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Research Article

Development and Evaluation of a Bivalent Oil-Adjuvanted Vaccine Against Necrotic Enteritis and Avian Colibacillosis in Poultry

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Abstract | Necrotic enteritis (NE) and avian colibacillosis are significant diseases affecting poultry, leading to economic losses due to reduced productivity and increased mortality. The present study investigated the stability, safety, and potency of combination *Clostridium perfringens* type A toxoid and Avian Pathogenic *Escherichia coli* (APEC) bacterins with oil adjuvants. Stability assessment of vaccine emulsion determined under artificially induced thermal conditions. Vaccine potency based on immune response in 12-week-old commercial layer chickens that were divided into four groups consisting of single vaccine NE or APEC, bivalent vaccine, and control unvaccinated. The prepared vaccine could withstand high thermal stress up to 37˚C for 24 days any adverse effects on the physicochemical properties of the emulsion. The vaccine showed significant physical changes in stability due to storage at 37˚C for more than 24 days, causing the separation of the oil and water phases. Bivalent NE-coli vaccine was found safe and produced antibody response against toxin alpha *C. perfringens* and APEC after 4 weeks of first vaccination measured by Enzyme-linked immunosorbent assay (ELISA). Immunogenicity studies showed that antibody responses to each antigen were significant higher between combination and single formulated vaccine. Combination vaccine induced optimum immune response against both antigens, in spite of the fact that single vaccine showed significantly higher than combination vaccine. However, this study illustrates that the bivalent vaccine formulation effectively stimulates immune responses, potentially providing protection against NE and colibacillosis in poultry. The efficiency of vaccine administration in the field may be higher compared to two single vaccine administrations.

Keywords | *Clostridium perfringens*, Necrotic enteritis, *E. coli*, Toxin, Vaccine

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INTRODUCTION

Necrotic enteritis (NE) and colibacillosis are major gastrointestinal and systemic disorders in poultry, primarily caused by toxigenic *Clostridium perfringens* and *Avian Pathogenic Escherichia coli* (APEC), respectively. These pathogens contribute significantly to economic losses in the poultry sector by impairing productivity and increasing bird mortality. In its acute form, NE can lead to mortality rates reaching as high as 50% in vulnerable flocks (Fancher *et al*., 2020; Lee and Lillehoj, 2021). On the other hand, colibacillosis exhibits a prevalence ranging from 9.52% to 36.73%, with adult laying hens particularly affected, showing rates up to 36.73%. Both diseases commonly present with clinical symptoms such as depression, diarrhea, and increased mortality. Acute colibacillosis cases are often marked by respiratory tract infections followed by septicaemia, leading to death (Kabir, 2010). These infections typically occur in broilers aged 2–6 weeks and layers aged 12–24 weeks, coinciding with the decline of maternal antibodies against bacterial infections (Kumar *et al*., 2019). The diseases frequently occur in poultry raised on litter, which as a source of infection (Malmarugan *et al*., 2012).

Clostridium perfringens is a gram-positive, anaerobic, spore-forming bacterium frequently isolated from the gastrointestinal tracts of poultry, as well as ruminants such as cattle, sheep, and goats (Miyakawa and Redondo, 2018). The organism produces a range of toxins and enzymes that serve as virulence factors contributing to its pathogenicity. Among the six primary toxins implicated in infections, alpha, beta, epsilon, iota, enterotoxin, and NetB—several are associated with disease in both humans and animals (Rood *et al*., 2018). Strains of *C. perfringens* responsible for necrotic enteritis were previously categorized as type A or C, based on their expression of beta toxin. However, recent findings have shown that NE-associated strains predominantly produce NetB toxin, and have thus been reclassified as type G (Johnston *et al*., 2021). Phospholipase C (plc), also known as alpha toxin, produced by *C. perfringens*, functions by breaking down the phospholipid components of host cell membranes. In contrast, NetB toxin contributes to pathogenesis through the formation of beta-barrel pores, leading to ionic imbalance and subsequent necrosis of the small intestinal mucosa (Timbermont *et al*., 2011).

Avian Pathogenic *Escherichia coli* (APEC) is the causative agent of colibacillosis, a systemic infection in broiler chickens, typically presenting with a characteristic triad of lesions: perihepatitis, pericarditis, and airsacculitis, which can progress to septicemia and result in early mortality. Various other factors, such as viral co-infections, toxins, immune response disorder, and nutritional deficiencies may exacerbate colibacillosis cases (Fancher *et al*., 2020). Moreover, *E. coli* acts as a reservoir for antimicrobial-resistant bacteria so that APEC prevention required various strategies (Christensen *et al*., 2021; Kurnia *et al*., 2018).

