P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2025.157

Immunogenicity of a Heat-Inactivated Local *Streptococcus suis* Vaccine Formulated with Montanide Adjuvants in Mice

Ni Komang Wahyu Centika Sari [1]*, I Gede Bagas Upaditha Adresya Kaler [2], I Gusti Ngurah Kade Mahardika [3], Yulianna Puspitasari [4], Ni Ketut Suwiti [2], I Nengah Kerta Besung [3], Ida Bagus Oka Winaya [5] and Kadek Karang Agustina [6]

[1]Master of Veterinary Medicine Student, Udayana University, Bali, Indonesia
[2]Department of Veterinary Basic Science, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Bali, Indonesia
[3]The Animal Biomedical and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Bali, Indonesia
[4]Departement of Microbiology, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60299, Indonesia
[5]Laboratory of Pathology, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Bali, Indonesia
[6]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Bali, Indonesia
***Corresponding author:** centikasari@gmail.com

Article History: 25-241 Received: 27-Aug-25 Revised: 12-Nov-25 Accepted: 23-Nov-25 Online First: 12-Dec-25

ABSTRACT

Streptococcus suis (S. suis) is an important zoonotic bacterium that causes severe infections in both pigs and humans, underscoring the urgent need for effective vaccines. This study evaluated the immunogenicity and protective efficacy of an inactivated vaccine derived from a local *S. suis* strain in BALB/c mice, using different inactivation methods and adjuvant formulations. Mice were randomly assigned to four groups: P1 (control; Montanide ISA 201 VG without antigen), P2 (ultrasonication–heat-inactivated vaccine with Montanide ISA 201 VG), P3 (ultrasonication–heat-inactivated vaccine with Montanide Gel 01), and P4 (ultrasonication–formaldehyde-inactivated vaccine with Montanide ISA 201 VG). Animals (n=6/group) were immunized at week 1 and received a booster at week 4. Antibody responses were monitored weekly for seven weeks using indirect ELISA, followed by challenge with 1×10^8 CFU of virulent *S. suis* at week 7. Clinical signs, gross lesions, and histopathology of major organs were assessed. All vaccinated groups developed significantly higher antibody titers compared with controls ($P<0.05$). The highest ELISA optical density (OD) values were observed in P2 (0.194±0.090) and P3 (0.192±0.113), with no significant difference between them, but both were significantly higher than P4 (0.178±0.132). Antibody levels increased markedly from week 2, peaking after booster immunization. Following the challenge, vaccinated mice exhibited milder histopathological lesions relative to controls, indicating partial protection.

Keywords: Challenge test, Heat inactivation, Humoral immune response, Inactivated vaccine, Montanide adjuvant, *Streptococcus suis.*

INTRODUCTION

Streptococcus suis (*S. suis*) is an emerging zoonotic pathogen causing severe infections, including meningitis, septicemia, and arthritis in humans, as well as septicemia, pneumonia, and sudden death in pigs (Benea et al. 2025). Over 1,600 human cases have been reported across 30 countries, with the highest incidence in regions of intensive pig farming (Gajdacs et al. 2020). In Indonesia, although reported human cases remain relatively low, *S. suis* continues to pose a significant public health risk. In Bali, the bacterium was detected in 44 of 77 confirmed cases of acute bacterial meningitis between 2014 and 2017 (Susilawathi et al. 2019). Consumption of raw or undercooked pork, such as the traditional dish Lawar Plek, and occupational exposure among pig farmers, butchers, and abattoir workers are recognized as major transmission routes (Besung et al. 2019; Tarini et al. 2022). These findings underscore the persistent threat of *S. suis* at the human–animal interface and highlight the need for improved surveillance, food safety, and preventive strategies, including vaccination (Kerdsin et al. 2022).

Cite This Article as: Sari NKWC, Kaler IGBUA, Mahardika IGNK, Puspitasari Y, Suwiti NK, Besung INK, Winaya IBO and Agustina KK, 2026. Immunogenicity of a heat-inactivated local *Streptococcus suis* vaccine formulated with montanide adjuvants in mice. International Journal of Veterinary Science 15(2): 474-480. https://doi.org/10.47278/journal.ijvs/2025.157

In pigs, *S. suis* is considered endemic and represents a major cause of morbidity and mortality, particularly in piglets aged 4–12 weeks (Segura 2020). Clinical signs range from fever and arthritis to neurological disorders and sudden death, resulting in substantial economic losses in the swine industry (Besung et al. 2019; Obradovic et al. 2021a). Control efforts are complicated by the pathogen's high genetic diversity and serotype variability, which limit cross-protection and hinder the development of broadly effective vaccines (Fang and Ning 2025). Furthermore, the increasing prevalence of antimicrobial resistance among *S. suis* isolates exacerbates disease management challenges, making vaccination the most sustainable and practical approach for improving swine health and reducing zoonotic transmission risks (Yan et al. 2024).

Recent developments highlight the need for a *S. suis* vaccine, including the emergence of hypervirulent strains (Liu et al. 2025; Wahied and Jakee 2025) and the influence of modern swine production systems that facilitate rapid pathogen transmission (Lv et al. 2025). Underdiagnosis remains common due to limited laboratory capacity and frequent misidentification with other *streptococci* (Zhang et al. 2020).

