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Oral and parenteral vaccination of broiler chickens with Recombinant NetB antigen from *Clostridium perfringens* confers significant protection against necrotic enteritis

Mohammad Ali Shamshirgaran[1] and Mehdi Golchin[1*]

Abstract

Background Necrotic enteritis is a devastating economic disease caused by *Clostridium perfringens* in poultry. NetB toxin from *C. perfringens* type G is the major responsible cause of necrotic enteritis. After the ban on growth-promoting antibiotics, alternative effective intervention approaches such as the vaccination of birds were considered critical to control necrotic enteritis. To date, no commercial vaccines with proven efficacy have been approved against necrotic enteritis. In this study, we evaluated the efficacy of the oral and parenteral vaccines based on NetB antigen from *C. perfringens* to choose the best prime-boosting vaccination strategy against necrotic enteritis. The broiler chickens were orally vaccinated with either previously developed recombinant probiotic bacterium, *Lactobacillus casei* strain expressing NetB toxoid, followed by a parenteral booster by the purified recombinant NetB toxoid (oral/parenteral), or the recombinant NetB toxoid alone (parenteral-only).

Results Immunizations of birds with these vaccines elicited strong specific anti-NetB antibody responses and provided significant protection against the infectious challenge. Additionally, the vaccinated birds represented significant mean body weight gains compared with birds in control groups during the experiment.

Conclusions The current study showed that oral and parenteral vaccines using NetB antigen from *C. perfringens* could provide significant protection against necrotic enteritis in broiler chickens.

Keywords *Clostridium perfringens*, *Lactobacillus casei*, Necrotic enteritis, NetB toxin, Probiotic, Vaccine

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Background

Necrotic enteritis (NE), an economic enteric disease of poultry, was first described in young cockerels in England [1]. NE occurs widely in all areas of the world particularly in poultry-producing countries [2] with a cost of around $0.0625 per bird and also the total global loss of up to $6 billion annually in NE outbreaks [3]. *Clostridium perfringens* (*C. perfringens*), a gram-positive spore-forming anaerobe, is the major causative agent responsible for NE [4]. Being broadly distributed in the environment, *C. perfringens* is present in the intestine of humans and animals [5]. Based on the six major toxins, the recent toxinotyping scheme was introduced by Rood et al. (2018), dividing *C. perfringens* into seven types (A to G), with the evidence that type G, producing two most important toxins, α-toxin and necrotic enteritis toxin β-like (NetB), being considered the principal cause of NE [6]. NetB toxin from *C. perfringens* is a secreted pore-forming toxin with a molecular weight of approximately 33-kDa, showing similarity with other pore-forming toxins such as β-toxin from *C. perfringens* and α-hemolysin from *Staphylococcus aureus* [7].

NE can occur in two forms: acute (clinical) and chronic (subclinical) [1]. The acute manifestation of the disease in chickens is characterized by clinical indications such as diarrhea, depression, and sternal recumbency, accompanied by marked necrotic lesions in the small intestine. Conversely, birds affected by the chronic form of the illness exhibit intestinal mucosal damage, as well as a notable decrease in both feed intake and weight gain [8, 9]. Birds usually are affected by NE in normal body conditions, with the mortality rate reaching up to 50% [9]. While birds typically exhibit susceptibility to NE within the age range of 2 to 6 weeks, instances of its occurrence have also been reported in commercial layers exceeding 3 months of age [10].

NE was routinely prevented by supplementation of feed with growth-promoting antimicrobial agents. In 2006, the usage of antimicrobial growth promoters was banned by the European Union due to many considerations about the adverse effects of these antimicrobial agents on public health and also the risk of emerging strains resistant to such antimicrobial agents. Since these growth promoters had prophylactic effects against several diseases in animals, such as NE in birds, the withdrawal of such antimicrobial agents resulted in an unprecedented increase in the deterioration of animal health and the occurrence of NE in broiler chickens [11, 12].

In the post-antibiotic era, several strategies were suggested as possible alternative approaches to control NE, such as the vaccination of birds using NetB antigen from *C. perfringens* to elicit specific immune responses and provide protective immunity against NE in birds. Being the most important toxin involved in NE disease, NetB toxin is frequently used in recent vaccine studies to immunize birds against NE. It was previously proved that NetB antigen from *C. perfringens* is capable of inducing immune responses, and could also provide significant protection against the virulent *C. perfringens* challenge experiment in broiler chickens [13–18].

Among different routes of administration of NE vaccines in broiler chickens, the parenteral and oral vaccines have been widely used to deliver foreign immunogenic antigens to broiler chickens. Some studies formerly reported that parenteral vaccination of birds with NetB toxin or toxoid could significantly lower gross lesions in the small intestine of vaccinated birds, and partially protect chickens against experimental NE challenge [13–17]. It was also shown in several studies that inoculation of chickens with live oral vaccine candidates based on bacterial strains such as *Salmonella* and *Lactobacillus* could elicit immune responses against different antigens of *C. perfringens*, and provide partial protective immunity against NE [18–22]. Live oral vaccines have the advantages of ease in administration, cost-effectiveness, induction of protective mucosal and systemic immune responses, and also the property of being adjuvant-free [23, 24]. On the other hand, the parenteral inactivated vaccines have the benefits of eliciting strong immune responses, being stable on storage, and low development costs [25]. Since the presence of NE is such a serious threat to broiler chickens, especially in highly populated poultry farms, it is essential to choose the more convenient route of vaccine administration to practically deliver foreign immunogens to broiler chickens.

In the present study, we aimed to immunize broiler chickens with either a combination of oral and parenteral vaccines or a parenteral vaccine alone based on the NetB antigen from *C. perfringens* to assess the efficacy of these vaccines against experimental induction of NE in broiler chickens.