Prevention of NE and colibacillosis by vaccination is important, considering the risk of antimicrobial resistance in digestive tract of poultry product is increase and spread, which has led to a global public health concern. Bivalent vaccines have the advantages of rapid prevention of several infectious bacterial diseases, laborsaving approach, and low production cost. This study was conducted to formulate a combined inactivated vaccine consisting of *C. perfringens* type A toxoid and bacterins from *E. coli* serotype O1, emulsified with an oil-based adjuvant. The vaccine formulation was evaluated for its physicochemical stability and immunogenic potency, with antibody responses measured by ELISA to assess its potential protective efficacy against the target diseases.

MATERIALS AND METHODS

Ethic Declaration

All procedures involving animals in this study were conducted in accordance with ethical standards and were approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University, Indonesia, under approval certificate number 231/KEH/SKE/VII/2024.

Bacterial Isolates Seed

The *C. perfringens* type A strain utilized in this research was isolated from a clinical case of necrotic enteritis in poultry. The Avian Pathogenic *Escherichia coli* (APEC) strain was obtained from a confirmed case of colibacillosis and had been previously characterized through biochemical and molecular identification techniques. Bacterial isolate for seed vaccine was stored in freeze-dried in ampoule at -20 ˚C. In this study molecular identification was performed as quality control procedure for bacterial seed vaccine preparation. DNA was purified from bacterial seed colonies on Blood Agar by Geneaid Genomic DNA Mini Kit, following the manufacturer's instructions. The cpa/plc gene was detected to confirm *C. perfringens* type A isolate using PCR primers (F) 5′-GCTAATGTTACTGCCGTTGA-3′ and (R) 5′-CCTCTGATACATCGTGTAAG-3′ (Navarro *et al*., 2018; van Asten *et al*., 2009). While target uspA gene were used for detection *E. coli* (uspAF;5′CCGATACGCTGCCAATCAGT-3′ and uspAR;5′-ACGCAGACCGTAGGCCAGAT-3′). Serotyping of APEC was confirmed using conventional single PCR with four specific primer pairs, which had been previously designed to detect and distinguish between serotypes O1, O2, O18, and O78 (Wang *et al*., 2014).

Laboratory Animal

A total of 140 commercial laying hens of the Hy-Line breed were included in this study, with 20 hens allocated

for safety testing of the formulated vaccines (Figure 1). Both local injection site reactions and systemic responses were monitored, following the guidelines outlined in the WOAH (WOAH Terrestrial Manual, 2022). The hens, aged 12 weeks, were housed in battery cages with a wire mesh floor. They were initially confirmed to be free from *C. perfringens* type A and *E. coli* serotype O1 antibodies. The laying hens were maintained in experimental cages and had access to feed and water ad libitum for the duration of the vaccine evaluation.

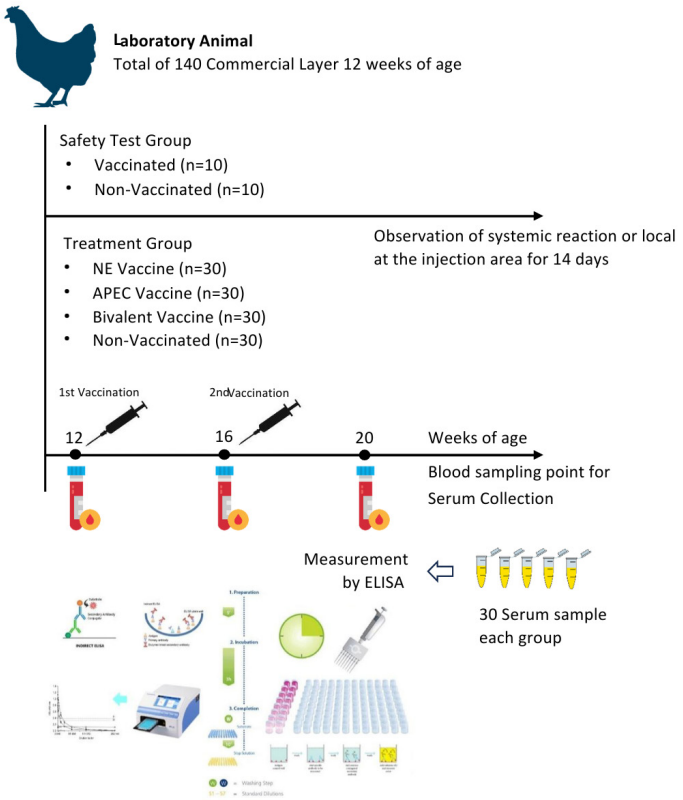

Figure 1: Flowchart schematic diagram of experimental design in laboratory animal for safety test and vaccination program.

