Research Article

Evaluation of a Novel Protein A (*Staphylococcus aureus*)–IgG Matrix Complex as an Adjuvant for a Poultry Necrotic Enteritis Toxoid Vaccine

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Abstract | Necrotic enteritis (NE) is a digestive disease caused by the toxins produced by *Clostridium perfringens*. This study aimed to develop an NE matrix vaccine prototype using staphylococcal protein A (SpA) and a sheep IgG complex. The NE-matrix vaccine was prepared by mixing *C. perfringens* alpha toxoids, sheep IgG, and SpA, and its potential to induced immune response was evaluated by determining associated CD4 and MHC II gene expression levels and antibody formation. Thirty 14-week-old ages layer chickens were divided into three groups: non-vaccinated control (NC), matrix NE vaccine (MV), and oil-based commercial NE vaccine (KV). Vaccination was performed twice, at a dose of 0.3 mL (9 MLD), and with an interval time of four weeks between each vaccination via the intramuscular route. CD4 and MHC II gene expression was observed 12 and 24 h after the first vaccination, whereas antibodies were measured four weeks after the first and four weeks after the second vaccinations. Our results showed that the expression of CD4 and MHC II-encoding genes increased 24 h after the first vaccination in the MV group. *C. perfringens* alpha toxin antibodies were detected four weeks after the first vaccination and increased four weeks after the second vaccination. Our results indicate the potential of this complex matrix-adjuvant in vaccine as novel adjuvant for toxoid, although the immunity development required a booster dose to reach optimal antibody titers, indicating a gradual immune activation rather than a delayed response.

Keywords | *Clostridium perfringens*, Necrotic enteritis, Matrix vaccine, Staphylococcal protein A, Sheep IgG, Toxoid

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INTRODUCTION

Proteins of animal origin are essential components required to improve the nutritional status of the Indonesian population. Chicken meat and eggs represent a primary source of these proteins (Khusun *et al*., 2022). As such, a sufficient supply of high-quality chicken meat and eggs is required to meet the nutritional requirements of the population (Wójcik *et al*., 2022). Quality chicken meat and eggs must be produced from healthy poultry; hence, maintenance management, including health management and disease control, is required (Wilde *et*

al., 2019). In poultry production, several digestive diseases commonly occur, and antibiotic growth promoters (AGPs) have traditionally been used as a preventive measure. The prohibition on the use of antibiotics as feed additives, such as AGPs, since the end of 2018, has led to an increase in the incidence of infectious diseases of the chicken digestive tract, including necrotic enteritis (NE) (M'Sadeq *et al*., 2015; Zhu *et al*., 2021).

Necrotic enteritis is an infectious disease of the digestive tract that can present as either acute, subacute, or subclinical (Akerele *et al*., 2022). This disease is caused by the alpha (α) toxins produced by *Clostridium perfringens* type A bacteria, and commonly occurs in both layer and broiler chickens (Lee and Lillehoj, 2021). Although NE is caused by bacteria, it is difficult to treat with antibiotics because it is not the bacteria themselves that directly cause the disease but rather the toxin they produce (Prescott *et al*., 2016).

Previous studies have demonstrated that chicken populations injected with toxoid vaccines comprising the inactivated α toxin were protected from NE throughout their lifetime, while antibodies could be passed down to their day-old chicks (DOCs) (Crouch *et al*., 2010). By providing protection through vaccination, the dependence on AGPs to control these bacterial challenges can be significantly reduced. Conventional NE vaccines commonly used oil-based adjuvants to enhance antigen stability and prolong immune stimulation. However, oil emulsions may induce local tissue irritation and require complex emulsification steps (Leanpolchareanchai and Teeranachaideekul, 2023). To address these limitations, this study explored an alternative adjuvant system based on *Staphylococcus aureus* Protein A (SpA) and immunoglobulin G (IgG), designed to improve antigen uptake via Fc receptor-mediated internalization by antigen-presenting cells (APCs).

The SpA–IgG complexes were hypothesized to form stable immune complexes recognizable by Fcγ receptors on macrophages and dendritic cells, thereby enhancing antigen presentation and T-cell activation. The SpA–IgG complexes have been shown to facilitate macrophage phagocytosis through Fc receptor interactions (Qtaishat *et al*., 2013), while SpA has demonstrated feasibility as a vaccine carrier that promotes antigen-antibody complex formation (Shi *et al*., 2021). Supporting evidence from our previous *in vitro* study confirmed the stability and specificity ot the SpA-IgG complex in binding *C. perfringens* α-toxin (Kurnia *et al*., 2024), providing a biochemical basis for its development as an antigen-delivery matrix. However, its cellular uptake through phagocytosis has not yet been evaluated, and future studies should confirm this mechanism experimentally.

The NE cases are still prevalent in Indonesia, and the types of NE vaccines available are very limited; hence, there is a need for studies to develop effective vaccines. The aim of this study was therefore to develop a prototype Necrotic Enteritis matrix vaccine using SpA and sheep IgG complex, and to assess its potency by measuring the expression of CD4 and Major Histocompatibility Complex (MHC) II genes, as well as the antibody responses formed in layer chickens.