Several vaccine strategies have been explored, including live-attenuated, subunit, DNA-based, and inactivated formulations. Among these, whole-cell antigen (WCA) vaccines are promising because they present diverse epitopes capable of inducing both humoral and cellular immune responses (Osterloh 2022). However, their efficacy depends heavily on the inactivation method and adjuvant formulation. Heat and ultrasonication treatments generally preserve antigenic integrity better than formaldehyde, which may alter critical epitopes (Wilton et al. 2014; Saylor et al. 2020). Likewise, Montanide-based adjuvants, such as ISA 201 VG and Gel 01, enhance immune responses through distinct mechanisms, modulating antigen presentation and T-cell polarization (Obradovic et al. 2021a; Facciola et al. 2022; Zhao et al. 2023). Despite these advances, limited data exist on how different inactivation techniques interact with Montanide adjuvants to shape vaccine efficacy.

To address this gap, the present study evaluated the immunogenicity and protective efficacy of vaccines derived from a local *S. suis* isolate, inactivated using distinct methods and formulated with either Montanide ISA 201 VG or Gel 01. Using a BALB/c mouse model, we assessed antibody responses, histopathological changes, and clinical outcomes following challenge to identify promising vaccine candidates for future application in swine populations.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were conducted in accordance with the guidelines of the Research Ethics Committee, Faculty of Veterinary Medicine, Udayana University, Denpasar, Indonesia (Approval No. B/178/UN14.2.9/PT.01.04/2024).

Animals and experimental design

Twenty-four female BALB/c mice (8 weeks old, 20–30g) were randomly allocated into four groups (n=6/group). Mice were acclimatized for seven days prior to experimentation. The treatment groups were as follows: P1 (control, Montanide ISA 201 VG adjuvant only), P2 (ultrasonication-heat inactivated vaccine + Montanide ISA 201 VG), P3 (ultrasonication-heat inactivated vaccine + Montanide Gel 01), and P4 (ultrasonication-formaldehyde inactivated vaccine + Montanide ISA 201 VG). Vaccinations were administered subcutaneously at week 1 and boosted at week 4, with a final concentration of 1×10^{12}CFU/mL per dose (0.4mL/mouse). Serum was collected weekly from week 1 to week 7 for antibody analysis.

Vaccine preparation

A local *S. suis* previously well characterized isolate (Besung et al. 2019; Susilawathi et al. 2019) was obtained from the Biomedical Laboratory, Faculty of Veterinary Medicine, Udayana University, was propagated in tryptone soy broth (Difco™, USA) until the exponential growth phase. For inactivation, suspensions designated for groups P2 and P3 were subjected to ultrasonication (70% amplitude, 20kHz, 30 minutes) followed by heat treatment at 80°C for 2h, whereas group P4 was treated with ultrasonication (Newton, CT, USA) followed by 0.2% formaldehyde incubation at room temperature for 24h. Complete inactivation was verified by the absence of bacterial growth on Mueller-Hinton agar (Difco™, USA) (Besung et al. 2019). The inactivated preparations were adjusted to a final concentration of 1×10^{12}CFU/mL, after which antigens were emulsified either with Montanide ISA 201 VG (50:50 v/v; Fairfield, NJ, USA) for groups P2 and P4, or with Montanide Gel 01 (7.5% Gel, 42.5% saline, 50% antigen) for group P3

Sample collection and ELISA

Blood samples (1mL) were obtained weekly via facial vein puncture (Enos and Moore 2022). Serum was separated by centrifugation (5000rpm, 10 minutes) and stored at 4°C. Antibody titers were determined using an indirect enzyme-linked immunosorbent assay (ELISA) following the procedure described by (Obradovic et al. 2021a). The antigen was prepared by diluting a precipitated *S. suis* suspension (1:10 in distilled water), followed by a further dilution of 1:250 in coating buffer. Microplates were coated with 50µL of the antigen solution and incubated overnight at 4°C. After washing with PBS-Tween 20, wells were blocked with 100µL of 10% skim milk for 1h at room temperature. Serum samples, diluted 1:100, were added (1µL/well) and incubated for 1h. After additional washing, 50µL of goat anti-mouse IgG (H+L) conjugated with alkaline phosphatase (1:1000; Sigma-Aldrich, USA) was added and incubated for 1h. The wells were then rewashed and developed with 50µL of p-nitrophenyl phosphate (pNPP) substrate (Sigma-Aldrich, USA) for 15 minutes at room temperature. Optical density was recorded at 405nm using a microplate reader (Bio-Rad, USA).

Challenge test

At week 7, mice were challenged intraperitoneally with 1×10^{8}CFU of virulent *S. suis*. Clinical signs and survival were monitored for 14 days. At termination, animals were euthanized, and major organs (brain, lungs, spleen, liver, heart, kidneys, middle ear) were collected for gross pathology and histopathology. Tissues were fixed in 10% formalin, processed, and stained with hematoxylin and eosin (H&E) (Koivukoski et al. 2023).

