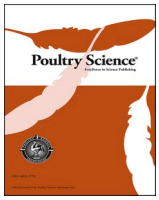
103 (2024) 104436

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj



Identification of immunogenic antigens and evaluation of vaccine candidates against *Clostridium perfringens*

Zewei Li [a,b], Yifei Chen [a,b], Haiping Xie [a,b], Quan Li [a,b], Shifeng Wang [c], Huoying Shi [a,b,d,*]

[a] College of Veterinary Medicine, Yangzhou University, Yangzhou, 225009 Jiangsu China
[b] Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China
[c] Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611-0880, USA
[d] Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou University (JIRLAAPS), Yangzhou, China

ARTICLE INFO

Keywords:
Necrotic enteritis
Immunogenic antigen
Subunit vaccine
Clostridium perfringens

ABSTRACT

Necrotic enteritis (NE) caused by *Clostridium perfringens* (*C. perfringens*) has resulted in significant losses for the poultry industry worldwide. Currently, there is no widely promoted vaccine for NE. In this study, immunoprecipitation (IP) was employed to isolate immunogenic proteins of *C. perfringens*, and 118 potential candidate antigens were identified through liquid chromatography-mass spectrometry/mass spectrometry (LC–MS/MS). From these, three candidate antigen proteins were selected based on their predicted antigenicity, hydrophilicity, stability, and transmembrane signalling properties: ArcB (an ornithine aminotransferase), TmpC (a probable membrane lipoprotein), and EntB (a possible enterotoxin). These three proteins were successfully produced in large quantities using *Escherichia coli* (*E. coli*), with confirmed good solubility. Both in *vitro* and *in vivo* research demonstrated that these antigens possess strong immunogenicity, eliciting robust antigen-specific humoral and cellular immune responses in chickens and mitigating NE symptoms caused by *C. perfringens*. The candidate antigens identified through immunoproteomics hold potential as subunit vaccines against *C. perfringens* infection.

Introduction

C. perfringens infection causes NE, a severe intestinal disease in poultry, characterised by reduced production performance and increased mortality rates (To et al., 2017). NE leads to an estimated $6 billion in losses annually for the global poultry industry (Gharib-Naseri et al., 2021). Although antibiotics are currently used to prevent and treat *C. perfringens* infection in clinical practice (Gaucher et al., 2015), their use may lead to the mutation and drift of antibiotic resistance genes, posing significant public health and safety risks (Innes et al., 2020). Consequently, there is an urgent need to develop new strategies for preventing and treating NE.

Vaccination is an effective method of controlling infectious diseases and has been highly successful in managing epidemics (Qiao et al., 2021; Burman et al., 2023). However, a widely promoted vaccine for *C. perfringens* has yet to be developed (Moore, 2024). Mishra et al. (2017) reported that a *C. perfringens* attenuated vaccine candidate, based on a non-virulent NetB-positive strain, failed to offer substantial protection against *C. perfringens* challenge in chickens following oral vaccination. Immunisation with toxoid vaccine candidates has only provided partial protection against experimental NE (Mot et al., 2013; Fernandes Da Costa et al., 2013). Subunit vaccines offer advantages such as high purity, good safety, minimal reactogenicity, and high specificity (Wang et al., 2019). Nevertheless, potential subunit antigens of *C. perfringens* have been rarely reported to date (Alizadeh et al., 2021).

With advancements in proteomics and immunology, reverse vaccinology has been utilised to screen immunogenic antigens on a large scale and develop new vaccine targets (Lau and Tan, 2023; Jiang et al., 2023). Meniaï et al. (2021) employed a comparative and subtractive reverse vaccine approach to analyse the genomes of 16 strains of *C. perfringens*, identifying 12 potential immunogenic proteins. Subsequently, five of these proteins were selected for protein expression, and their immunogenicity was confirmed (Heidarpanah et al., 2023b). In this study, 118 potential immunogenic candidate antigens were identified from the whole bacterial and secretory proteins of *C. perfringens* using an immunoproteomics approach. Three proteins with predicted high

Scientific section: Immunology, Health and Disease.

* Corresponding author at: College of Veterinary Medicine, Yangzhou University, Yangzhou, 225009, Jiangsu, PR China.
E-mail addresses: LZW950302@163.com (Z. Li), YFChen19990927@163.com (Y. Chen), 408597961@qq.com (H. Xie), liquan2018@yzu.edu.cn (Q. Li), shifengwang@ufl.edu (S. Wang), hyshi@yzu.edu.cn (H. Shi).

https://doi.org/10.1016/j.psj.2024.104436
Received 30 April 2024; Accepted 12 October 2024
Available online 13 October 2024

antigenicity—EntB (a probable enterotoxin), TmpC (a probable membrane lipoprotein), and ArcB (an ornithine carbamoyltransferase)—were selected by reverse vaccinology. Their immunogenicity and protective efficacy against the *C. perfringens* challenge were assessed in a chicken model, laying a solid foundation for the development of safe and effective *C. perfringens* vaccines.