MATERIALS AND METHODS

Analysis of clostridium perfringens alpha toxin antigen

The antigen used in this study was the alpha toxin of *C. perfringens* derived from bacterial isolate archives, which were isolated from West Java, were characterized for toxinotyping based on previous studies (Kurnia *et al*., 2022). Alpha toxin was produced by growing *C. perfringens* in trypticase-glucose-yeast extract (20 g of trypticase, 30 g of yeast extract, and 0.5 g of cysteine hydrochloride per liter, pH 7.2) (TGY) under anaerobic conditions, at 37 °C overnight. The toxin was concentrated using ammonium sulfate precipitation (70% saturation), centrifuged at 10,000 rpm for 10 min, and dialyzed against Tris-HCl (10 mM, pH 7.5). The protein concentrations were determined using the Bradford method (Kielkopf *et al*., 2020). Subsequently, Ion Exchange Chromatography using Q Sepharose™ Fast Flow ion exchange resin (Cytiva, US) was performed by stepwise NaCl elution ranging from 0.1 to 1 M. The purified fraction showing the highest hemolytic activity, and appropriate molecular weight protein was considered to contain the active toxin and was subsequently used as the antigen. Hemolysis tests and protein concentration measurements were subsequently conducted to determine the amount of phospholipase C enzyme or alpha toxins. Hemolytic activity was quantified by incubating sheep erythrocytes (6×10^{11} cells/ml) with purified toxin in TBS at 37 °C for 30 min. Unlysed cells were removed by centrifugation (1,100 × g, 3 min), and hemolysis was quantified spectrophotometrically at A_{550} by measuring released hemoglobin. Untreated erythrocytes served as the negative control (0% lysis), while Triton X-100–treated cells served as the positive control (100% lysis). The alpha toxin antigen was inactivated by adding 0.6% formaldehyde (Kurnia *et al*., 2024). Confirmation of inactivated toxin (toxoid) based on safety tests performed in mice by intraperitoneal (i.p.) route and toxoid were then stored at -20 °C until further research.

Production of anti-clostridium perfringens toxin immunoglobulin (Ig) G in sheep

Three 8-month-old male sheep weighing 50-65 kg were used for hyperimmunity induction. Hyperimmune

conditions were achieved by administering two doses of *C. perfringens* toxoid vaccine with aluminum-precipitated adjuvant (APT) via the subcutaneous route, at a dose of 2 ml, with a one-month interval. This was followed by four subcutaneous injections of toxoid alone with a three-day interval subcutaneously with escalating volumes of 3, 5, 7.5, and 10 ml. Hyperimmune serum was collected two days after the last immunization. Blood (100 mL) was collected from the jugular vein (Kurnia *et al*., 2024).

Sheep-specific Immunoglobulin G against *C. perfringens* toxin was purified by the addition of rivanol (2-ethyoxy-6,9-diaminoacridine lactate), followed by ammonium sulfate precipitation (Vargas *et al*., 2012). The concentration and purity of IgG were determined by spectrophotometer UV-Vis Spectrophotometry Genesys, Thermo Scientific at a wavelength of 260 and 280 using the formula (mg/ml) = 1.55 × A280 – 0.76 × A260 for measure the concentration of IgG and =260/280 for measure the purity of IgG. The strength of sheep-specific IgG against *C. perfringens* toxin was evaluated using the AGP test. Agarose 0.1% was prepared, poured onto glass slides (5 ml), and allowed to solidify. Subsequently, the agar was punctured with a gel puncher to produce one well in the center and six wells around it. Twenty-five µl of toxoid antigen was then added to the center well, while 25 µl of serum was added to each of the six wells around it, prior to incubation at 25°C for 18-48 h. Positive results were indicated by the formation of a precipitation line between antigen and antibody (serum) wells.

Production of *Staphylococcus aureus* strain cowan I and SpA detection.

Staphylococcus aureus strain Cowan I was cultured in blood agar media, while SpA detection was performed using serum soft agar (SSA) media. SSA media was prepared by mixing 9 ml of Brain-heart Infusion Broth (BHIB), 1 ml of liquid agar base, and 50 µl of rabbit serum to obtain a concentration of 10%. *S. aureus* inoculates were then incubated at 37^{0}C and observed after 18 h. If the *S. aureus* strain contains protein A, bacterial colonies on SSA media form compact shapes, whereas bacterial colonies that do not contain protein A have a diffuse appearance. *Staphylococcus* were grown in blood agar at 37 ˚C for 24 h. Bacterial cells were collected by centrifugation (10000 × g for 20 min) and washed with PBS (pH 7.4). The cells were inactivated using 0.5% formalin (Kurnia *et al*., 2024). Stabilized SpA was then prepared to contain bacterial cells at a concentration of approximately 10^9 cell/ml.