Statistical Analysis

Body weight and clinical parameters were analyzed descriptively. Antibody titers were analyzed using Generalized Linear Model (GLM) Repeated Measures ANOVA, followed by Tukey's post hoc test. Mauchly's test of sphericity and within-subject effects were applied when necessary (Wahied and Jakee 2025). Analyses were performed using SPSS v26 (IBM), with $P<0.05$ considered significant. Graphs were prepared in GraphPad Prism V8.

RESULTS

The overall health status of the mice was assessed by monitoring local reactions at the injection site, clinical signs, and changes in body weight. Mild, transient redness at the injection site was observed in groups receiving Montanide™ ISA 201 VG (P1, P2, and P4) following the first vaccination; these reactions resolved spontaneously within a few hours. In contrast, three mice in the Montanide™ Gel 01 group (P3) developed localized swelling and mild necrosis at the injection site two weeks after the booster dose. These lesions subsided within two weeks without intervention.

Throughout the seven-week observation period, all groups maintained normal coat condition, behavior, and food intake, with no systemic adverse effects recorded. Body weight increased progressively across all groups as presented in Fig. 1, and two-way ANOVA indicated no significant effect of treatment on weight gain ($P>0.05$), suggesting that vaccination did not negatively impact growth performance.

Serum antibody responses were evaluated using indirect ELISA. Mean optical density (OD) of mouse serum in ELISA using sediment of *S. suis* extract as coating antigen of all treatment groups in seven weeks observation is presented in Table 1 and Fig. 2. The adjuvant-only control group (P1) displayed the lowest mean optical density (OD) values, whereas vaccinated groups demonstrated significantly higher titers ($P<0.001$). Among vaccinated groups, P2 (ultrasonication–heat-inactivated vaccine with Montanide™ ISA 201 VG) and P3 (ultrasonication–heat-inactivated vaccine with Montanide™ Gel 01) induced the highest antibody responses, which were statistically comparable ($P=0.939$). Both P2 and P3 elicited significantly higher titers than P4 (formaldehyde-inactivated vaccine with Montanide™ ISA 201 VG) ($P<0.05$).

Table 1: Mean Optical Density (OD)/Standard Deviation (SD) of mice serum in ELISA using sediment of *S. suis* extract as coating antigen of all treatment groups in seven weeks observation (W)

	W1	W2	W3	W4	W5	W6	W7	Average
P1	$0.029/0.003^{Aa}$	$0.03/0.003^{Aa}$	$0.036/0.003^{Aa}$	$0.056/0.010^{Aa}$	$0.066/0.008^{Aa}$	$0.079/0.011^{Aa}$	$0.132/0.042^{Aa}$	$0.061/0.006^{a}$
P2	$0.093/0.055^{Aa}$	$0.133/0.041^{Ab}$	$0.145/0.032^{Bd}$	$0.166/0.020^{Cc}$	$0.224/0.055^{Cb}$	$0.258/0.065^{Db}$	$0.339/0.047^{Db}$	$0.194/0.011^{c}$
P3	$0.046/0.029^{Aa}$	$0.104/0.033^{Bb}$	$0.142/0.031^{Bc}$	$0.182/0.052^{Bc}$	$0.248/0.037^{Cb}$	$0.262/0.058^{Cb}$	$0.358/0.119^{Cb}$	$0.192/0.020^{c}$
P4	$0.094/0.061^{Aa}$	$0.102/0.042^{Ab}$	$0.133/0.087^{Ab}$	$0.136/0.101^{Ab}$	$0.181/0.066^{Bb}$	$0.238/0.067^{Bb}$	$0.361/0.220^{Bb}$	$0.178/0.037^{b}$

Note: Group P1 (vaccinated with adjuvant Montanide ISA 201 VG only), P2 (ultrasonication-heat inactivated vaccine with Montanide ISA 201 VG), P3 (ultrasonication-heat inactivated vaccine with Montanide Gel 01), and P4 (ultrasonication-formaldehyde inactivated vaccine with Montanide ISA 201 VG). Different superscript letters indicate statistical significance ($P<0.05$). Capital letters $^{A, B, C, \text{and } D}$ mean statistical differences among weeks within the same treatment; Lower case letters $^{a, b, c, \text{and } d}$ mean statistical differences between treatments (P1, P2, P3, P4) within the same week.

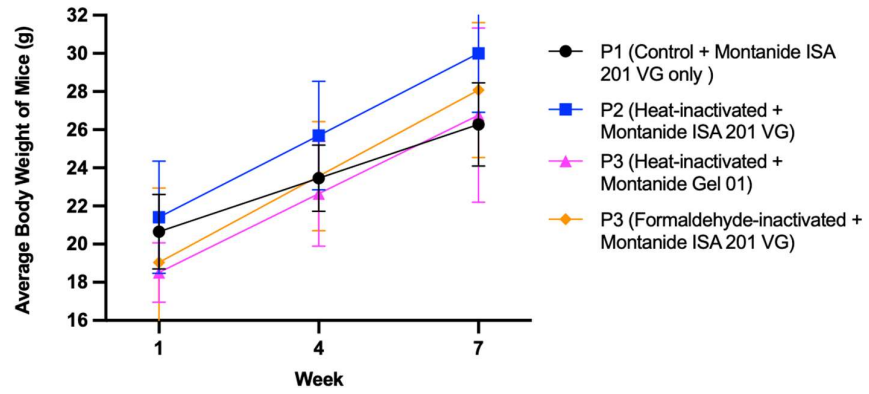

Fig. 1: Average body weight of mice (g) from week 1 to week 7. Data are shown as mean ± SD (n = 6 per group). Error bars represent the standard deviation (SD) of the mean.