Preparation of Formulation Vaccine

The process of producing the *C. perfringens* type A toxoid antigen starts with the formulation of a culture medium, which includes yeast extract, peptone, lactalbumin hydrolysate, and sodium chloride (NaCl). A sterile 50% (w/v) aqueous glucose solution is added to achieve a final concentration of 1.0% glucose in the medium. The freeze-dried working seed ampoule is rehydrated with the broth medium and cultured on blood agar plates under strict anaerobic conditions at 37°C for 22 hours. Colony growth is monitored through both macroscopic and microscopic examinations as part of quality control. A subculture is then transferred into 200 mL of the cultured medium and used as inoculum in 2 L fermenters for the subsequent stage of production. During fermentation, anaerobic conditions are maintained, and the pH is adjusted to 7.2-7.4 using 5N NaOH. Finally, the cultured medium is centrifuged at 7,000 × g for 20 minutes at 4°C to separate the bacterial cells from the supernatant. The supernatant containing the toxin was subsequently tested for potency in mice. The toxin was deemed suitable if its potency reached ≥100 minimum lethal doses (MLD)/mL or higher (Saadh *et al.*, 2022). Toxin potency based on biological activity was determined in mice (body weight: 18–22 g) that were intravenously injected with 0.1 mL of minimum diluted 1/10 toxin. Once toxin potency reached ≥100 MLD/mL, 0.6% formalin was added for inactivation (Diancourt *et al.*, 2019).

The freeze-dried working seed ampoule *E. coli* serotypes O1 was suspended in Brain Heart Infusion Broth (BHIB) medium and grown in blood agar then incubated at 37°C for 18 h. To obtain bacterins antigen, bacterial pure colonies were then sub cultured in 100 ml medium and used as inoculum in 1 L final medium incubated in shaking incubator for 6 hours at 37°C. Bacterial cell concentration was determined using spectrophotometry at a wavelength of 600 nm. The *E. coli* bacterial antigen was deemed suitable for vaccine formulation if the bacterial concentration was more than 10^9/ml. The inactivation process was carried out by adding 0.3% formalin, followed by sonication using ultrasonic waves for 20 minutes (Gu *et al.*, 2018).

A bivalent vaccine was formulated by combining *C. perfringens* type A toxoid with sonicated bacterins of *E. coli* serotype O1 in a 1:1 ratio, followed by mixing with an adjuvant. The emulsion was prepared using a rotor-stator homogenizer (Ultra-Turrax T 50 digital, IKA®-Werke, Germany), incorporating 30% total antigen and 70% oil-based adjuvant (ISA 71VG Montanide™, Seppic, France), in accordance with the manufacturer's recommended 30:70 (w:w) ratio (Jang *et al.*, 2013). The aqueous phase was gradually introduced during mixing at a maximum speed of 5000 rpm. The vaccine volume was standardized to 0.3 ml per dose for intramuscular administration. Prior to immunization, all vaccine formulations underwent sterility and safety evaluations following the British Pharmacopoeia and WOAH guidelines (British Pharmacopeia, 2022; WOAH Terrestrial Manual, 2022). Sterility assessment ensured the absence of contaminants, including aerobic and anaerobic bacteria, fungi, and mycoplasmas. Safety testing was conducted on 20 commercial laying hens, each receiving a double dose of the vaccine via intramuscular injection.

The stability of the emulsion was assessed using pH and viscosity under deliberately generated heat settings such as 4°C, 25°C, and 37°C. Measurement of emulsion viscosity by Brookfield DV-I Primer Viscometer with LV spindle no 2 at 60 rpm. Observation of stability was performed with period of sampling at 10, 20 and 30 days. Measurements were done in triplicate, the values reported were the average (Alade *et al.*, 2021; Sarmad *et al.*, 2019).

Determination of Vaccine Potency

A total of 120 chickens were randomly divided into four treatment groups, with each group comprising 30 birds. Group 1 was vaccinated with the necrotic enteritis (NE) vaccine, Group 2 received a vaccine targeting colibacillosis, Group 3 was given a combination NE-Coli vaccine, and Group 4 acted as the unvaccinated control, injected intramuscularly with 0.3 mL of sterile saline. Blood samples were taken at three time points: before the vaccination at 12 weeks of age, four weeks after the primary immunization (at 16 weeks of age), and four weeks after the booster dose (at 20 weeks of age). Sera were separated and stored at -20°C for future analysis. The efficacy of the vaccines was assessed by evaluating the antibody responses using an enzyme-linked immunosorbent assay (ELISA).