Production of necrotic enteritis matrix vaccine prototype

The matrix vaccine prototype was prepared by mixing three components to form a matrix complex: SpA, sheep IgG specific to *Clostridium perfringens* toxin (2.1 mg/ml), and the toxoid. SpA was labelled with monospecific sheep IgG anti-alpha-toxin with the same of volume (ratio 1:1, v/v) by incubation in suspension for 3 h at 37°C. The suspension was then centrifuged at 5000× g for 5 min and washed twice with 0.02 M phosphate (pH 7.3)-buffered 0.85% saline (PBS; pH 7.3). The cells were resuspended in PBS to a final volume of 1 ml. The antibody conjugated staphylococci were then stored at 4˚C. The complex matrix was prepared using a 30% toxoid and 70% stabilized SpA-sheep IgG suspension (v/v), which was then incubated at 25˚C for 3 h, followed by centrifugation at a speed of 10,000 g for 20 min. Subsequently, the supernatant was discarded, and the pellet was resuspended in PBS to a concentration equivalent with Mac Farland 0.5, after which it was stored at 4˚C until ready for use.

Efficacy testing of necrotic enteritis matrix vaccine prototype on layers

Thirty commercial layers aged 14 weeks were divided into three groups of 10 layers per group: The non-vaccinated group (KN), the Necrotic Enteritis matrix vaccine group (MV), and the oil-based commercial Necrotic Enteritis vaccine group (KV). Chickens in the KN group were intramuscularly injected with phosphate-buffered saline (PBS) solution at 14 weeks via the intramuscular (IM) route at a dose of 0.3 ml per chicken. Chickens in the MV group were injected with the NE matrix vaccine prototype at the age of 14 weeks via the IM route at a dose of 0.3 ml per chicken. Chickens in the KV group were injected with the with the commercial vaccine at the age of 14 weeks via the IM route at a dose of 0.3 ml per chicken. Both vaccine formulations (MV and KV) contained equivalent antigen potency standardized to 9 Minimal Lethal Doses (MLD) per 0.3 ml dose, ensuring comparable antigenic input across treatments and valid evaluation of adjuvant effects. Boosters in the MV and KV groups were administered four weeks after the first immunization using the same route and dose.

Blood sampling for the analysis of CD4 and MHC II gene expression was conducted 12 and 24 h after the first vaccination. Blood sampling for antibody detection was performed four weeks after the first and four weeks after the second vaccinations. Blood samples were collected prior to vaccination.

Expression of CD4 and MHC II encoding genes

The expression of CD4- and MHC-II-encoding genes was determined in blood samples collected at 12 and 24 h following the first immunization. Genomic RNA was extracted using the Total RNA Mini Kit® (Blood/Cultured Cell) (Geneaid), in accordance with the manufacturer's protocol. cDNA was synthesized using the ReverTraAce

cDNA Synthesis Kit (Toyobo). Amplification was carried out using quantitative (q)PCR with RT-qPCR performed using the SensiFAST™ SYBR Lo-ROX Kit® (Bioline) and specific primers (Table 1) (Kurnia *et al*., 2022).

The housekeeping gene β-actin was used as the endogenous control to normalize target gene expression across samples. The stability of β-actin expression was verified by comparing Ct variation among treatment groups (mean Ct difference < 1.0), confirming its suitability as a reference gene under the present experimental conditions.

Gene expression analysis was conducted based on the relative comparison of the cycle threshold (Ct) values of the target sample to those of the calibrator/control sample. ΔCt (sample) = Ct target (sample) – Ct endogenous control (sample) ΔCt (calibrator) = Ct target (calibrator) – Ct endogenous control (calibrator) ΔΔCt = ΔCt (sample) – ΔCt (calibrator). The Relative Expression Level (RQ) represents how many times the expression of the target gene in the sample compares to the expression of the target gene in the calibrator; RQ = $2^{-\Delta\Delta Ct}$. The expression results indicated the number of times gene expression increased or decreased compared to the control (Nugroho *et al*., 2022; Poetri *et al*., 2023).

Detection of antibodies against clostridium perfringens toxin using indirect enzyme-linked-immunosorbent-assay (ELISA)

The ELISA method for detecting antibodies against *Clostridium perfringens* toxin following vaccination was based on a previous publication (Natalia, 2014). The optical density (OD) values were determined at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (THERMO multiscan EX). Analysis was performed by transforming the OD values into S/P ratios using the formula = (sample OD – negative control OD) / (positive control OD – negative control OD). The cutoff point for ELISA was determined based on the S/P ratio values of serum samples from the negative control (non-vaccinated) using the following formula: mean + 3 standard deviations. In this study, the cutoff S/P ratio was set at 0.52. Therefore, serum samples are considered positive for antibodies if the S/P ratio value was ≥ 0.52.

Data analysis

Quantitative data were analyzed using analysis of variance (ANOVA), followed by Tukey's post-hoc test if the results showed significant differences (P <0.05), using GraphPad Prism 9.

RESULTS

The purification of clostridium perfringens toxin antigen

We validated that protein purification using Ion Exchange Chromatography is effective for producing pure toxins from *Clostridium perfringens* type A bacteria. The 0.2 mol NaCl fraction was proven to release toxin protein with the highest purity compared to other fractions, with the highest hemolytic activity reaching 85.4%, and a total protein content of 25.8 mg in a 30 ml fraction solution (Table 2).

Table 1: Primers used for gene expression analysis of genes encoding CD4 and MHC II.