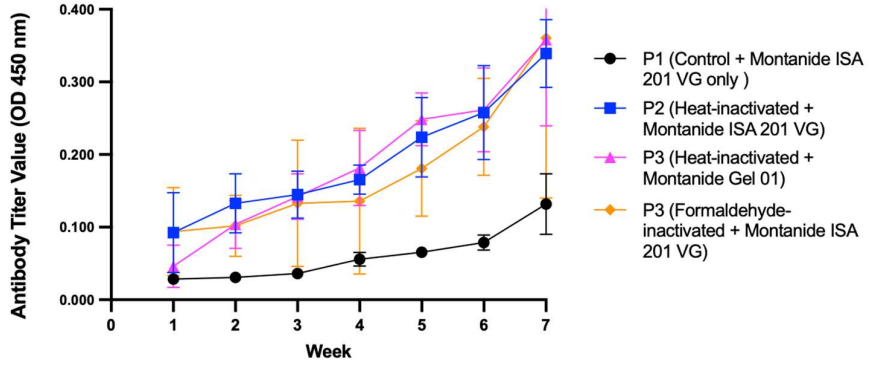

Fig. 2: Weekly antibody titers (OD values) from week 1 to week 7 in female BALB/c mice. Data are shown as mean ± SD (n = 6 per group). Error bars represent the standard deviation (SD) of the mean.

Longitudinal analysis revealed a marked increase in antibody titers from week 2 onwards, with a pronounced rise following the booster immunization. Generalized Linear Model (GLM) Repeated Measures ANOVA confirmed significant effects of both treatment and time ($P<0.05$), as well as a significant interaction between the two ($P<0.05$). Bonferroni-adjusted pairwise comparisons showed no significant differences in titers between weeks 1 and 3 ($P>0.05$), whereas titers from weeks 4 to 7 were significantly higher than those in earlier weeks ($P<0.05$). No significant differences were detected among weeks 5, 6, and 7 ($P>0.05$), indicating that antibody responses plateaued after the booster dose.

After challenge with virulent *S. suis*, no mortality or overt clinical signs were observed in either vaccinated or control groups during the 14-day monitoring period. However, bacterial culture revealed widespread *S. suis* colonization in multiple organs of control mice (P1), whereas colonization was substantially reduced in vaccinated groups.

Representative histopathological changes in challenged mice (H&E, 400X) are indicated in Fig. 3. The number of animals showing inflammation/lesion in various organs and the number of examined animals from each treatment group are presented in Table 2. Histopathological examination demonstrated severe lesions in control mice, including meningoencephalitis, bronchopneumonia, myocarditis, interstitial nephritis, and otitis media. In contrast, vaccinated mice exhibited markedly milder tissue damage. P2 (heat-inactivated vaccine + Montanide™ ISA 201 VG) showed only mild meningeal and pulmonary inflammation, with no cardiac or ear lesions. P3 (ultrasonication-inactivated vaccine + Montanide™ Gel 01) presented mild meningitis, pneumonia, and occasional myocarditis or otitis media. P4 (formaldehyde-inactivated vaccine + Montanide™ ISA 201 VG) exhibited moderate meningitis and pneumonia, with sporadic myocarditis but no kidney or ear involvement.

Table 2: Number of animals showing inflammation/lesion in various organs and the numbers of examined animals from each treatment group

Organ	P1 (A/B)	P2 (A/B)	P3 (A/B)	P4 (A/B)
Brain	1/2	1/2	1/2	1/2
Middle Ear	2/2	1/2	2/2	0/2
Lung	2/2	1/2	2/2	2/2
Liver	2/2	2/2	2/2	2/2
Heart	2/2	2/2	1/2	1/2
Spleen	2/2	2/2	2/2	2/2
Kidney	2/2	2/2	2/2	2/2

Note: A represents the number of animals showing lesions in each organ; B represents the number of total animals necropsied from each treatment group; P1-P4 as in Table 1.

These findings indicate that vaccination reduced bacterial colonization and attenuated tissue pathology, with ultrasonication–heat-inactivated formulations (P2 and P3) providing superior protection compared to formaldehyde-inactivated vaccines (P4).

DISCUSSION

S. suis is a major zoonotic pathogen with significant implications for both swine health and public health worldwide (Segura et al. 2017). Vaccination remains one of the most effective preventive strategies; however, the success of inactivated vaccines is largely dependent on two key factors: the method of antigen inactivation and the choice of adjuvant formulation (Kozak and Hu 2023). Optimizing both is therefore critical for generating robust and durable protective immunity.