To evaluate the immune response, ELISA was conducted using two different antigen coatings. For *C. perfringens* type A toxoid, phospholipase C type XIV (Sigma) was employed as the coating antigen at a concentration of 1 µg/mL, following the method outlined by Crouch *et al.* (2010). The detection of antibodies against O1 Avian Pathogenic *E. coli* was performed using soluble protein from the supernatant of sonicated whole-cell bacteria, as described by Sadeyen *et al.* (2014). A known positive serum was obtained from chickens previously immunized at 10 weeks of age with the same antigen, filtered, and combined with an oil-based adjuvant. This serum was collected four weeks after the booster dose. A negative control serum was collected from birds before any immunization.

To measure antibody titers, ELISA plates (Nunc Maxisorp, 96-well, flat-bottom) were coated with antigen diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Afterward, unbound antigens were removed by washing three times with PBS containing 0.05% (v/v) Tween-20. Wells were then blocked using PBS supplemented with 0.2% casein and kept at 4°C overnight. After blocking, appropriately diluted serum samples were added to each well and incubated either at room temperature or 4°C. After gentle shaking, the wells were washed three times. Subsequently, each well received an optimized concentration of peroxidase-labeled anti-chicken IgG (Sigma). Following a 1-hour incubation at room temperature, the wells were washed once more, and the ABTS substrate was added to facilitate color development. After another 1-hour incubation at room temperature, the absorbance was recorded at 450 nm using a Biochrom EZ Read 2000 microplate reader. The optical density (OD) values were then transformed into sample-to-positive (S/P) ratios using the following equation:

$$\frac{S}{p} = \frac{OD\ sample - OD\ negative\ control}{OD\ positive\ control - OD\ negative\ control}$$

The resulting S/P ratios were employed for evaluating the immune responses. The ELISA threshold value (cut-off point) was determined by calculating the mean OD of known negative sera plus three standard deviations, as per the method described by Heier *et al.* (2001).

Data Analysis

Quantitative data were analyzed using GraphPad Prism software version 9.1.2 (GraphPad Software, San Diego, CA, USA). Data were presented as means, along with either the standard error of the mean (SEM) or the standard deviation (SD), obtained from repeated independent trials. To determine statistical differences among groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test for multiple comparisons. A p-value less than 0.05 was considered statistically significant.

RESULTS

Molecular Confirmation of Bacterial Seed

In this study, pure growth of colony was confirmed by PCR for each gene target marker. Molecular confirmation of avian pathogenic *E. coli* bacterial seed shows amplification PCR product of approximately 884 bp for uspA gene and 263 bp based on serotype specific O1 (Figure 2A and 2B). DNA from *C. perfringens* type A strain produced an amplification product of approximately 324 bp and did not show other amplification product beta (β), and epsilon (cpε) compared with type C and type D strain (Figure 2C).

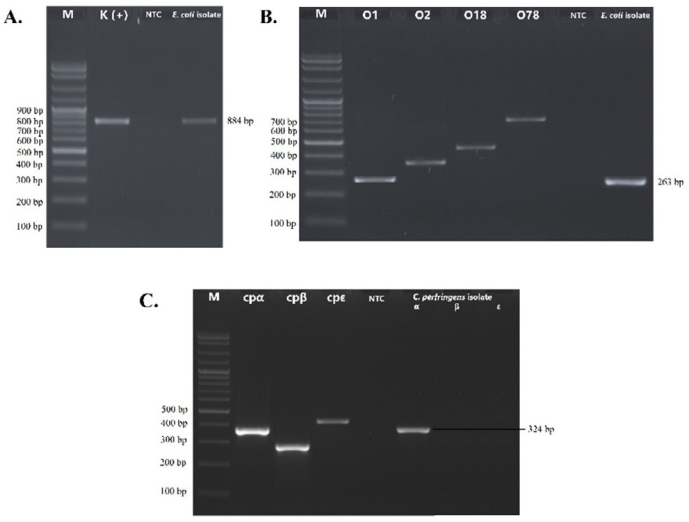

Figure 2: Polymerase chain reaction (PCR) results of amplification of bacterial avian pathogenic *E. coli* and toxin *Clostridium perfringens* genes. (A) Confirmation of *uspA* genes *E. coli* isolate; (B) Amplification PCR result on serotype avian pathogenic *E. coli*; (C) Amplification toxin genes encoding alpha (cpα), beta (cpβ), and epsilon (cpε) of *C. perfringens* isolate bacteria.