Target genes	Primers	Sequences (5`-3`)	References
CD4	CD4-F	TGTGGAACTGTCACCTCGTG	(Kurnia *et al*., 2022)
	CD4-R	CACATGCATGCAAGGCCAAT	
MHCII	MHCII-F	GCTGTACTTCAGCTCGGGAC	
	MHCII-R	ATGTCCTTGTTGACGTGGCT	
Beta actin	B. aktin-F	GAGAAATTGTGCATGACATCA	
	B. aktin-R	CCTGATACCTCTCAATGCCA	

Table 2: Results of *Clostridium perfringens* toxin purification by ion exchange chromatography.

Anion exchange chromatography fraction	Volume (mL)	Total proteins (mg)	Hemolytic activity* (%)	Specific activity (Hemolytic Units/mg protein)
Pre (Before)	100	417	-	-
Fraction 0	90	116.1	-	-
Fraction 1 (0.1 M NaCl)	30	21	42.33	0.605
Fraction 2 (0.2 M NaCl)	30	25.8	85.40	0.993
Fraction 3 (0.4 M NaCl)	30	69	12.40	0.054
Fraction 4 (0.6 M NaCl)	30	42	2.20	0.016
Fraction 5 (0.8 M NaCl)	30	24	3.95	0.049
Fraction 6 (1 M NaCl)	30	31.2	2.66	0.026

*Hemolytic activity (%) was calculated as the ratio between the optical density (OD_{550}) of the sample and that of the positive control (Triton X-100–treated erythrocytes), representing 100% hemolysis.

Antibodies from sheep against Clostridium perfringens Toxin

In this study, antibodies against the pure toxin of *Clostridium perfringens* were successfully generated from three hyperimmunized sheep, as evidenced by the formation of precipitin lines in the agar gel precipitation test (AGPT) (Figure 1A). The same results were demonstrated in AGPT testing against IgG purified from antibodies against *Clostridium perfringens* bacterial toxins. The pure IgG results showed a thick single precipitate line (Figure 1B). These findings revealed a strong reaction between the toxin antigen and IgG antibodies.

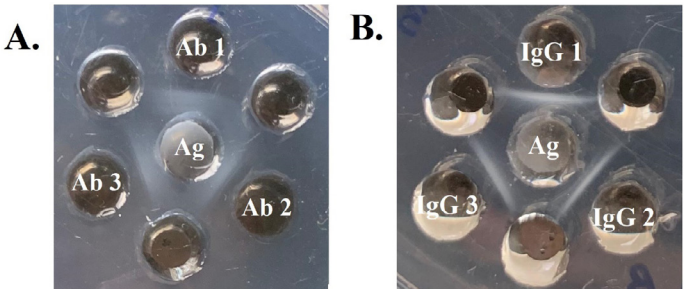

Figure 1: Testing results of antibodies and IgG against *Clostridium perfringens* using the AGPT method. (A) antibodies (serum); (B) purified IgG. Ab1 Ab2 Ab3 IgG1 IgG2 IgG3 Ag.

IgG concentrations were calculated as shown in Table 3. Based on the test results, the highest IgG concentration was obtained from the purification of sheep 2 serum, followed by sheep 3, and sheep 1 (Table 3). This correlates with the AGPT IgG test results, where the clearest line was observed for IgG from Sheep 2, followed by sheep 3, and sheep 1. However, a different result was observed regarding the purity of IgG, where the best purity ratio was obtained from sheep 1, followed by sheep 2, and sheep 3. Good purity IgG approached 0.580.

Table 3: Results of IgG concentration calculation in serum, and purification results of sheep-origin IgG conducted during hyperimmunization.

Sheep code	OD_{260}/ OD_{280}	IgG Concentration (mg/mL) with dilution factor 1:5	Purity ratio
Sheep 1	1.081/1.823	10.02	0.593
Sheep 2	2.194/3.212	16.56	0.683
Sheep 3	2.630/3.290	15.50	0.791

Characteristics of *Staphylococcus aureus* strain cowan I

The presence of SpA was evidenced by the formation of compact colonies on SSA medium containing rabbit serum (Figure 2A). For comparison, *Staphylococcus* bacteria lacking protein A were used as controls. Colonies of these control bacteria showed diffuse growth on SSA medium containing rabbit serum (Figure 2B).

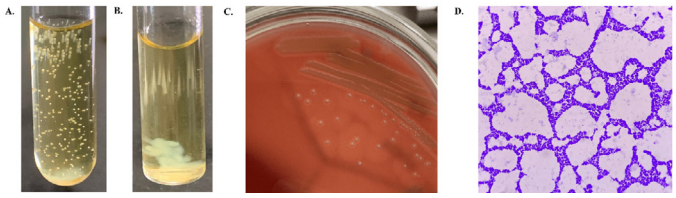

Figure 2: Identification of the study isolate *Staphylococcus aureus* strain Cowan I. (A) Compact colonies of S. *aureus* Cowan I on SSA; (B) Diffuse colonies of Protein A-negative control; (C) Growth on Blood Agar; (D) Microscopic view (Gram-staining).

The proliferation of *Staphylococcus aureus* strain Cowan I, which grew in a compact form on SSA media in this study, was further confirmed macroscopically on BA media (Figure 2C), as well as microscopically. Microscopic observations confirmed that the growth on the medium was that of *Staphylococcus* (Figure 2D). In the bacterial turbidity test, the grown bacteria yielded a value equivalent to that of the McFarland 5.