Results

Protection of chickens against experimental NE

Two experiments were conducted to assess the efficacy of two distinct vaccination strategies against NE. The strategies evaluated were: (1) oral vaccination of birds with *L. casei* displaying the NetB antigen on its surface, followed by a single parenteral booster; and (2) parenteral vaccination of birds using purified NetB antigen. The protection of immunized broiler chickens against the in-vivo challenge of NE was evaluated by observing and scoring the gross lesions in the small intestine during necropsy and measuring body weight gains during the infection with virulent *C. perfringens* strain. The results of two experiments showed that broiler chickens vaccinated with the oral/parenteral vaccine in experiment 1 and the parenteral vaccine alone in experiment 2 were

significantly protected against experimental challenges of NE ($P < 0.05$) (Fig. 1). In contrast, control birds that received the LCV control strain (*L. casei* harboring an empty vector) or adjuvant-only had severe and frequent intestinal lesions. Additionally, the birds that was unvaccinated but subjected to the challenge infection (NV-C group) exhibited the most severe lesions (score 3). However, no statistically significant differences were found when comparing the NV-C birds with those in the vector-only group ($P = 0.5698$) and the adjuvant-only group ($P = 0.9998$).

The body weight gains of broiler chickens were assessed on days 25, 30 and 35 of age, and showed no significant differences between the vaccinated and control groups before the NE challenge ($P > 0.05$) (Fig. 2). However, significant weight gains were observed in birds received the oral/parenteral (54.41%) or parenteral-only (47.64%) vaccines during the NE infection compared with birds that received vector (31.18%) or adjuvant (25.82%) alone, respectively ($P < 0.05$). The birds that did not receive vaccination and were not challenged with virulent *C. perfringens* (NV-NC group) showed the highest weight gain (56.08%).

High anti-NetB antibody responses in vaccinated chickens

An indirect ELISA assay was carried out using the sera collected from vaccinated birds to assess the anti-NetB IgY responses elicited by oral/parenteral and parenteral-only vaccines. Vaccination with either oral/parenteral or parenteral-only vaccines resulted in significant serum antitoxin antibody responses, as compared with pre-immunized birds, after the first vaccination ($P < 0.05$) (Fig. 3). Specifically, birds vaccinated with the parenteral-only vaccine revealed higher anti-toxin IgY responses. Birds received vector-only and adjuvant-only showed no anti-NetB antibody responses against the purified recombinant NetB antigen ($P > 0.05$). The specificity of the antitoxin antibody responses in birds vaccinated with either oral/parenteral or parenteral-only vaccines were also confirmed via western blotting, which showed the development of the respective protein band corresponding to the NetB antigen (approximately 33 kDa) on the nitrocellulose membrane in both experiments (Fig. 4). The sera collected from chickens that received only the vector or adjuvant did not show the presence of IgY antibodies that cross-reacted with the purified recombinant NetB antigen.

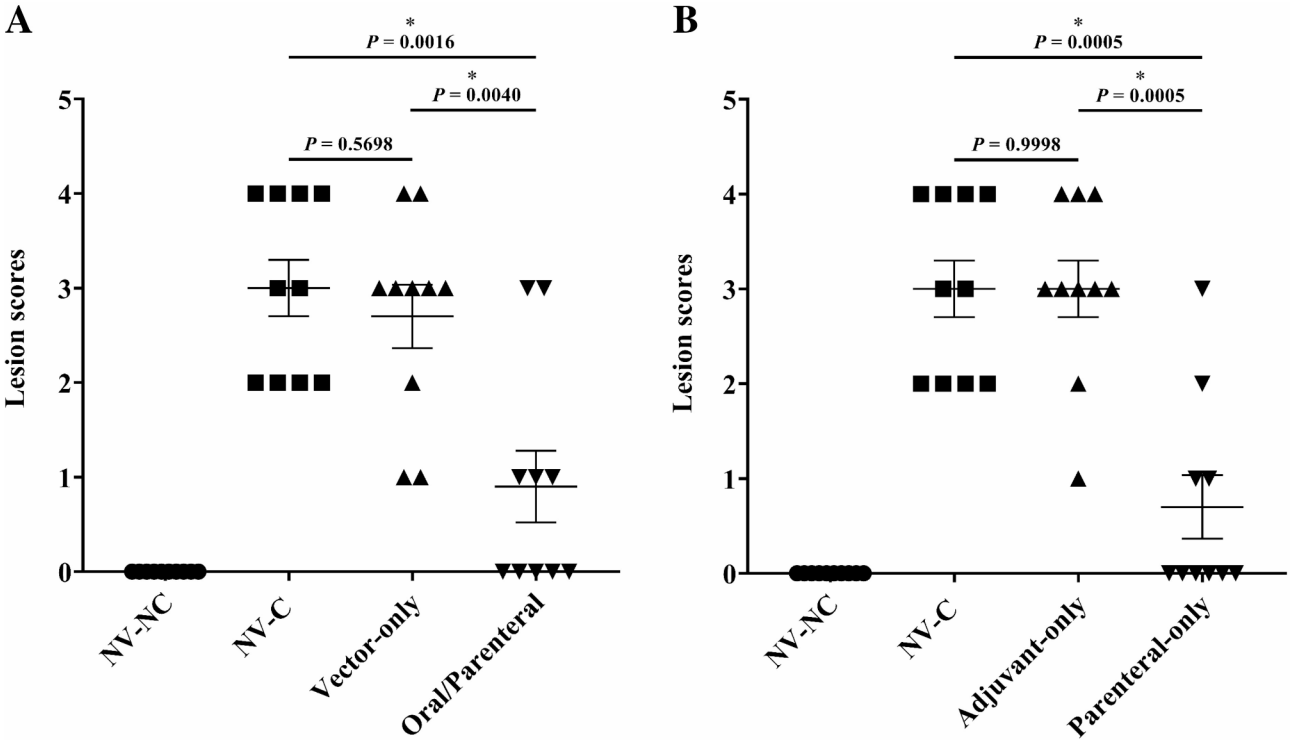

Fig. 1 Gross lesion scores of the small intestine of broiler chickens. Birds, in experiment 1, were orally immunized with the LC-NetB vaccine strain on days 4 and 14 of age, followed by a subcutaneous booster with recombinant NetB toxoid. In experiment 2, birds were immunized subcutaneously with recombinant NetB toxoid vaccine on days 4, 14, and 22 of age. Control groups consist of birds treated with vector-only or adjuvant-only in experiments 1 and 2, respectively. On day 31 of age, all birds were experimentally challenged with a virulent *C. perfringens* strain for 4 consecutive days. All chickens were euthanized, necropsied, and then the small intestine was examined for lesions scoring. The bars indicate the mean lesion score in each group. Asterisk represents significant differences compared with the respective control group ($P < 0.05$). There was no significant difference between the control groups. All data were analyzed in triplicate