Vaccine Formulation and Stability

The antigen obtained from the production process yielded *C. perfringens* alpha toxin with a potency of 100 MLD/ml. This was determined based on the mortality of mice injected with 0.1 ml of harvested toxin diluted in PBS at a 1:9 ratio. The inactivation process using 0.6% formalin successfully inactivated toxin into a toxoid, as confirmed by safety testing in mice. The APEC antigen was successfully produced at a concentration suitable for vaccine formulation, as confirmed through spectrophotometric analysis. The final concentration reached $10^2 \times 10^9$ CFU/ml, determined by correlating the Total Plate Count (TPC) with the optical density (OD) readings at 600 nm, using a standard calibration curve. The spectrophotometer reading produced an OD value of 1.271A, which was applied to the correlation coefficient equation y = 73.537x + 8.8925 with R^2 value 0,985, and OD represents x.

The mixture of all prepared vaccine including NE-Coli vaccine, showed no bacterial contamination while tested for sterility in Thioglycolate (TGC) and Brain Heart Infusion (BHI) broth medium. Safety testing on chickens revealed no abnormalities appears, either systemic reaction or local at the injection area with observation for 14 days. The stability of the prepared vaccine was observed for it physicochemical changes that can be detected when stored at various temperature conditions with sampling every 10 days. Physicochemical changes occurred during storage based on viscosity from 43,5 to 37,5 mPa-s on day 30 at temperatures 25°C. Physicochemical changes also occurred at storage 37°C by pH from 6,8 to 6,5 and viscosity from 33,2 to 28,79 mPa-s on day 30. Bivalent NE-coli vaccine emulsion appeared stable at storage conditions at 4°C with no significant physicochemical changes occurring. This indicates a change in stability when stored at these temperatures as shown in Table 1.

Table 1: Effect of thermal conditions at different time period on the stability of prepared vaccine.

Temperature-stored	Parameter	Sampling (days)		
		10	20	30
37°C	pH	6,8	6,6	6,5
	Optical appearance[a]	-	25	25
	viscosity (mPa-s)	33,2±0,2	30,9±0,3	28,79±1,3
25°C	pH	6,8	6,6	6,6
	Optical appearance[a]	-	-	-
	viscosity (mPa-s)	43,5±2,2	38,3±1,56	37,5±0,6
4°C	pH	6,8	6,9	6,8
	Optical appearance[a]	-	-	-
	viscosity (mPa-s)	44,8±0,26	44,6±1,3	44,5±1,6

[a] Emulsion vaccine optical appearance of water and oil phase layer separation at day.

Evaluation of Antibody Response by ELISA

Humoral immune responses to *Clostridium perfringens* type A toxin and *Escherichia coli* serotype O1 were assessed using ELISA, as illustrated in Figure 3. Antibody levels against both antigens showed a marked increase by the fourth week following the initial vaccination and continued to rise, reaching their highest levels four weeks after the booster dose. Conversely, the control group (unvaccinated) did not exhibit any significant changes in mean S/P ratio values ($p > 0.05$). The cutoff S/P ratio values indicating a positive antibody response were set at 0.43 for *C. perfringens* type A toxin and 0.45 for *E. coli* serotype O1. The distribution of the antibody response shows that in all the vaccinated group at 4 weeks post the first vaccination, relatively diverse between individuals. S/P ratio 63,3 % above cut off value for single NE vaccinated group and 53,3% for Bivalent NE-coli vaccinated group against *C. perfringens* type A toxin antigens. While a 70% above cut off value for single *E. coli* vaccinated group and 40% for Bivalent NE-coli vaccinated group against *E. coli* serotypes O1 antigens.