Prototype of NE matrix vaccine

The visual differences between the prototype of the NE matrix vaccine and the oil-based commercial NE vaccine are presented in Figure 3. The NE matrix vaccine exhibited large granular and clumpy particles (Figure 3A) compared with the fine particles of the commercial NE vaccine (Figure 3B). The visualization of the NE matrix vaccine, which is a complex of IgG, SpA, and *C. perfringens* toxins, is illustrated in Figure 3C.

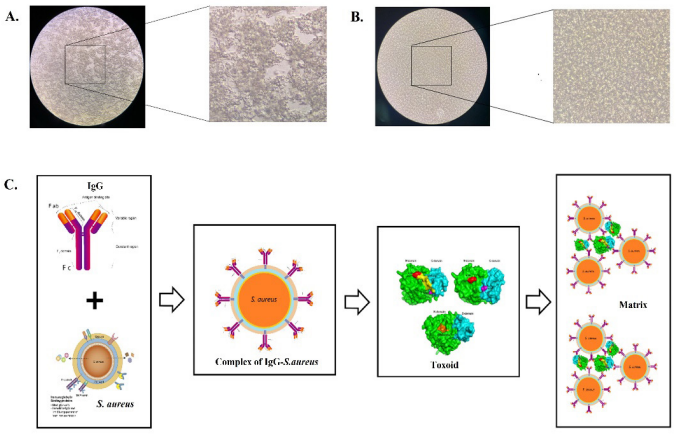

Figure 3: Microscope (magnification 100x) evaluation of vaccines resulting from the mixing of active ingredients with two different types of adjuvant bases: (A) Necrotic Enteritis matrix vaccine prototype; (B) commercial NE vaccine; (C) illustration of SpA, IgG, and the toxin binding complex.

Expression of CD4 and MHCII encoding genes

The expression of CD4- and MHC-II-encoding genes is presented in Figure 4 and Table 4. At 12 h post-vaccination, the expression of the CD4 encoding gene in KN was

higher than that in MV and KV. The CD4 encoding gene expression increased from 12 h to 24 h in both MV and KV, but not in KN. After 24 h, the highest CD4 expression was observed in the MV group.

Table 4: Fold change in the expression of CD4- and MHC II-encoding genes after vaccination.

The encoding genes	Groups	Fold change (mean ± SD)*	
		12 h	24 h
CD4	KN	1.08 ± 0.08[a]	1.08 ± 0.06[a]
	MV	0.10 ± 0.07[b]	3.21 ± 0.56[b]
	KV	0.24 ± 0.10[bc]	1.83 ± 0.33[c]
MHC II	KN	1.07 ± 0.07[a]	1.09 ± 0.16[a]
	MV	0.94 ± 0.29[a]	5.12 ± 0.42[b]
	KV	3.47 ± 0.36[b]	1.48 ± 0.33[ac]

KN, non-vaccinated group; MV = Necrotic Enteritis matrix vaccine group; KV, commercial Necrotic Enteritis vaccine. *Different superscript letters (a, b, c) indicate significant differences (P <0.05) in each group within a time point.

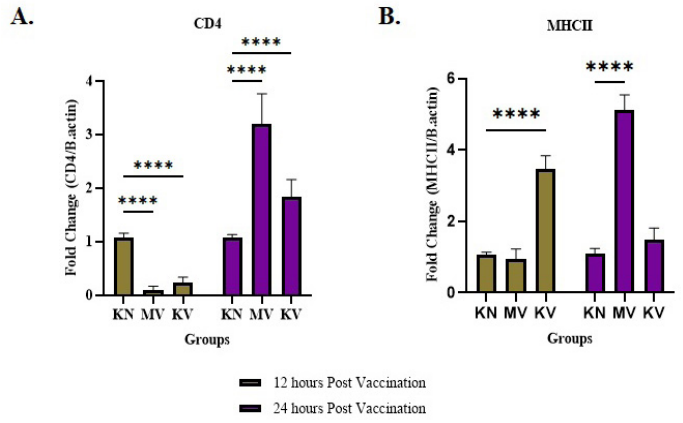

Figure 4: Observation of (A) CD4 and (B) MHCII (B) gene expression after vaccination. Data are presented as mean ± SD. * indicates a significant difference (P < 0.05) between groups at each time point (12 h or 24 h post-vaccination). KN = non-vaccinated group; MV = Necrotic Enteritis matrix vaccine group; KV = commercial Necrotic Enteritis vaccine.

At 12 h post-vaccination, the highest expression of the MHC II-encoding gene was found in the KV group. The expression patterns of MHC II-encoding genes differed between MV and KV. In MV, gene expression increased from 12 to 24 h, whereas the opposite occurred in KV, and no increase in expression was observed in KN.

Statistically, MV was significantly higher than KV for CD4 and MHC II at 24 h (P < 0.05), whereas KV exceeded MV for MHC II at 12 h (P < 0.05) but not for CD4 (P > 0.05). These results indicate that MV induces a delayed but stronger activation of CD4 and MHC II pathways compared to KV.