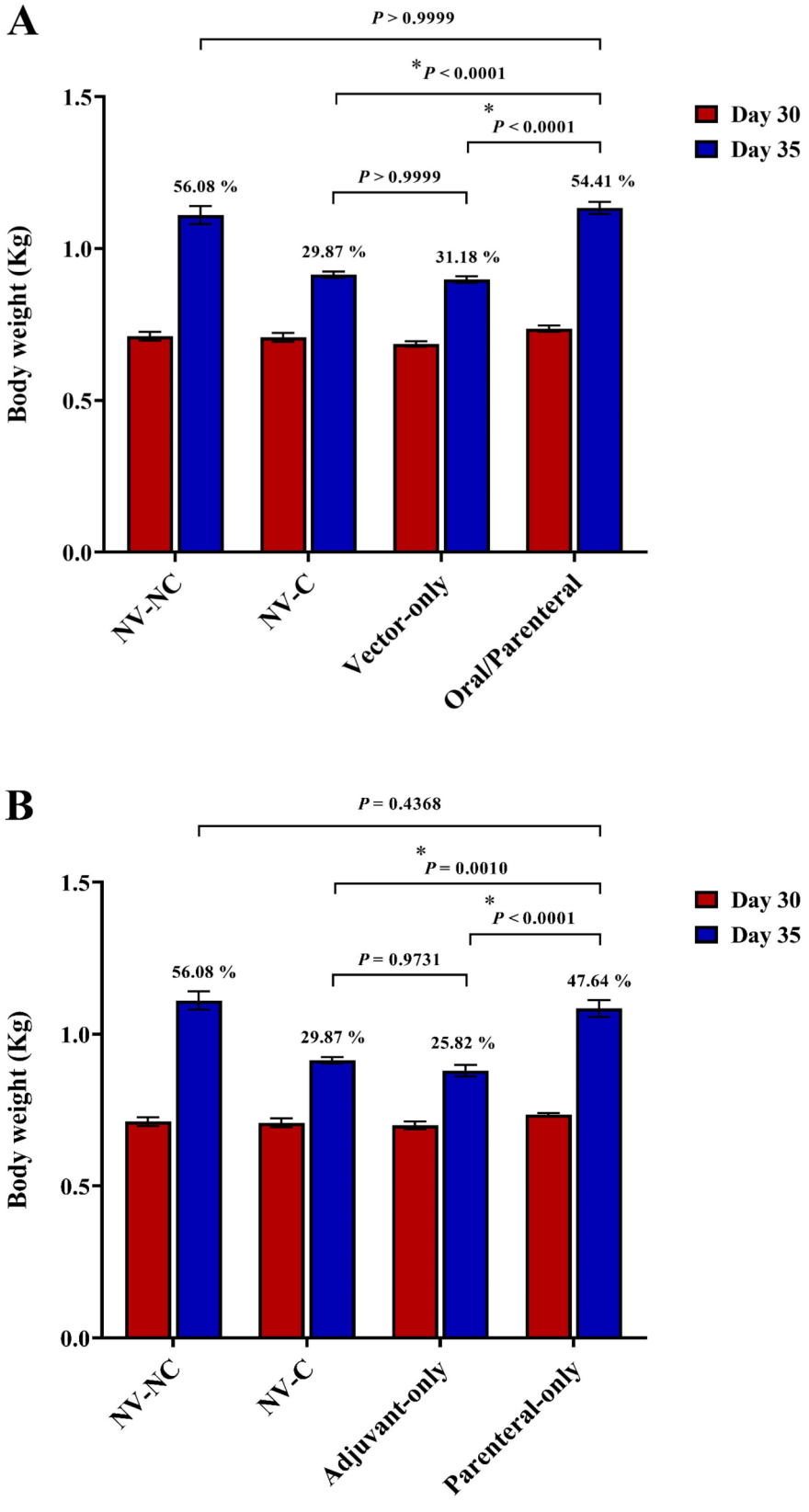

Fig. 2 (See legend on next page.)

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Fig. 2 The mean body weights of broiler chickens pre- and post-challenge experiment. Individual body weights were measured at 5-day intervals from day 25 to 35 of age. No significant statistical differences were observed in mean body weight gains between vaccinated and control birds before the challenge experiment (data not shown). Birds immunized with oral/parenteral or parenteral-only vaccines showed significant body weight gains during the challenge experiment compared with the respective control birds ($P < 0.0001$). There were no significant differences in mean body weight gains between control birds. Each value represents mean ± SEM. Asterisk shows a significant statistical difference compared with the respective control birds ($P < 0.0001$). All data were analyzed in triplicate