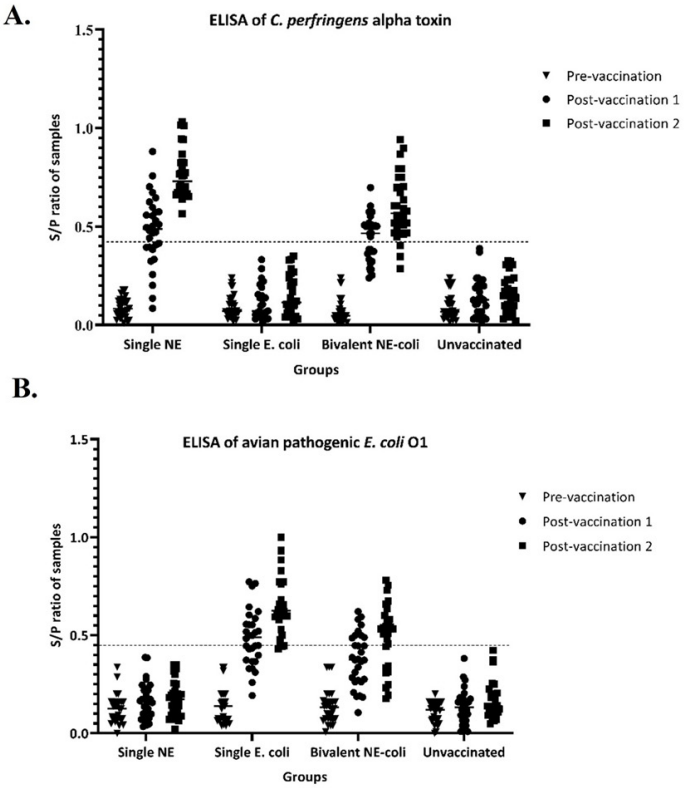

Figure 3: Distribution and percentage S/P ratio of the individual antibody response to vaccination. (A) S/P ratio of ELISA against *C. perfringens* alpha toxin; (B) S/P ratio of ELISA against avian pathogenic *E. coli* O1.

However, S/P ratios increased 4 weeks after the second vaccination and homogeneity begin to appear in each vaccinated group. A 100% positive of S/P ratio was achieved for single NE vaccinated group and 90% for Bivalent NE-coli vaccinated group against *C. perfringens* type A toxin anti-

gens. A 93,3% positive of S/P ratio was achieved for single *E. coli* vaccinated group and 70% for Bivalent NE-coli vaccinated group against *E. coli* serotypes O1 antigens. Statistical analysis of the S/P ratio against the increase in antibody response revealed that the prepared Bivalent NE-coli vaccine showed a significant difference in the increase in antibody response ($p<0.05$) compared to the single NE vaccine either *E. coli* vaccine. Significant differences ($p<0.05$) were observed post-primary and post-booster vaccinations compared to controls.

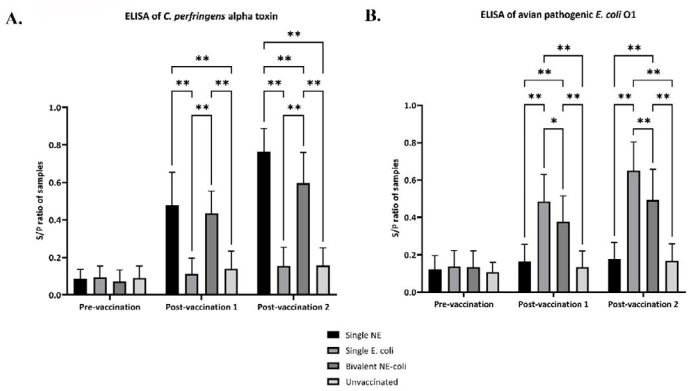

Figure 4: Antibody response after vaccination with prepared vaccine at day 0 pre-vaccination, 4 weeks post-1st vaccination, and 4 weeks post-2nd vaccination in layer chickens. Data represent the mean value of each vaccine group and statistical analysis was carried out by ANOVA, followed by Tukey's post-hoc test for multiple comparisons. Asterisk refers to statistical significant difference between the indicated groups (*$p < 0.05$).

DISCUSSION

This study evaluated the stability, safety, and immunogenicity of an oil-adjuvanted bivalent vaccine containing *C. perfringens* type A toxoid and APEC bacterins for poultry use. The vaccine demonstrated good safety, as no adverse local or systemic reactions were observed, and the laying performance of the chickens remained unaffected. In general, vaccines elicit protective immunity by activating both humoral and cellular immune responses. Inactivated (killed) vaccines, which are often formulated with adjuvants, typically require booster doses to achieve prolonged immunity (Bastola *et al*., 2017). Adjuvants play a crucial role by enhancing immune responses, prolonging antigen presentation, and shielding immunogens from degradation caused by external environmental factors. Because vaccine components especially antigens are highly sensitive to heat, light, and radiation, maintaining cold chain storage is essential to preserve their efficacy and stability. In this study, the prepared vaccine showed a decrease in viscosity when stored at 25°C on sampling 20th day. Observation of significant physical changes in stability occurred due to storage at 37°C for more than 24 days, which caused the separation of the emulsion to form thin layer of oil and water phases. The emulsions formulated in this study were of the water-in-oil (W/O) type, where antigen-containing aqueous droplets (internal phase) are dispersed within a continuous oil phase. This formulation allows the antigen to be retained at the site of injection, facilitating a slow and sustained release. Such prolonged antigen availability likely supports long-term humoral immune responses by continuously stimulating B cells. However, one drawback of W/O emulsions is their high viscosity, which can make administration more challenging. In contrast, oil-in-water (O/W) emulsions are easier to inject due to their lower viscosity and are generally well tolerated, though they tend to induce only short-lived immune responses (Jansen *et al*., 2006; Zhang *et al*., 2018).