Evaluation of matrix vaccine

The presence of antibodies resulting from NE vaccination was determined using indirect ELISA with a positive antibody S/P ratio cutoff of 0.52. Pre-vaccination samples were negative across all groups. The S/P ratios gradually increased after the first and second vaccinations in the MV and KV groups (Figure 5A and Table 5). After two vaccinations, all chickens in the KV group (100%) and most chickens in the MV group (88.88%) developed detectable antibody titers (Figure 5B), representing the proportion exceeding the positive threshold rather than a reduction in sample number; all 10 chickens per group were included in the analysis. Statistical analysis (P < 0.05) confirmed that KV induced significantly higher antibody responses than MV at both post-vaccination time points. The observed decrease in MHC II expression in KV from 12 to 24 h likely reflects a transient response associated with rapid antigen delivery and mild inflammatory effects of the oil-based adjuvant, rather than a sustained antigen-processing event.

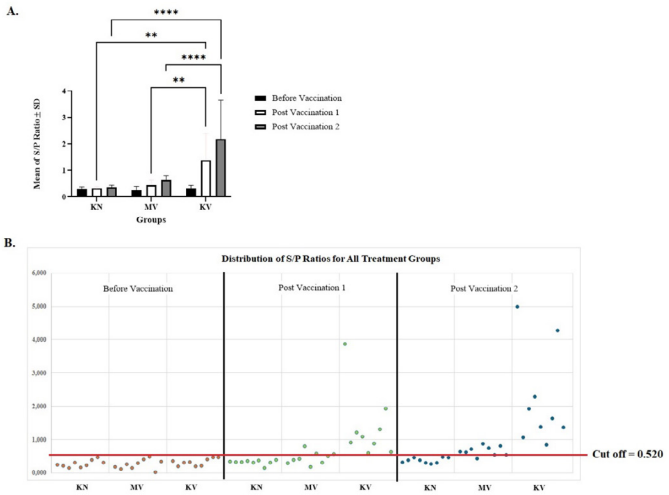

Figure 5: ELISA results showing antibody response in the form of the S/P ratio. (A) Mean S/P ratio of antibodies against *Clostridium perfringens* toxin; (B) Distribution and percentage of the S/P ratio of the antibody response to vaccination. Data are presented as mean ± SD. * indicates a significant difference (P < 0.05) between groups at each time point (12 h or 24 h post-vaccination). KN = non-vaccinated group; MV = Necrotic Enteritis matrix vaccine group; KV = commercial Necrotic Enteritis vaccine.

DISCUSSION

In this study, we developed and evaluated a necrotic enteritis (NE) matrix vaccine prototype composed of *Staphylococcus aureus* protein A (SpA), sheep-derived IgG, and *Clostridium perfringens* toxoid. The matrix vaccine concept was intended to explore an alternative adjuvant platform to enhance antigen presentation through Fc-binding interactions, potentially reducing reliance on conventional

oil-based adjuvants. Microscopic visualization of the NE-matrix vaccine revealed clumpy, granular particles that appeared coarser than the fine particles of the commercial NE vaccine, which consists of an inactivated oil-in-water emulsion vaccine. These morphological differences likely reflect variations in composition and may influence biodistribution and local immune activation at the injection site, potentially contributing to the slower antibody response observed in the matrix vaccine (MV) group.

Table 5: Mean S/P ratio values ascertained from ELISA test results for Necrotic Enteritis antibody detection.

Time of sampling	Age of chicken (weeks)	S/P ratio of treatment groups (mean ± SD)*		
		KN	M	KV
Before vaccination	14	0.28±0.10[a]	0.26±0.15[a]	0.33±0.11[a]
Post vaccination 1	18	0.33±0.07[a]	0.46± 0.19[a]	1.39±0.08[a]
Post vaccination 2	22	0.37±0.08[a]	0.65±0.08[b]	2.20±1.46[ab]

KN, non-vaccinated group; MV = Necrotic Enteritis matrix vaccine group; KV, commercial Necrotic Enteritis vaccine. *Different superscript letters (a, b) indicate significant differences (P <0.05) in each group (KN, M or KV).

Following NE vaccination, the immune response of chickens was observed using several parameters, including the expression of CD4-and Major Histocompatibility Complex (MHC) II-encoding genes and the presence of specific antibodies against *C. perfringens* toxin. At 12 h post-vaccination, the highest expression of CD4 encoding genes in the blood was observed in the unvaccinated group (KN). This could be because most of the CD4 encoding genes are already used in the protein formation process shortly after the vaccine is injected into the body. However, 24 h post-vaccination, the expression of CD4 encoding genes increased in the MV and KV groups, but not in the KN group. The increase in CD4 gene expression in the MV group was 3.21±0.56-fold higher than that in the KN group, whereas in the KV group, it was 1.83±0.33-fold higher compared to the KN group. This pattern aligns with previous reports describing early activation of T-cell subsets following antigen stimulation (Olteanu *et al*., 2012).