Discussion

After the ban on the usage of antibiotics as growth promoters in chickens' feed by the European Union, many investigators suggested approaches that could use alternatively in the post-antibiotic era to control the occurrence of NE disease in birds effectively, and also eliminate the need for antimicrobial agents due to concerns raised about the antibiotic-resistant strains. Vaccination of birds with different antigens of *C. perfringens* was formerly shown to provide partial protection against experimentally-induced NE in broiler chickens [26]. Several studies indicated that vaccination of birds with the major causative agent of NE, NetB antigen from *C. perfringens*, could elicit systemic immune responses, and also lower the intestinal lesions observed in NE-challenged birds [13, 14, 16, 27]. Parenteral immunization and oral delivery of antigens are two common vaccination strategies frequently employed in vaccine studies targeting NE disease. Several studies have demonstrated that parenteral vaccination of chickens with two or three doses of NetB toxoid could partially protect birds against NE challenge [13, 14, 16, 17]. This immunization approach, although demonstrating promise in identifying protective antigens suitable for vaccine formulations [28] and in eliciting robust protective immune responses in vaccinated birds, should be integrated into more commercially feasible vaccine platforms prior to implementation in farm [29]. Conversely, oral administration of live vaccines delivering immunogenic antigens to broiler chickens could have more benefits compared with killed vaccines due to convenient administration especially for farms with large poultry populations [30]. These live vaccines also have the advantages of inducing more permanent immune responses with no adverse effects observed post-immunization in vaccinated birds [30–32]. Some researchers have previously shown that immunization of broiler chickens with oral vector-based vaccine candidates such as *Salmonella*- and *Lactobacillus*-based vaccines delivering different antigens from *C. perfringens* could elicit anti-toxin antibody responses, and also provide partial protective immunity against experimental NE challenge [18, 20–22, 33, 34].

The probiotic bacterium used as the vaccine strain in this study was *L. casei*, a safe lactic acid bacterium [24]. The benefits of supplementation of chicken's diet with probiotics were represented previously [35]. As a probiotic bacterium, *L. casei* could lower the severity of lesions observed in the small intestine of birds following *C. perfringens* challenge [36], and also play a role in mucosal immunity stimulation and regulation, modification of the microbiota of the intestine, and increase the growth performance [24, 35, 37]. Moreover, probiotics were widely used as vaccine vehicles to deliver foreign immunogens to birds and provide protection against several diseases such as avian influenza [38–40], chicken anemia [41], infectious bursal disease [42], and Newcastle disease [43].

When selecting the optimal route for vaccine administration against NE in poultry, several critical factors warrant consideration. These encompass the practicality of administration in large-scale flocks, the ability to elicit robust immune responses, and the capacity to generate a diverse array of immune responses, including systemic, humoral, and mucosal immunity. Oral vaccines present potential advantages due to their ease of administration, particularly in flocks with large populations; however, they may induce suboptimal immune responses that necessitate enhancement through parenteral booster vaccinations [20]. Conversely, parenteral vaccines are capable of inducing stronger immune responses; yet, their implementation may be impractical given the logistical challenges associated with frequent needle-based immunizations in large flocks. Therefore, implementing a combination vaccination strategy that incorporates both oral and parenteral routes may represent a promising approach. This dual strategy could leverage the advantages of each administration method, thereby enhancing the overall immunogenicity and efficacy of vaccination against NE disease in poultry. The combination vaccination strategy, wherein a parenteral booster is administered subsequent to oral immunization, has been investigated in several studies, demonstrating significant immune responses [44–47]. In this study, immunization of birds with combination of oral and parenteral vaccines induced significant immune responses and provided protective immunity. Prior to the experiment, the birds were confirmed to be free of related infections through comprehensive physical examinations and continuous monitoring of clinical signs. Additionally, the absence of specific antibodies associated with the infection was verified using ELISA. Moreover, although parenteral vaccination of broiler chickens with the NetB toxoid elicited significant immune responses and offered protection against NE, the three frequent injections could be an unfeasible approach especially in flocks with large

