367 ± 0.127^{d}
Week 7	0.449 ± 0.207^{de}
Week 8	0.452 ± 0.223^{de}
Week 9	0.470 ± 0.231^{e}

[abcde] Means that different superscript letters in a column indicate significant differences ($p < 0.05$).

DISCUSSION

The control group (P1) and the groups vaccinated with *S. suis* formulated with Montanide™ ISA 201 VG (P2) and Montanide™ Gel 01 adjuvants (P3) demonstrated consistent body weight increases each week throughout the observation period, indicating that the vaccination process and the use of these adjuvants did not adversely affect the animals' growth ($p > 0.05$). These results supported the potential of Montanide™ ISA 201 VG adjuvant and Montanide™ Gel 01 adjuvant as viable components for *S. suis* vaccine development (Obradovic et al., 2021).

Streptococcus suis contains a variety of antigenic molecules, including surface proteins, capsular polysaccharides (CPS), and peptidoglycans, which are recognized by the host immune system as foreign. These molecules act as *S. suis* antigens due to their ability to trigger immune responses upon recognition, specifically surface-associated proteins such as muramidase, released protein, suilysin, and fibronectin-binding proteins, along with CPS, which are the major immunogenic components responsible for stimulating antibody production (Xia et al., 2019). Among surface components, proteins such as M-proteins and CPS are the primary immunogenic components commonly targeted during antibody production (Gao et al., 2024). The IgG antibodies formed after vaccination bind specifically to *S. suis* antigens, thereby neutralizing the bacteria, inhibiting their adhesion to host tissues, and promoting bacterial clearance through opsonization and subsequent phagocytosis (Brouwer et al., 2023). The antigens present in *S. suis* were capable of eliciting the production of *S. suis*-specific IgG antibodies.

In the present study, anti-swine IgG was employed as a secondary antibody in an indirect ELISA to detect specific IgG antibodies produced by pigs in response to *S. suis* antigens. The use of anti-swine IgG enabled accurate identification of IgG-class antibodies and ensured specificity within the detection system. Antibody titer testing using this indirect ELISA provided a reliable method for assessing the humoral immune response in vaccinated or infected piglets by quantifying antigen-specific IgG concentrations in serum samples. Immunoglobulin G plays a pivotal role in preventing *S. suis* infections, serving as the predominant immunoglobulin involved in systemic immune responses and exhibiting broad effector functions (Li et al., 2020). These antibodies recognize and bind to specific bacterial antigens, thereby neutralizing pathogens, blocking their adherence to host cells, and promoting opsonization (Dong et al., 2023). Additionally, IgG is capable of activating the complement cascade, thereby enhancing bacterial lysis (Jensen et al., 2020). The presence of sufficient levels of antigen-specific IgG following vaccination or natural exposure indicated the development of a protective immune response that can reduce disease severity and help prevent reinfection (Rumsey et al., 2022).

The administration of *S. suis* vaccine formulated with either Montanide ISA 201 VG or Montanide Gel 01 adjuvants significantly ($p < 0.05$) enhanced antibody titers compared to the control group. This enhancement can be attributed to the role of adjuvants in strengthening the immune response by effectively stimulating the host immune system (Bastola et al., 2017). Both adjuvants function by retaining the antigen at the injection site for a prolonged period, inducing a local inflammatory response, and activating antigen-presenting cells, thereby promoting optimal antibody production (Heegaard et al., 2016). However, the absence of a statistically significant difference between the two adjuvants suggested that Montanide ISA™ 201 VG and Montanide™ Gel 01 possess comparable capacities to modulate the humoral immune response, despite potential differences in formulation or mechanisms of action. The absence of

statistically significant differences in antibody titers suggested that Montanide™ ISA 201 VG and Montanide™ Gel 01 are equally effective in enhancing antibody responses against *S. suis* antigens (Obradovic et al., 2021).

A significant weekly increase in antibody titers in pigs following vaccination with *S. suis* indicated that the vaccine effectively elicited a robust adaptive immune response. This increase in antibody titers reflected the immune system's ability to recognize the antigen and produce specific antibodies against *S. suis*. The immune response is typically mediated by the activation of B cells, which are stimulated by the antigen, with support from T-helper cells. These cells subsequently differentiate into plasma cells responsible for antibody production (Murphy and Weaver, 2016). The progressive rise in antibody titers over time further suggested that the immune system underwent a maturation process in response to repeated antigen exposure, notably when the vaccine formulation included adjuvants that enhance long-term immune stimulation (Reed et al., 2013). The current results supported the conclusion that the vaccination protocol successfully triggered the anticipated immunological responses in pigs, serving as an early indicator of the vaccine's efficacy against the target pathogen.

CONCLUSION

The *Streptococcus. suis* vaccine formulated with either Montanide™ ISA 201 VG or Montanide™ Gel 01 adjuvants demonstrated safety (No adverse effects on health or growth performance) and effectiveness in inducing a robust antibody production in Landrace pigs. The mean antibody titer induced by the vaccine with Montanide™ ISA 201 VG was 0.404 ± 0.201, while the Montanide™ Gel 01 formulation yielded a titer of 0.404 ± 0.199. Both adjuvants elicited comparable immune responses, with no statistically significant difference in antibody titers. These findings underscored the potential of Montanide™ ISA 201 VG adjuvant and Montanide™ Gel 01 as reliable adjuvants for inactivated *S. suis* vaccines. However, further studies, including challenge trials, are necessary to evaluate the protective efficacy of Montanide ISA 201 VG adjuvant and Montanide Gel 01 adjuvant under field conditions.

DECLARATIONS

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Authors' contributions

I Gede Bagas Upaditha Adresya Kaler was responsible for conceptualizing the idea, designing the experiment, and collecting and analyzing the data. Ni Komang Wahyu Centika Sari, Ni Ketut Suwiti, and I Gusti Ngurah Kade Mahardika contributed to the preparation of the vaccine and organization of the laboratory. I Nengah Kerta Besung was involved in conceptualizing the idea, performing statistical analysis, and drafting the initial manuscript. All authors reviewed and approved the final edition of the manuscript.

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Competing interests

The authors declared there are no conflicts of interest.

Availability of data and materials

The authors confirmed that all data supporting this study's findings are available upon reasonable request from the corresponding author.

Ethical considerations

This article was written originally without any copy from the data of published articles and books.

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