The higher CD4 expression in the MV group may suggest enhanced antigen presentation by antigen-presenting cells (APCs), possibly driven by a higher antigen load. An increased antigen load could stimulate greater CD4 protein synthesis to facilitate the coordination of adaptive immune responses (Koblischke *et al*., 2017). In the KN group, there was no increase in CD4 encoding gene expression, likely due to the absence of immune stimulation. CD4+ T cells play a central role in orchestrating adaptive immunity by activating activate innate immune cells, B lymphocytes, and cytotoxic T cells (Luckheeram *et al*., 2012). Nonetheless, increased CD4 expression alone does not always equate to functional T-cell proliferation or protective immunity. Previous reports has shown that transcriptional upregulation of CD4 can occur independently without corresponding functional activation (Li *et al*., 2023), and T-cell proliferation is not always directly coupled to differentiation (Laouar and Crispe, 2000). Because antigen uptake by APCs is a key determinant of adaptive immunity, the observed molecular responses suggest that the SpA–IgG complex effectively engaged early antigen-processing pathways. This interpretation is consistent with previous findings that antigen/antibody ratios influence macrophage presentation and antibody output (Manca *et al*., 1991), while dendritic cell uptake correlates with T-cell proliferation and antibody responses (Zhu *et al*., 2023). Nonetheless, additional functional assays such as T-cell proliferation or cytokine release studies will be essential to confirm this hypothesis.

Temporal differences in MHC II-encoding gene expression between two vaccine groups further underscore distinct antigen delivery dynamics. At 12 h post-vaccination, MHC II expression was highest in the KV group, likely reflecting the oil-in-water adjuvant's depot effect, which facilitates rapid antigen delivery to lysosomes and promotes early peptide loading. In contrast, MHC II expression in the MV group increased only at 24 h post-vaccination, suggesting slower peptide binding and presentation, which may have contributed to the delayed humoral response observed. These findings reinforce the importance of antigen presentation kinetics for subsequent activation of CD4+ T cells and initiation of antibody production (Shrestha *et al*., 2018).

Unlike natural infections, vaccines administered parenterally via intramuscular routes do not pass through the early response mediated by the mucosal system; thus, adaptive immunity due to vaccination occurs more quickly (Kang and Compans, 2009). Adaptive immune responses begin approximately 24 h after the vaccine enters the body, which is consistent with the results of this study. CD4 and MHC II upregulation in the MV- and KV-vaccinated groups were detectable at 12 h post-vaccination, indicating early molecular activation preceding measurable antibody production, aligning with established immunological timelines.

The proposed mechanism of SpA-mediated enhancement of phagocytosis and antigen presentation is supported by prior studies. The Fc domain of IgG3 efficiently induces phagocytosis of *S. aureus* expressing SpA compared to IgG1 (Boero *et al*., 2022), whereas SpA's non-specific binding may inhibit phagocytosis and B-cell clonal expansion (Tsai *et al*., 2022). Immunization with SpA

mutants improves anti-SpA antibody quality and enhance phagocytic activity (Mandelli *et al*., 2024). These reports validate the rationale for employing SpA–IgG as a matrix framework but also highlight the need for experimental confirmation of the proposed cellular mechanism. Although this study did not include in vitro phagocytosis assays using chicken macrophages, such investigations will be necessary to elucidate the mechanistic basis of immune activation. Moreover, β-actin was employed as the qPCR reference gene, but its stability under the experimental conditions (MV, KV, and KN groups at 12 h and 24 h post-vaccination) was not assessed. Future work should therefore include reference gene stability assessments to ensure the accuracy of relative expression analyses.

The gene expression results confirmed immunogenic activation, but the antibody response elicited by the prototype vaccine was notably weaker than that induced by the commercial oil-based vaccine (KV). The presence of specific NE antibodies after vaccination was determined by ELISA, with a cutoff value for positive S/P ratio being ≥0.52. In both MV- and KV-vaccinated groups, the mean S/P ratios at week 8 after the first vaccination were higher than those at week 4. This was because there was a booster at week 4, resulting in a secondary immune response that caused an increase in the S/P ratio values at week 8. Higher S/P ratios at week 8 than at week 4 indicated an increase in antibodies. Based on the distribution of positive S/P ratio values, the percentage of chickens with specific NE antibodies reached 88.88% at week 8 in the MV group, and 100% in the KV group. This indicates the need for at least one NE booster vaccine to achieve optimal vaccination coverage. Vaccination boosters aim to increase titers to achieve protective immunity and suppress NE infections (Hesse *et al*., 2018).

The relatively lower humoral response observed in the MV group may be explained by differences in vaccine composition and antigen delivery mechanisms. The commercial oil-in-water emulsion NE vaccine (KV) forms a depot at the injection site, allowing sustained antigen release and efficient B cells stimulation. This depot effect likely facilitates more rapid antigen delivery to APCs, promoting earlier MHC II expression and subsequent antibody production (Yang *et al*., 2005; O'Hagan *et al*., 2021). In contrast, the matrix formulation lacks a depot effect and instead relies on the SpA–IgG complex to facilitate recognition by APCs. This mode of delivery may delay the initiation of the antibody response, as indicated by the lower S/P ratio values after the first and second vaccinations. Despite this delay, the matrix vaccine elicited a measurable booster effect, indicating the induction of immunological memory and the ability to induce a secondary adaptive response. It is important to note that no direct quantification of antigen particles presented by APCs *in vivo* was performed, limiting definitive conclusions regarding adjuvant efficacy versus antigen load.