In this study, vaccine safety was evaluated by monitoring injection site reactions and body weight changes. Mice receiving Montanide™ ISA 201 VG exhibited only mild, transient redness that resolved spontaneously, while those vaccinated with Montanide™ Gel 01 showed localized swelling and mild necrosis at the injection site. Such adverse reactions are consistent with local inflammatory responses triggered by vaccine

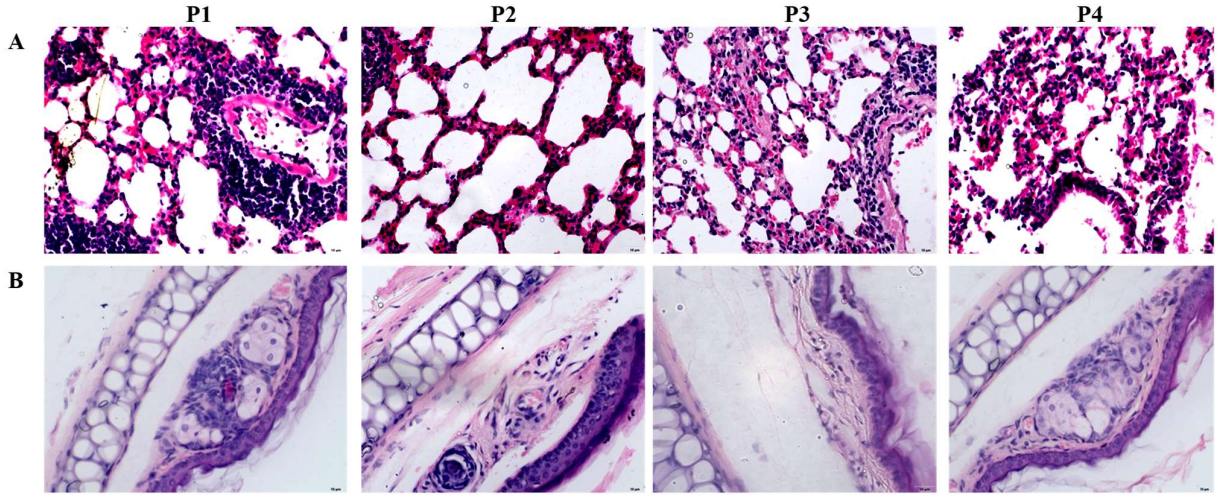

Fig. 3: Representative histopathological changes in challenged mice (H&E, 400×). In row A (lung tissue), P1 shows bronchitis, P2 demonstrates bronchopneumonia, P3 reveals interstitial pneumonia, and P4 shows more severe bronchopneumonia. In row B (middle ear tissue), P1 exhibits mild otitis media with limited inflammatory cells along the mucosal lining, P2 demonstrates otitis media, P3 shows more severe otitis media with inflammatory accumulation, and P4 reveals otitis media with thickened mucosa.

adjuvants and antigen deposition (Marshall et al. 2018). Importantly, these local effects did not translate into systemic toxicity, as evidenced by the absence of abnormal behavior, reduced feed intake, or significant differences in body weight across groups ($P>0.05$). This finding aligns with previous studies showing that severe systemic inflammation typically correlates with growth retardation and altered energy metabolism (Momose et al. 2010; Liang and Loré 2016).

Serological analysis demonstrated that all vaccinated groups developed significantly higher antibody titers than the adjuvant-only control, indicating successful induction of antigen-specific humoral immunity. This response reflects the uptake and processing of antigens by antigen-presenting cells (APCs), activation of $CD4^+$ T-helper cells, and subsequent differentiation of B cells into antibody-secreting plasma cells producing *S. suis*-specific IgG (McKee et al. 2010; Pollard and Bijker 2020).

Among the formulations tested, ultrasonication–heat-inactivated vaccines combined with Montanide™ ISA 201 VG (P2) or Montanide™ Gel 01 (P3) elicited the highest antibody titers, with no significant difference between them, suggesting comparable adjuvant efficacy. Both induced significantly stronger responses than the formaldehyde-inactivated vaccine (P4). These results are consistent with previous studies showing that formaldehyde inactivation may cause protein cross-linking and epitope modification, thereby diminishing immunogenicity (Wilton et al. 2014; Westcott et al. 2023). In contrast, heat-based inactivation—particularly when combined with ultrasonication—appears to better preserve conformational epitopes critical for B-cell recognition, explaining the enhanced antibody responses observed in P2 and P3.

The findings of the present study are consistent with the recent report that has evaluated the effect of the inactivation method on vaccine efficacy. Another study by (Sharmin et al. 2024) demonstrated that the heat-inactivated WCA vaccine effectively protected mice against highly resistant *Acinetobacter baumannii* and induced a significant increase in IgG antibody titers. These parallel findings further validate the enhanced immunogenicity observed in the P2 and P3 formulation of the current study.

The adjuvants used in this study, oil-based Montanide™ ISA 201 VG and polymer-based Montanide™ Gel 01, both significantly enhanced humoral responses compared with controls, confirming their immunostimulatory capacity in pigs (Kaler et al. 2025). Although these adjuvants act through distinct immunological mechanisms, they produced comparable antibody titers. ISA 201 VG has been reported to be biased toward Th1/Th17 responses, which favor cell-mediated immunity, while Gel 01 tends to promote a Th2-skewed response, enhancing antibody production (Zhao et al. 2023). Despite these mechanistic differences, both adjuvants effectively supported the generation of strong *S. suis*-specific IgG responses, demonstrating their suitability for use in WCA vaccine formulations.