Materials and methods

Animal and ethical statement

Female BALB/c mice, aged 6 weeks, were purchased from the Comparative Medicine Center at Yangzhou University (Jiangsu, China). Two-month-old female New Zealand white rabbits were obtained from the Songlian Experimental Animal Breeding Facility in Shanghai, China. Specific pathogen-free (SPF) chicken embryos were acquired from Jiangsu Boehringer Ingelheim Vital Biotechnology Co., Ltd. One-day-old chicks were hatched using SPF chicken embryos in our laboratory. All animals were housed in a conventional facility with unlimited access to food and water, and their condition was monitored daily. All animal studies conducted at Yangzhou University were approved by the Jiangsu Provincial Experimental Animal Management Committee (license numbers SYXK (SU) 2021-0027, SYXK (SU) 2017-0044, SYXK (SU) 2021-0026). This process complies with international law and the ethical and welfare standards of Jiangsu Province for the use of experimental animals.

Bacterial strains and growth conditions

The *C. perfringens* strain NE-1, used for antigen screening and challenge, is a virulent strain isolated from chickens with necrotic enteritis and is a G-type strain positive for α and NetB toxins. *E. coli* BL21(DE3) Rosetta (Tiangen, Beijing, China) was used for recombinant protein expression. Plasmids pET-28a(+) and pET-32a(+) were employed for recombinant plasmid construction and gene expression.

Indirect ELISA

The antibody titres against whole bacterial proteins, secretory proteins, and recombinant proteins of *C. perfringens* were determined using the indirect enzyme-linked immunosorbent assay (ELISA), following a previously published method with some modifications (Heidarpanah et al., 2023a). Whole bacterial proteins, secretory proteins, or recombinant proteins of *C. perfringens* were diluted to a concentration of 500 ng/mL in a coating buffer (0.1 M carbonate buffer at pH 9.6). Then, 100 μL was added to each well of the ELISA microtitre plate, followed by incubation at 4°C overnight. The plate was blocked with 5% non-fat milk powder in phosphate-buffered saline with 0.5 % Tween 20 (PBST) at 37°C for 3 h after rinsing with PBST. The plate was washed again before adding the diluted test serum in PBST to each well and incubating at 37°C for 2 h. After washing, 100 μL of horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Boster Biological Technology, Wuhan, China) was added to each well, followed by incubation for 1 h at 37°C. After further washing, 100 μL of 3, 3′,5, 5′-Tetramethylbenzidine (TMB, Solarbio, Beijing, China) substrate solution was added to each well, and the reaction was allowed to proceed for 15 min at 37°C. The reaction was stopped by adding 50 μL of 2 M H_2SO_4 solution. The optical density (OD) value was measured at 450 nm. Positive sample wells were defined as those with an OD value more than 2.1 times that of the negative control wells.

Separation of subcellular fractions of C. perfringens

The crude membrane and cytoplasmic fractions of *C. perfringens* were prepared as previously described (Teng et al., 2008). *C. perfringens* was cultured overnight in BHI broth. Cells were harvested by centrifugation at 5000 × g at 4°C for 5 min. The supernatant was filter-sterilised using a 0.22 μm filter. A saturated ammonium sulfate solution was added to the supernatant to a final concentration of 50 %, and the mixture was incubated at 4°C overnight. The next day, the solution was centrifuged at 8000 × g for 20 min at 4°C, and the supernatant was discarded. The precipitate was dissolved in 0.05 M Tris-HCl (pH 7.5, Solarbio, Beijing, China) buffer before gradually adding more saturated ammonium sulfate solution to achieve a final concentration of 40 % ammonium sulfate. The solution was centrifuged under the same conditions, and the precipitate was dissolved in 0.05 M Tris-HCl to obtain concentrated secretory proteins. The cell pellet was washed once with 10 mL PBS (pH 7.4), then resuspended in 10 mL PBS and treated with 1 mM protease inhibitor. The bacterial suspension was lysed by sonication. The whole-cell lysate was centrifuged at 9000 × g for 30 min at 4°C to remove intact cells and debris. The supernatant from this step was ultracentrifuged at 300,000 × g for 2 h at 4°C to extract the cytoplasmic fraction. The pellet was resuspended in 1 mL PBS, centrifuged at 300, 000 × g for 30 min at 4°C, then resuspended in 200 μL PBS to extract the membrane fraction.