In the early stages of vaccine preparation, *C. perfringens* type A and its alpha-toxin were inactivated using formaldehyde—a small, water-soluble aldehyde widely used for its ability to neutralize toxicity, prevent bacterial proliferation, and detoxify bacterial toxins. Importantly, the resulting vaccine formulation did not induce any adverse effects in the tested animal models. Moreover, it proved to be cost-efficient while effectively stimulating the production of neutralizing antibodies and providing protective immunity following administration (Saadh *et al*., 2022). Bivalent vaccine which contains toxoid antigens and APEC bacterial cells in this study induces an enhanced immune response, particularly a humoral immune response. When exposed to an antigen whether naturally or through vaccination the body reacts by eliminating the antigen via the immune system (Pollard and Bijker, 2021). Antibody responses in the single NE-vaccinated, single *E. coli*-vaccinated, and bivalent NE-*E. coli* groups were assessed using ELISA at four weeks following both the first and second immunizations. As shown in Figure 4, statistically significant differences ($P < 0.05$) were observed among the groups. Both the NE-only and bivalent vaccine groups demonstrated a marked increase in antibody levels against *C. perfringens* alpha-toxin ($P < 0.05$), whereas the group vaccinated solely with the *E. coli* vaccine did not exhibit a measurable antibody response to the alpha-toxin. The immunogenic alpha-toxoid vaccine stimulates hens to produce specific anti-alpha-toxin antibodies, which persist at substantial levels throughout the laying cycle (Crouch *et al*., 2010). In this study, the bivalent vaccine combining alpha-toxoid with *E. coli* serotype O1 antigens significantly enhanced the antibody response against alpha-toxin four weeks after the initial immunization. These findings align with previous research indicating that alpha-toxin can induce strong immune protection and a robust antibody response in layer chickens (Ferreira *et al*., 2016). Nevertheless, a different perspective has been presented by Keyburn *et al*., who reported that alpha-toxin may not be an essential virulence factor in necrotic enteritis

(NE), and that NetB offered only minimal protection. This is supported by findings showing that NetB alone was insufficient to significantly boost antibody levels within two weeks following vaccination (Fernandes da Costa *et al*., 2013). Although alpha-toxin continues to be a major virulence element, other proteins such as NetB and the cytotoxin TpeL have also been investigated as potential subunit vaccine targets, albeit offering only partial immunity. Notably, recombinant alpha-toxin has demonstrated potential in improving immune responses against NE challenges (Fernandes da Costa *et al*., 2013; Valipouri *et al*., 2022). In this study, the addition of *E. coli* serotype O1 antigens along with alpha-toxoid led to a pronounced improvement in antibody production four weeks after the initial vaccine dose. Administering a single dose of the bivalent NE–*E. coli* vaccine was enough to induce a significant antibody response targeting *C. perfringens* alpha-toxin, potentially due to the presence of other immunostimulatory elements. Interestingly, birds given two doses of the bivalent vaccine produced antibody levels comparable to those immunized with the NE-only vaccine.

The bivalent NE–*E. coli* vaccine also induced strong antibody responses against *E. coli* serotype O1 antigens, at levels similar to those generated by the monovalent *E. coli* vaccine. ELISA results demonstrated that the humoral immune response to *E. coli* antigens significantly increased by the fourth week post-vaccination. Both vaccine types enhanced immunity by at least 70% compared to baseline for single *E. coli* and 40% for bivalent NE–*E. coli* formulations.

The relatively broad distribution of individual antibody titers in the bivalent group observed four weeks post-vaccination could be attributed to potential antigenic competition between the components. Nonetheless, prior studies on combination vaccines suggest that despite variability in individual immune responses, chickens can still adapt to concurrent infections and potentially mitigate disease severity (Van Goor *et al*., 2017; Zhang *et al*., 2025). A significant difference in antibody responses ($P < 0.05$) was observed among all vaccinated groups compared to the control group, as illustrated in Figure 4 following vaccination.