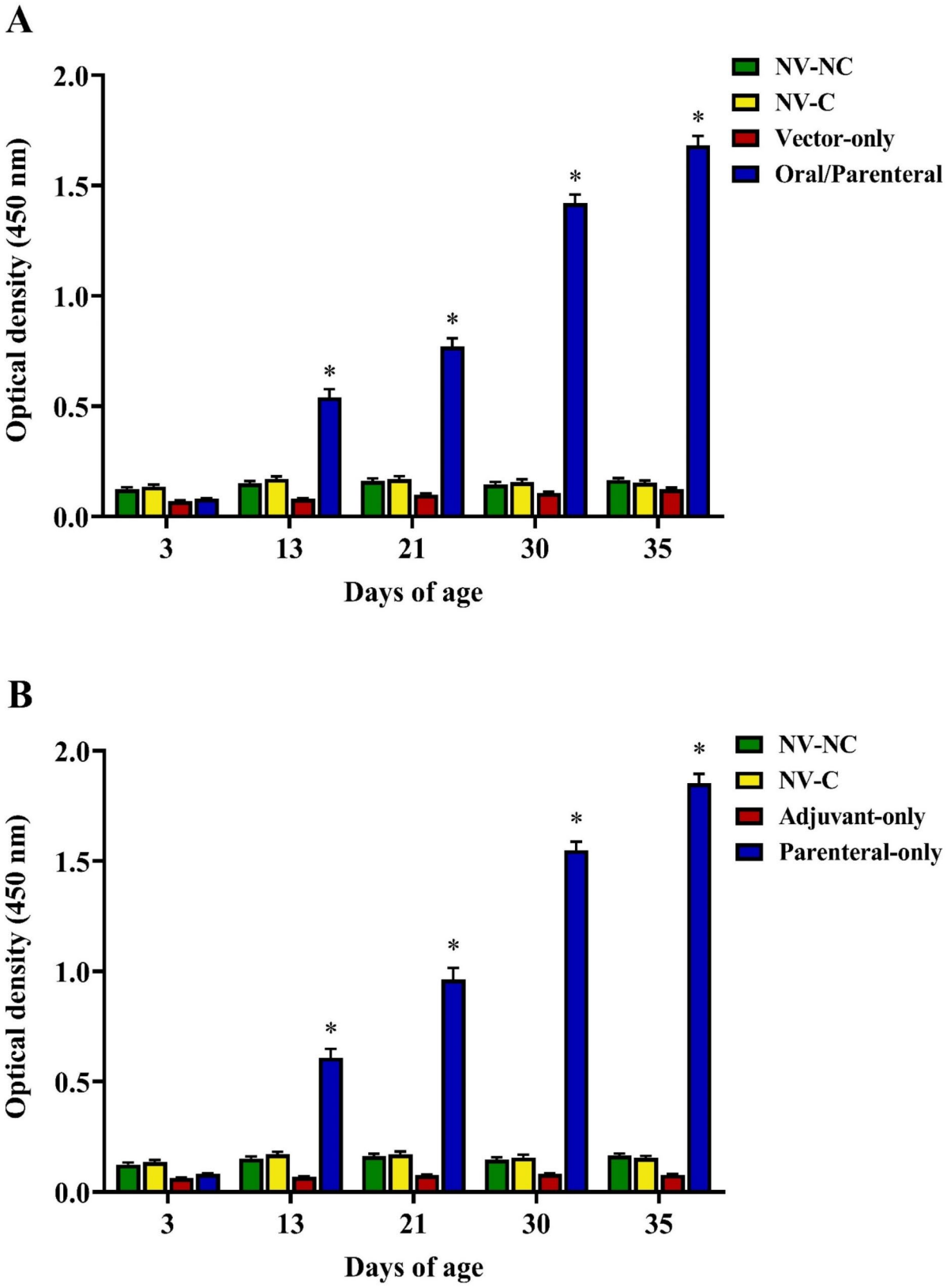

Fig. 3 (See legend on next page.)

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Fig. 3 Serum anti-NetB antibody responses of broiler chickens. The purified NetB toxoid was used as the coating antigen at a density of 30 µg/ml, and the sera collected from each group of birds were pooled and used as the source of the primary antibodies at 1:100 dilutions in an indirect ELISA assay. As the source of the secondary antibody, Goat anti-chicken IgY antibody-HRP was used (1:3000 in blocking buffer). Birds vaccinated with oral/parenteral (**A**) or parenteral-only (**B**) vaccines elicited significant anti-NetB antibody responses after each immunization compared with the respective control birds. No significant anti-NetB IgY responses were observed in control birds. * indicates significant values ($P < 0.0001$). Values represent mean ± SEM ($P < 0.05$). All samples were analyzed in triplicate

density. In addition, while parenteral-only vaccination elicited more robust immune responses and yielded enhanced protection compared to the combined vaccination approach, the differences observed between the two methodologies were not substantial enough to justify overlooking the practical challenges associated with the exclusive implementation of parenteral vaccination.

We previously indicated that the recombinant probiotic vaccine strain, *L. casei* displaying NetB antigen from *C. perfringens* on the cell surface, could elicit significant anti-NetB IgY responses, and also provide significant protection against the experimental challenge of NE in broiler chickens [18]. In the present study, we used either the previously prepared recombinant *L. casei* vaccine strain as an oral vaccine candidate in combination with the recombinant NetB toxoid as a booster, or the recombinant NetB toxoid alone as the parenteral vaccine candidate in two separate experiments. Although the data indicated that the combined vaccination approach did not provide additional benefits over the parenteral-only strategy, the immune responses and protective effects obtained from the oral/parenteral method were approximately comparable to those achieved with the parenteral-only approach. Frequent parenteral vaccinations are often impractical for farm applications; however, the use of oral vaccinations with the safe probiotic bacterium, *L. casei*, could present a promising approach. This probiotic has the potential to be lyophilized, allowing for administration through feed or drinking water, thereby enhancing its practicality for large-populated poultry flocks. The strategy of employing a combined immunization approach effectively addresses the practical challenges associated with parenteral vaccinations while simultaneously enhancing the immune efficacy achieved through oral-only vaccination.

Conclusion

In conclusion, the current study highlights that different vaccination strategies based on NetB antigen from *C. perfringens* could elicit anti-NetB IgY responses, and also provides partial protective immunity against NE challenge. Our findings demonstrated that parenteral vaccines could elicit more robust antibody responses and also provide stronger protection against NE compared with the combination of the oral and parenteral routes. However, other properties such as costs, ease of vaccination, being adjuvant-free, and inducing long-lasting immune responses should be considered to choose an ideal vaccine for NE. While the NetB toxin produced by *C. perfringens* type G is recognized as the principal antigen associated with NE pathogenesis in poultry, it is important to note that some clinical isolates either lack or exhibit reduced expression of this toxin [48, 49], suggesting that the vaccination approach employed in this study may prove insufficient in providing protection against these isolates. To address this limitation, it is essential to develop a more comprehensive vaccination strategy that targets multiple antigens, thereby enhancing the efficacy of immunization against a wider array of *C. perfringens* strains.

Materials and methods

Bacterial strains and growth conditions

The recombinant *Lactobacillus casei* (*L. casei*) strain expressing the modified NetB antigen from *C. perfringens* (LC-NetB), and LCV strain were constructed as described by authors in the previous study [18]. The strains were anaerobically grown in deMan, Rogosa, and Sharpe (MRS) medium (Himedia, Thane, India) at 37 °C. Erythromycin (5 µg/ml) was used whenever needed. *C. perfringens* strain CP58 [18] was used in the challenge experiment. *C. perfringens* was grown in brain heart infusion (BHI) medium (Merck, Darmstadt, Germany) and 5% sheep blood agar to differentiate the respective colonies and evaluate the hemolytic activity, respectively. The Cooked meat medium (CMM) (Merck, Darmstadt, Germany) and fluid thioglycolate (FTG) medium (Merck, Darmstadt, Germany) were also used to achieve large quantities of *C. perfringens* required for animal inoculation in the challenge experiment. All *C. perfringens* cultures were grown at 37 °C under anaerobic condition with no shaking. The previously constructed recombinant *Escherichia coli* (*E. coli*) BL21 strain expressing NetB antigen from *C. perfringens* was used for NetB protein expression and purification as described in the previous study by authors [18].