Comparable results have also been observed in studies exploring Montanide-based adjuvants in bacterial vaccines. The adjuvant Montanide 201 VG has been reported to boost humoral and cellular immunity in an inactivated *S. suis* vaccine and protective capacity of the vaccine against a challenge (Obradovic et al. 2021b). Likewise, Montanide Gel 01 has been shown to enhance antibody production and promote long-term protective immunity in multiple swine pathogens, including *Haemophilus parasuis and Pseudorabies* (Li et al. 2020; Hua et al. 2022). These findings align closely with our results, where both adjuvants produced strong antigen-specific responses and mild local reactions, supporting their suitability for WCA vaccine formulation targeting *S. suis*.

Longitudinal analysis revealed that antibody titers began rising significantly after week 2 and peaked following booster immunization at week 5, after which they plateaued between weeks 5 and 7. This pattern reflects the induction of memory B-cell responses and the transition from a primary to a secondary immune response, characterized by clonal expansion, affinity maturation, and stabilization of antibody production (Marzi et al. 2023; Syeda et al. 2024). These findings emphasize the importance of booster doses in sustaining protective antibody levels, particularly for vaccines based on inactivated whole-cell antigens.

Despite robust antibody responses, challenge experiments demonstrated only partial protection. No mortality or overt clinical signs were observed in any group; however, histopathological evaluation revealed persistent lesions in vaccinated mice, albeit markedly milder than those in controls. These results indicate that humoral immunity alone may be insufficient to achieve full protection against *S. suis*. Similar findings have been reported in previous studies, where rapid disease progression and septicemia limited the protective contribution of antibodies alone (Besung et al. 2019).

For improved vaccine efficacy, future strategies should focus on incorporating conserved protective antigens capable of inducing stronger T-cell-mediated immunity, optimizing adjuvant systems to enhance balanced Th1/Th2/Th17 responses, and promoting durable immunological memory (Schijns et al. 2021). In addition, evaluating vaccine performance directly in swine—the natural host—will be essential to validate translational relevance and confirm protective efficacy under field conditions.

Conclusion

Vaccination with heat-inactivated *Streptococcus suis* formulated with either Montanide™ ISA 201 VG (P2) or Montanide™ Gel 01 (P3) elicited the highest antibody titers, significantly exceeding those induced by the formaldehyde-inactivated vaccine (P4) and the adjuvant-only control (P1), with no significant difference between P2 and P3. Booster immunization further enhanced antibody responses, and histopathological analysis demonstrated partial protection, as vaccinated mice developed markedly milder lesions compared to controls. These findings indicate that heat-inactivated vaccines combined with Montanide adjuvants represent promising candidates for *S. suis* prevention. However, further optimization of antigen formulations, evaluation of cellular immune responses, and validation in target animal species are warranted to achieve broader and more durable protection.

DECLARATIONS

Funding: This research was funded by Udayana University through the Assignment Agreement Letter for the Implementation of Research Activities (SP3) under the PNBP Research Grant for the Fiscal Year 2024, Contract Number: B/255.147/Un14.4.A/Pt 01.03/2024. The authors gratefully acknowledge the support from the Rector and the Head of the Institute for Research and Community Service (LPPM), Udayana University.

Acknowledgement: The authors would like to express their sincere gratitude to the Laboratory of Immunology, Faculty of Veterinary Medicine, Udayana University, Indonesia, for the valuable support and facilities provided throughout this research.

Conflict of Interest: The authors declared there were no conflicts of interest.

Data Availability: Relevant datasets are available from the corresponding Author upon request.

Ethics Statement: All animal procedures were approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Indonesia, under approval letter number B/178/UN14.2.9/PT.01.04/2024.

Author's Contribution: Ni Komang Wahyu Centika Sari: Conceptualization, Writing-original draft, Resources, Formal analysis, Investigation, Project administration. I Gede Bagas Upaditha Adresya Kaler, Yulianna Puspitasari, Ni Ketut Suwiti, Ida Bagus Oka Winaya and Kadek Karang Agustina: Investigation, Writing - Review & Editing. I Gusti Ngurah Kade Mahardika: Methodology, Resources, Writing - Review & Editing. I Nengah Kerta Besung: Conceptualization, Resources, Funding acquisition, Investigation, Project administration, Validation, Writing - Review & Editing.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

Publisher's Note: All claims stated in this article are exclusively those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated/assessed in this article or claimed by its manufacturer is not guaranteed or endorsed by the publisher/editors.