Previous research demonstrated that a trivalent *E. coli* vaccine containing serotypes O1, O2, and O78 conferred strong protective immunity in layer chickens. Vaccines formulated with both heat- and formalin-inactivated whole-cell virulent *E. coli* strains were also shown to be effective in protecting chickens of various ages from different manifestations of avian colibacillosis (El Sayed *et al*., 2021; Keita *et al*., 2022). Among the more than 180 identified *E. coli* serogroups—classified based on the O-antigen component of the cell wall lipopolysaccharide (LPS)—serogroups O1, O2, and O78 are the most commonly isolated from avian pathogenic *E. coli* (APEC) infections worldwide.

Notably, serogroup O1 is of particular concern due to its zoonotic potential, raising both economic and public health issues that affect poultry production and human health alike (Nishi *et al*., 2021). In other studies, APEC vaccines produced via ultrasonication were shown to offer a degree of cross-protection, likely due to the presence of conserved internal immunogenic proteins. These specific antigens are associated with enhanced stimulation of antigen-presenting cells (Alempour and Rajabi, 2024). Furthermore, lipopolysaccharide (LPS) interacts with Toll-like receptor 4 (TLR-4), triggering the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β). This mimics the host's response to bacterial invasion, promoting cytokine secretion and inflammatory signaling, ultimately leading to antibody production (Nahla MS *et al*., 2014).

Based on the findings of this study, the formulated bivalent vaccine comprising *C. perfringens* type A toxoid and APEC serotype O1 with an oil-based adjuvant shows promising potential as an effective alternative for the prevention and control of necrotic enteritis and colibacillosis in poultry farming. However, the use of combination vaccines may reduce logistical burdens, but antigen competition could reduce immunogenicity. The safety, efficacy, and immunogenicity of combined vaccine may be affected by interaction, not only between the antigens but also other component such as adjuvant, stabilizer, and preservatives. Further study with the formulation of additional APEC serotype antigens needs to be carried out considering the complexity of colibacillosis disease caused by other serotypes

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrated that the formulated bivalent NE-Colivac vaccine meets the essential criteria for vaccine formulation, safety, and immunogenicity. It effectively stimulated a robust immune response in layer chickens against both necrotic enteritis and avian colibacillosis. A notable rise in antibody levels was recorded in vaccinated groups when compared to those receiving single-antigen vaccines and unvaccinated controls, showcasing the high immunogenic strength of the bivalent formulation. Through induction of a humoral immune response, NE-Colivac could provide robust protection against *Clostridium perfringens* type A and avian pathogenic *Escherichia coli* (APEC), contributing to a decrease in both incidence and severity of these economically detrimental poultry diseases. Additional studies are encouraged to assess the durability of immunity under field conditions and the potential integration of this vaccine into broader poultry health programs.

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NOVELTY STATEMENTS

We have developed and formulated a bivalent vaccine combining Clostridium perfringens type A toxoid (NE) and APEC (serotype O1) antigen in a single, physically stable emulsion. The bivalent emulsion's stability was systematically evaluated at 4 °C, 25 °C, and 37 °C over 30 days, with pH and viscosity measured every 10 days. We demonstrate that only storage at 4 °C maintained pH (6.8–6.8) and viscosity (≈43.5–42 mPa·s) without significant change, whereas higher temperatures induced viscosity degradation and pH decline—data not previously reported for either monovalent NE or APEC vaccines.

Using ELISA S/P ratios, we show that the bivalent vaccine induces significant antibody responses against both antigens ($p < 0.05$), achieving 90–100 % seropositivity by eight weeks post-prime. Although peak antibody levels were slightly lower than those elicited by respective monovalent vaccines, the bivalent formulation produced a more homogeneous and sustained response post-booster, indicating potential for simultaneous protection against necrotic enteritis and colibacillosis in poultry.

AUTHOR'S CONTRIBUTIONS

Agustin Indrawati, Safika, Christian Marco Hadi Nugroho, Muhammad Ade Putra, Otto Sahat Martua Silaen, Desak Gede Budi Krisnamurti, Max U.E Sanam, and Ryan Septa Kurnia were involved in conceptualizing the study, designing the experiments, gathering and analyzing the data, as well as drafting the manuscript. Agustin Indrawati and Ryan Septa Kurnia also provided oversight during the research process and contributed to the critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript for submission.

Conflict of Interest

None of the authors have any conflicts of interest to disclose.

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