Animal housing and conditions

One-day-old Ross 308 broiler chickens were obtained commercially from Mahan Chicken Production Complex in Kerman, Iran, where breeder birds are maintained under strict standard conditions and certified free of pathogens. Prior to the experiment, all birds underwent physical examinations to ensure their health. The flock

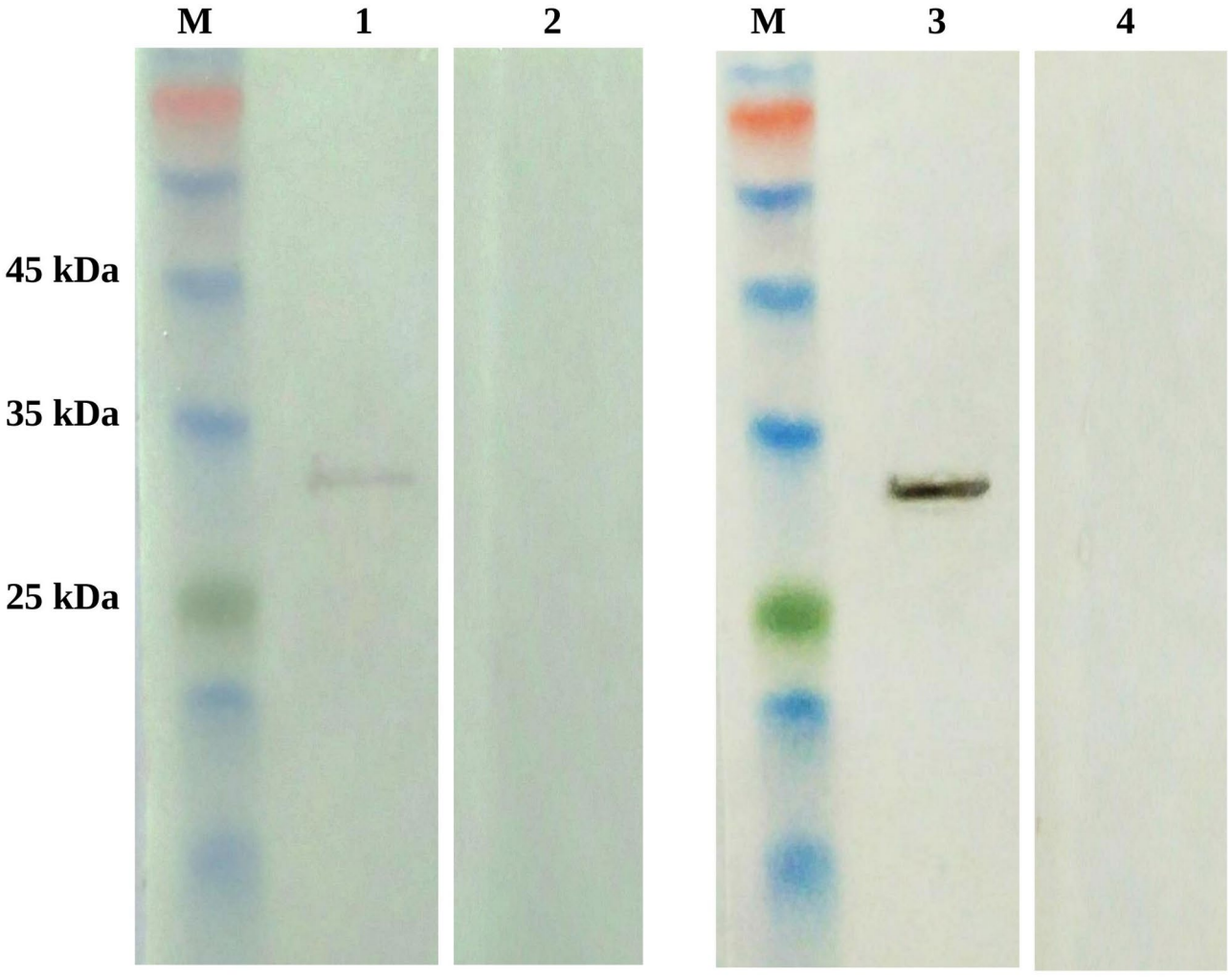

Fig. 4 Immunoblot of the NetB toxin from *C. perfringens*. Sera collected from chickens immunized with oral/parenteral or parenteral-only vaccines were pooled, and then used as the source of the primary antibody at 1:100 dilutions. Goat anti-chicken IgY antibody-HRP (at a 1:3000 dilution) was used as the secondary antibody. A 33-kDa protein band of the NetB toxoid shows the reactivity of the chicken anti-NetB IgY antibodies collected from birds immunized with oral/parenteral (lane 1) or parenteral-only (lane 3) vaccines with purified NetB toxin. No specific reaction was observed between purified NetB toxin and the sera obtained from birds received vector-only (lane 2) or adjuvant-only (lane 4). Lane M, the ExcelBand 3-Color Prestained Protein Marker (Smobio, Hsinchu, Taiwan). Full-length blots are illustrated in Additional Fig. 1

history did not indicate any prior *C. perfringens* vaccination of the parent birds. The animal housing was similar to previous study [22]. Briefly, the chicks were placed in a single room and randomly assigned to pens (4 pens per experiment with 10 chicks each) with separating walls, with a density of 20 chicks/m^2 to prevent contact between birds within each group. All birds were provided with ad libitum access to feed and water. The temperature conditions within the facility used for animal rearing commenced at 32 °C for the initial week and subsequently underwent a gradual decrease at a rate of 0.5 °C per day until it reached a steady-state of 25 °C, which was sustained throughout the entire duration of the experimental period. The light program was adjusted to provide continuous light during the first week, followed by a 16-hour light/8-hour dark cycle until the end of the experiment. All animal experiments were assessed with the approval of the Research Ethics Committees of the Faculty of Shahid Bahonar, University of Kerman (Code: IR.UK.VETMED. REC.1400.007).