REFERENCES

Benea SN, Moroti R, Deaconu T, Ciont C, Benea MA and Savulescu Fiedler I, 2025. *Streptococcus suis*: A possible emerging zoonotic pathogen in Romania. Microorganisms 13(2): 335. https://www.mdpi.com/2076-2607/13/2/335

Besung INK, Suarjana IGK, Agustina KK, Winaya IBO, Soeharsono H, Suwiti NK and Mahardika GN, 2019. Isolation and identification of *Streptococcus suis* from sick pigs in Bali, Indonesia. BMC Research Notes 12(1): 795. https://doi.org/10.1186/s13104-019-4826-7

Enos KE and Moore DM, 2022. Hematology of laboratory animals. Schalm's Veterinary Hematology 118: 1058-1072. https://doi.org/10.1002/9781119500537.ch118

Facciola A, Visalli G, Lagana A and Di Pietro A, 2022. An overview of vaccine adjuvants: Current evidence and future perspectives. Vaccines 10(5): 819. https://doi.org/10.3390/vaccines10050819

Fang L and Ning J, 2025. Decadal advances and future prospects in subunit vaccine development against *Streptococcus suis* infection. Frontiers in Immunology 16: 1680732. https://doi.org/10.3389/fimmu.2025.1680732

Gajdacs M, Nemeth A, Knausz M, Barrak I, Stajer A, Mestyan G, Melegh S, Nyul A, Toth A, Agoston Z and Urban E, 2020. *Streptococcus suis*: An underestimated emerging pathogen in Hungary? Microorganisms 8(9): 1292. https://doi.org/10.3390/microorganisms8091292

Hua T, Chang C, Zhang X, Huang Y, Wang H, Zhang D and Tang B, 2022. Protective efficacy of intranasal inactivated pseudorabies vaccine is improved by combination adjuvant in mice. Frontiers in Microbiology 13: 1-14. https://doi.org/10.3389/fmicb.2022.976220

Kaler IGBUA, Sari NKWC, Suwiti NK, Mahardika IGNK and Besung INK, 2025. Antibody responses in pigs induced by inactivated vaccine against *Streptococcus suis* formulated with Montanide ISA 201 and Montanide Gel 01 adjuvants. World's Veterinary Journal 15(2): 445-451. https://doi.org/10.54203/scil.2025.wvj44

Kerdsin A, Segura M, Fittipaldi N and Gottschalk M, 2022. Sociocultural factors influencing human *Streptococcus suis* disease in Southeast Asia. Foods 11(9): 1190. https://www.mdpi.com/2304-8158/11/9/1190

Koivukoski S, Khan U, Ruusuvuori P and Latonen L, 2023. Unstained tissue imaging and virtual hematoxylin and eosin staining of histologic whole slide images. Laboratory Investigation 103(5): 100070. https://doi.org/10.1016/j.labinv.2023.100070

Kozak M and Hu J, 2023. The integrated consideration of vaccine platforms, adjuvants, and delivery routes for successful vaccine development. Vaccines 11(3) https://doi.org/10.3390/vaccines11030695

Li X, Qiu L, Qiu G, Yang X and Zheng X, 2020. Evaluation of red clover isoflavone extract as a vaccine adjuvant for piglets against *Haemophilus parasuis*. Veterinární medicína 65(9): 387-393. https://doi.org/10.17221/44/2020-VETMED

Liang F and Loré K, 2016. Local innate immune responses in the vaccine adjuvant-injected muscle. Clinical & Translational Immunology 5(4) https://doi.org/10.1038/cti.2016.19

Liu F, Zhang S, Erdeljan M, Zhang Y, Chen Z, Li J, Ding L, Zhang L, Sun W, Yu J and Wu J, 2025. *Streptococcus suis*: Epidemiology and resistance evolution of an emerging zoonotic bacteria. One Health 21: 101098. https://doi.org/10.1016/j.onehlt.2025.101098

Lv R, Zhang W, Sun Z, Si X, Dong H and Liu X, 2025. Current prevalence and therapeutic strategies for porcine *Streptococcus suis* in China. Applied and Environmental Microbiology 91(3): e0216024. https://doi.org/10.1128/aem.02160-24

Marshall JS, Warrington R, Watson W and Kim HL, 2018. An introduction to immunology and immunopathology. Allergy, Asthma & Clinical Immunology 14(2): 49. https://doi.org/10.1186/s13223-018-0278-1

Marzi R, Bassi J, Silacci-Fregni C, Bartha I, Muoio F, Culap K, Sprugasci N, Lombardo G, Saliba C, Cameroni E, Cassotta A, Low JS, Walls AC, McCallum M, Tortorici MA, Bowen JE, Dellota EA, Jr., Dillen JR, Czudnochowski N and Piccoli L, 2023. Maturation of SARS-CoV-2 Spike-specific memory B cells drives resilience to viral escape. iScience 26(1): 105726. https://doi.org/10.1016/j.isci.2022.105726

McKee A, MacLeod M, Kappler J and Marrack P, 2010. Immune mechanisms of protection: can adjuvants rise to the

challenge? BMC Biology 1(8): 37. https://doi.org/10.1186/1741-7007-8-37

Momose H, Mizukami T, Ochiai M, Hamaguchi I and Yamaguchi K, 2010. A new method for the evaluation of vaccine safety based on comprehensive gene expression analysis. Journal of Biomedicine and Biotechnology 2010: 1-7. https://doi.org/10.1155/2010/361841

Obradovic MR, Corsaut L, Dolbec D, Gottschalk M and Segura M, 2021a. Experimental evaluation of protection and immunogenicity of *Streptococcus suis* bacterin-based vaccines formulated with different commercial adjuvants in weaned piglets. Veterinary Research 52(1): 133. https://doi.org/10.1186/s13567-021-01004-x