Although a toxoid-only formulation was not included, the antigen dose was standardized across formulations (9 MLD/0.3 ml), ensuring valid comparison of adjuvant performance. Future investigations should therefore incorporate such a control to delineate adjuvant-specific contributions. Although the agar gel precipitation test (AGPT) verified the specificity of IgG against *C. perfringens* toxin, potential IgG aggregation or altered Fc-binding characteristics were not examined. These parameters should be evaluated in subsequent studies to better define the physicochemical and immunological properties of the matrix.

The matrix vaccine's weaker humoral response may also reflect inherent limitations in directly activating B cells or promoting germinal center (GC) formation. SpA–IgG–based matrix vaccines can influence B cell activation, as SpA binds VH3-type BCRs, triggering proliferation and antibody secretion (Shi *et al*., 2021). However, Fc-mediated interactions of SpA can also bind immunoglobulins non-specifically, potentially reducing specific antibody responses and limiting effective B cell clonal expansion (Tsai *et al*., 2022). Since GC formation is critical for affinity maturation and development of long-lived plasma cells (Janeway *et al*., 2001; Ahmadivand *et al*., 2025), this limitation likely contributed to the lower S/P ratios observed. Optimization of antigen-to-IgG ratios and incorporation of components that enhance GC responses may be necessary to achieve robust protective antibody production.

Despite these limitations, the present study establishes a clear proof-of-concept for SpA–IgG matrix as an adjuvant platform, demonstrating its capacity to initiate immune activation at the gene expression level (Shi *et al*., 2021; Tornaletti *et al*., 2025). Although the commercial oil-based vaccine (KV) elicited a stronger and faster antibody response, the molecular evidence obtained here underscores that the matrix formulation effectively triggers early immune signaling, thereby validating its immunostimulatory potential. These findings highlight both the feasibility and the constraints of the current design, serving as a foundation for further refinement. Future optimization should focus on adjusting the antigen-to-IgG ratio, incorporating complementary carrier systems, and verifying the proposed phagocytic mechanism to improve antibody production and overall vaccine efficacy.

Finally, the use of inactivated *S. aureus* Cowan I strain as a source of SpA may raise concerns about confounding immune reactions. However, the bacterial cells were

completely inactivated using formalin, and no clinical or serological signs of non-specific responses were observed. Even so, we acknowledge that potential immune responses to *S. aureus* antigens could not be completely ruled out without specific serological assays. Future studies will therefore include anti-*S. aureus* antibodies screening and cytokine profiling to confirm the specificity of the immune response and safety of this novel matrix formulation.

CONCLUSION

The prototype necrotic enteritis (NE) matrix vaccine developed in this study was able to elicit an adaptive immune response in layer chickens, as indicated by transient upregulation of CD4 and MHC II gene expression and measurable antibody formation after booster administration. However, the humoral response induced by SpA–IgG–toxoid matrix was considerably weaker and delayed compared to the commercial oil-based vaccine. This limitation suggests that while the SpA–IgG–toxoid complex can enhance antigen presentation at the cellular level, its ability to induce robust and sustained antibody titers remains suboptimal. Therefore, the current formulation should be regarded as a preliminary proof of concept requiring substantial optimization, particulary in improving antigen delivery and adjuvant strategy to achieve protective antibody levels comparable to commercial standards. Future studies should specifically address these aspects by refining antigen release kinetics, confirming the phagocytic mechanism, and exploring combinations with complementary adjuvants to achieve an immune response comparable to current commercial standards.

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NOVELTY STATEMENT

This study is the first to develop a prototype necrotic enteritis (NE) vaccine in poultry using a novel matrix complex composed of *Staphylococcus aureus* protein A (SpA), sheep-derived IgG, and *Clostridium perfringens* toxoid. Unlike conventional oil-based vaccines, this matrix design facilitates antigen presentation through Fc-binding of SpA, thereby enhancing phagocytosis and immune activation. The combined strategy of toxin inactivation, generation of specific sheep IgG, and incorporation into an SpA-based matrix system has not been previously reported for NE prevention. This innovative approach provides new insights into alternative vaccine formulations that could reduce reliance on antibiotics and improve gut health management in poultry production.

AUTHOR'S CONTRIBUTION

RVA, IA, PON and KRS conceptualized the study, prepared the original draft, and visualized the study. RVA, IA, KRS, NCMH developed methodology. RVA, KRS, NCMH, PMA, SA performed formal analysis. KRS and NCMH investigated the data, reviewed and edited the manuscript, and performed data curation. IA, PON and SA supervised the study and administrated the project.

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethical statement

All experimental protocols were reviewed and approved by the Animal Ethics Commission of the Faculty of Veterinary Medicine and Biomedical Sciences at the IPB University (approval number 096/KEH/SKE/VIII/2023). The Hy-line Brown Max layer chickens used in this study were maintained under constant temperature, humidity, and availability of feed and water.

Generative AI and AI-assisted technology statement

The authors used generative AI solely to enhance the manuscript's readability and language and take full responsibility for its content.

Conflicts of interest

The authors have declared no conflict of interest.

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