Vaccine preparation

The oral LC-NetB vaccine strain and LCV control strain were prepared as described previously [18]. Briefly, a single colony of the strains grown on antibiotic-supplemented MRS agar was inoculated into an MRS medium containing 5 µg/ml of Erythromycin and then incubated for 18 h at 37 °C under anaerobic condition. The next day, 2% of the culture was inoculated into 100 ml MRS medium and grown at 37 °C under anaerobic condition

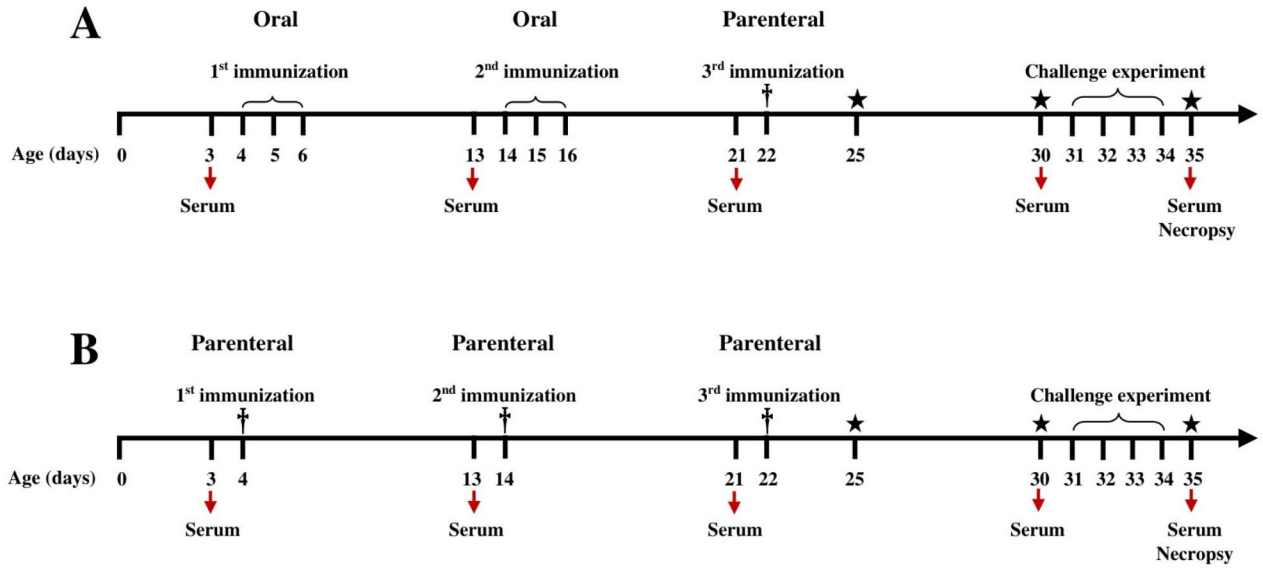

Fig. 5 Schematic outline of vaccination and experimental NE induction in broiler chickens. In experiment 1, birds were immunized orally with the LC-NetB vaccine strain on days 4 and 14 of age, and each immunization continued for 3 consecutive days, followed by a subcutaneous booster with recombinant NetB toxoid vaccine on day 22 of age (**A**). In experiment 2, birds were subcutaneously vaccinated with recombinant NetB toxoid vaccine emulsified in Freund's adjuvant on days 4, 14, and 22 of age (**B**). All birds were bled before each immunization, and also 8 and 13 days after the last vaccination. A gross pathological examination of the small intestine was performed the next day after the challenge experiment. The body weights of all birds were individually measured from day 25 to 35 of age, at 5-day intervals (Asterisk). Birds were challenged with the virulent *C. perfringens* on day 31 of age for four consecutive days

until the OD600 ≈ 1 was achieved. After centrifugation at 5000 × *g* for 10 min at 4 °C, the pellets were suspended in PBS solution. The final bacterial concentration of 9.00 ± 0.04 log10 CFU/ml was yielded using plate counting. The oral vaccine and control strains were prepared on each day of inoculation and were incubated on ice until immunization. The NetB toxoid was expressed by the recombinant *E. coli* strain and purified as described previously [18]. The purified NetB toxoid emulsified in Freund's adjuvant (Sigma-Aldrich, St. Louis, USA) was used as the parenteral vaccine.

Chicken vaccination

Two vaccination experiments, as shown in Fig. 5, were carried out based on the recombinant purified NetB antigen as follows: experiment 1, oral vaccination with LC-NetB vaccine strain, followed by a single subcutaneous booster of recombinant purified NetB toxoid (oral/parenteral group), with a group of control birds received LCV strain (Vector-only group); experiment 2, parenteral vaccination with recombinant purified NetB toxoid emulsified in Freund's adjuvant (parenteral-only group), with control chickens received adjuvant alone (Adjuvant-only group). Additionally, NV-NC group of birds was included in the study that was neither vaccinated nor infected, as well as NV-C group that was unvaccinated but subjected to the challenge infection. In experiment 1, chickens were orally immunized with 0.5 ml of 1×10^9 CFU/ml suspension of LC-NetB strain on day 4 of age for 3 consecutive days. Chickens in the control group were administered the same volume of the LCV strain. All chickens were deprived of feed and water for 12 h prior to each inoculation to facilitate the inoculation of the vaccine or control strains. Feed and water were given 30 min after the immunization. On day 14 of age, birds received the second dose of the inoculum, 1 ml of LC-NetB vaccine or LCV control strains (1×10^9 CFU/ml), for 3 consecutive days. As the last boost immunization, birds were vaccinated subcutaneously with 50 µg purified recombinant NetB antigen emulsified in complete Freund's adjuvant on day 22 of age. In experiment 2, birds were subcutaneously vaccinated with 100 µl of the purified recombinant NetB antigen (50 µg per injection) emulsified in complete Freund's adjuvant on days 4, 14, and 22 of age. Complete Freund's adjuvant was used for the first dose of vaccine and incomplete Freund's adjuvant was used for the remaining immunizations. Control birds also received the same volume of the respective adjuvants in each immunization.