Obradovic MR, Segura M, Segales J and Gottschalk M, 2021b. Review of the speculative role of co-infections in *Streptococcus suis*-associated diseases in pigs. Veterinary Research 52(1): 49. https://doi.org/10.1186/s13567-021-00918-w

Osterloh A, 2022. Vaccination against bacterial infections: Challenges, progress, and new approaches with a focus on intracellular bacteria. Vaccines 10(5) https://doi.org/10.3390/vaccines10050751

Pollard AJ and Bijker EM, 2020. A guide to vaccinology: from basic principles to new developments. Nature Reviews Immunology 21(2): 83-100. https://doi.org/10.1038/s41577-020-00479-7

Saylor K, Gillam F, Lohneis T and Zhang C, 2020. Designs of antigen structure and composition for improved protein-based vaccine efficacy. Frontiers in Immunology 11 https://doi.org/10.3389/fimmu.2020.00283

Schijns V, Majhen D, van der Ley P, Thakur A, Summerfield A, Berisio R, Nativi C, Fernández-Tejada A, Alvarez-Dominguez C, Gizurarson S, Zamyatina A, Molinaro A, Rosano C, Jakopin Ž, Gursel I and McClean S, 2021. Rational vaccine design in times of emerging diseases: The critical choices of immunological correlates of protection, vaccine antigen and immunomodulation. Pharmaceutics 13(4) https://doi.org/10.3390/pharmaceutics13040501

Segura M, 2020. *Streptococcus suis* Research: Progress and Challenges. Pathogens 9(9): 707. https://www.mdpi.com/2076-0817/9/9/707

Segura M, Fittipaldi N, Calzas C and Gottschalk M, 2017. Critical *Streptococcus suis* virulence factors: Are they all really critical? Trends in Microbiology 25(7): 585-599. https://doi.org/10.1016/j.tim.2017.02.005

Sharmin N, Khoda M and Uddin M, 2024. Immunogenicity and efficacy of heat inactivated whole-cell vaccine to increase murine survival after extensively drug-resistant *Acinetobacter baumannii* infection. International Journal of Human and Health Sciences 8: 275-282. https://doi.org/10.31344/ijhhs.v8i3.723

Susilawathi NM, Tarini NMA, Fatmawati NND, Mayura PIB, Suryapraba AAA, Subrata M, Sudewi AAR and Mahardika GN, 2019. *Streptococcus suis*–associated meningitis, Bali, Indonesia, 2014–2017. Emerging Infectious Diseases 25(12): 2235-2242. https://doi.org/10.3201/eid2512.181709

Syeda MZ, Hong T, Huang C, Huang W and Mu Q, 2024. B cell memory: From generation to reactivation: Amultipronged defense wall against pathogens. Cell Death Discovery 10(1): 117. https://doi.org/10.1038/s41420-024-01889-5

Tarini NMA, Susilawathi NM, Sudewi AAR, Soejitno A, Fatmawati NND, Mayura IPB, Lestari AAW, Suputra G, Subrata IK, Astiti C, Besung INK and Mahardika GN, 2022. A large cluster of human infections of *Streptococcus suis* in Bali, Indonesia. One Health 14: 100394. https://doi.org/10.1016/j.onehlt.2022.100394

Wahied RM and Jakee ELJ, 2025. Comparative immunological studies for evaluation enterotoxemia vaccine in rabbit. International Journal of Veterinary Science 14(4): 791-796. https://doi.org/10.47278/journal.ijvs/2024.075

Westcott MM, Blevins M, Wierzba TF, Morse AE, White KR, Sanders LA and Sanders JW, 2023. The immunogenicity and properties of a whole-cell ETEC vaccine inactivated with psoralen and UVA light in comparison to formalin. Microorganisms 11(8) https://doi.org/10.3390/microorganisms11082040

Wilton T, Dunn G, Eastwood D, Minor PD, Martin J and Perlman S, 2014. Effect of formaldehyde inactivation on poliovirus. Journal of Virology 88(20): 11955-11964. https://doi.org/10.1128/jvi.01809-14

Yan Z, Pan R, Zhang J, Sun J, Ma X, Dong N, Yao X, Wei JC, Liu K, Qiu Y, Sealey K, Nichols H, Jarvis M, Upton M, Li X, Ma Z, Liu J and Li B, 2024. Immunogenicity and protective capacity of sugar ABC transporter substrate-binding protein against *Streptococcus suis* serotype 2, 7, and 9 infection in mice. Vaccines 12: 544. https://doi.org/10.3390/vaccines12050544

Zhang X, Wu Z and Wang K, 2020. Diagnosis of *Streptococcus suis* Meningoencephalitis with metagenomic next-generation sequencing of the cerebrospinal fluid: a case report with literature review. BMC Infectious Diseases 20(1): 884. https://doi.org/10.1186/s12879-020-05621-3

Zhao T, Cai Y, Jiang Y, He X, Wei Y, Yu Y and Tian X, 2023. Vaccine adjuvants: mechanisms and platforms. Signal Transduction and Targeted Therapy 8(1): 283. https://doi.org/10.1038/s41392-023-01557-7