In-vivo NE challenge model

The broiler chickens were provided with an antibiotic-free starter diet containing 21.5% protein for the first 20 days. Subsequently, the diet was changed to a formulated wheat-based grower diet containing 48% fishmeal as the primary protein source from day 22 of age onward. For

the infectious challenge experiment, the *C. perfringens* strain CP58 was prepared and used from day 31 to 34 of age, after an 8-hour period of water and feed deprivation, as previously described [50]. At the end of the experiment on day 35 of age, all birds were euthanized using CO2 inhalation. Briefly, CO2 was administered using a compressed gas canister equipped with a flowmeter and pressure regulator. Once the switch was activated, a continuous flow of CO2 was directed into the euthanasia chamber. To prevent overcrowding, the number of birds introduced into the euthanasia chamber was adjusted according to the chamber's size. To ensure correct procedure, the entire body of the chicken was placed into the euthanasia chamber, and the gas was distributed at a displacement rate of 20% of the chamber's volume per minute. A secondary physical method of euthanasia, specifically cervical dislocation, was performed after the animal had become unconscious. Further necropsy was performed to assess any pathological changes.

The effectiveness of protection against the *C. perfringens* inoculation was evaluated by analyzing the gross lesions in the small intestine and determining the average body weight gains of the birds during the challenge experiment. The small intestines of all birds were visually examined, and the observed lesions were assessed using a scoring system previously described by Keyburn et al. (2013b). The scoring system was as follows: 0 = no visible gross lesions; 1 = thin or friable walls; 2 = focal necrosis or ulceration (1–5 foci); 3 = focal necrosis or ulceration (6–15 foci); 4 = focal necrosis or ulceration (16 or more foci); 5 = patches of necrosis (2–3 cm long); 6 = diffuse necrosis the same as that of field cases. The scorer bias was avoided by employing blind scoring. All chickens were also weighted individually on days 25, 30, and 35 of age.

Sample collection

Blood samples were collected from all chickens via left-wing vein puncture on days 3, 13, 21, 30, and 35 of age. Blood samples collected on day 3, prior to the start of the experiment, were used to determine the presence of any infection-related antibodies in the chicks. Following centrifugation at 2000 × g for 10 min, the resulting sera were stored at -20 °C for further vaccine studies.

Measuring antibody responses

An indirect ELISA assay was conducted to determine the presence of antitoxin immunoglobulin Y (IgY) responses in birds that were vaccinated with either oral/parenteral or parenteral-only vaccines against the *C. perfringens* challenge infection. To perform the assay, polystyrene 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with the purified recombinant NetB toxoid (25 µg/ml) in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, USA) (pH 7.4) at a volume of 100 µl/well, and then incubated overnight at 4 °C. To prevent nonspecific binding, a blocking step was carried out using 3% skimmed milk (Sigma-Aldrich, St. Louis, USA) that was diluted in PBS, and incubated at 37 °C for 2 h. Serum samples, which were obtained from vaccinated birds and diluted in PBS (1:100), were added to the wells in duplicate and the plates were incubated for 1 h at 37 °C. Subsequently, the secondary antibody (goat anti-chicken IgY antibody-HRP; Genscript, Piscataway, USA) was added to the wells. The secondary antibody was diluted in blocking buffer (1:3000) and incubated for 1 h at 37 °C. The development of color was carried out using 3,3′,5,5′ tetramethylbenzidine (TMB) substrate, and the reaction was halted by adding 1 M sulfuric acid. Finally, the optical absorption was measured at 450 nm using an ELISA plate reader (Biotek, Vermont, USA).

To validate the reaction of the anti-NetB antibodies obtained from vaccinated birds with the purified NetB toxoid, immunoblotting was performed. First, the recombinant NetB protein (10 µg) was run on a 12% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. A blocking step was performed using 3% skimmed milk, followed by incubation at room temperature for 2 h. The resulting blots were reacted with pooled serum antibodies obtained from birds immunized with either oral/parenteral or parenteral-only vaccines. In addition, the purified recombinant NetB antigen was also reacted with pooled sera from control birds that received the LCV strain or adjuvant alone to confirm that no cross-reaction occurred between the antibodies and the recombinant NetB protein. Then, goat anti-chicken IgY antibody-HRP, as secondary antibodies, was used and the respective color was developed using 4-chloro-1-naphthol substrate (Sigma-Aldrich, St. Louis, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 (Graph-Pad Software, San Diego, CA). Antibody titers and body weight values were analyzed using one-way analysis of variance followed by Tukey's posttest. Lesion scores were analyzed using a two-tailed Mann–Whitney test. Values were expressed as means ± SEM, and the significant level was considered at $p < 0.05$. All data were analyzed in triplicate.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04624-z.

Supplementary Material 1: Challenge data of vaccinated and control birds. The lesion scores of birds challenged with virulent ***C. perfringens*** are shown using the 1–6 scoring system.

Supplementary Material 2: Body weight data of birds (kg). Body weights of birds measured individually on days 25, 30, and 35 of age are

shown.

Supplementary Material 3: ELISA data of vaccinated and control birds. The ELISA data of the sera obtained from chickens on days 3, 13, 21, 30, and 35 of age are shown in 450 nm optical density.

Supplementary Material 4: **Additional fig. 1**. Uncropped full-length blots. Lane M, the ExcelBand 3-Color Prestained Protein Marker; lane 1, birds vaccinated with the oral/parenteral route; lane 2, birds received vector-only strain; lane 3, birds immunized with the parenteral-only vaccine; lane 4, birds administered adjuvant-only. Unrelated lanes are indicated with ×.

Supplementary Material 5

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Author contributions
Mohammad Ali Shamshirgaran: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing– original draft preparation. Mehdi Golchin: Conceptualization, Resources, Project administration, Supervision, Visualization, Writing– review & editing. All the authors read and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article [and its additional files].

Declarations

Ethics approval
All animal experiments were assessed and approved by the Research Ethics Committees of Faculty of Shahid Bahonar University of Kerman (Code: IR.UK.VETMED.REC.1400.007).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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