Preparation of polyclonal antibodies against whole bacterial proteins and secretory proteins of C. perfringens in rabbits

The whole bacterial proteins and secretory proteins of *C. perfringens* were subcutaneous injections to 3 female New Zealand white rabbits. An initial vaccination was administered on day 0, and 3 booster injections were received on days 14, 21 and 28. For the primary immunisation, 800 μg of *C. perfringens* whole bacterial proteins or secretory proteins were coupled with complete Freund's adjuvant (Sigma, USA), respectively, whereas incomplete Freund's adjuvant was used in vaccine formulations to booster immunisations. Blood was drawn from both vaccinated and unvaccinated animals a week following the final vaccination, and serum was extracted through centrifugation (3,000 rpm, 15 min). As previously stated, indirect ELISA was applied to assess serum antibody titres. Following the product specification (Solarbio, Beijing, China), rabbit hyperimmune serum with antibody titres greater than 128,000 has been dialysed utilising binding buffer. Antibodies were subsequently purified by protein A/G affinity chromatography.

Immunoprecipitation

Protein A/G agarose beads were used for the immunoprecipitation (IP) experiment in accordance with the product specification (Solarbio, Beijing, China). In summary, wash 50 μL of protein A/G agarose beads twice, utilising 0.5 mL of binding buffer. The beads were then combined with purified antibodies and incubated at room temperature for 30 min, followed by thorough washing with wash buffer. Next, 200 μg of *C. perfringens* protein was added to the mixture, and the complex was incubated overnight at 4°C. The beads were washed with wash buffer, and the pull-down antigens were eluted by boiling for 10 min at 100°C. The supernatant was collected after centrifugation at 12,000 rpm for 10 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Protein digestion and LC-MS/MS analysis were performed at GeneCreate's Proteomics Platform in Wuhan, China.

Bioinformatics analysis

The VaxiJen v2.0 program was used to predict the antigenicity of candidate proteins based on Gram-positive bacterial signals (https://www.ddg-pharmfac.net/vaxijen/vaxijen/vaxijen.html). The program predicts protein antigenicity using amino acid sequences with a default threshold of >0.4 (Safi et al., 2023). The Expasy online tool was used to predict protein stability and hydrophilicity (Fatoba et al., 2022). The average hydrophilicity coefficient (GRAVY) indicates hydrophilic proteins with negative values and hydrophobic proteins with positive

values. Protein stability is indicated by an instability index, with values <40 indicating stability and values >40 indicating instability. Signal peptide prediction was performed using the SignalP-5.0 online website (https://services.healthtech.dtu.dk/services/SignalP-5.0/) (Almagro Armenteros et al., 2019). The number of transmembrane helices in the protein was predicted using the TMHMM 2.0 server (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) (Santos Junior et al., 2020).

Sequence analysis

The reference strain and *C. perfringens* protein amino acid sequences were aligned using DNAMAN (v6.0) software. Linear epitopes of *C. perfringens* antigens were predicted using the SVMTriP online tool (https://sysbio.unl.edu/SVMTriP/prediction.php) (Yao et al., 2012).

Expression of recombinant antigen proteins

The primers were designed using the full genome sequence of *C. perfringens* strain 13 (BA000016.3), and the *entB, tmpC*, and *arcB* genes were amplified. The PCR products were inserted into expression vectors pET28a(+) or pET32a(+) using a homologous recombination approach to create recombinant expression plasmids pET28a-ArcB, pET32a-TmpC, and pET28a-EntB. The plasmids were then transformed into *E. coli* BL21(DE3) Rosetta to express the recombinant proteins. The bacterial strains were cultured in Luria broth (LB, Oxoid) medium at 37°C with either ampicillin (Sangon Biotech, Shanghai, China) or kanamycin (Sangon Biotech, Shanghai, China) until they reached the logarithmic growth phase, indicated by an OD600 of approximately 0.6. The cultures were induced for 4 h at 37°C using 0.5 mM IPTG. High-affinity Ni-NTA resin (GenScript; Nanjing, China) was used to purify the recombinant proteins. The BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to quantify the purified recombinant proteins.

Preparation of polyclonal antibodies against recombinant proteins

The recombinant protein was appropriately diluted and combined with an equal volume of QuickAntibody-Mouse 3W adjuvant (Biodragon, Suzhou, China). Female BALB/c mice, aged 6 weeks, were immunised with 50 μg of recombinant antigen intramuscularly in the leg every two weeks. One week after the second inoculation, a small blood sample was collected, and serum was separated to determine antibody titres. When the antibody titre exceeded 1:100,000, the serum was collected for subsequent experiments.

Antigenicity and subcellular localization analysis

Western blot analysis was performed to examine the antigenicity of *C. perfringens* recombinant antigens expressed by *E. coli*. Purified recombinant proteins (10 μg) were separated using SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with rabbit polyclonal antibodies against whole cell or secretory proteins of *C. perfringens* (1:1000). Following incubation with HRP-conjugated goat anti-rabbit IgG antibody (Bioss, Beijing, China), the membrane was analysed using ECL western blotting substrate.

Western blot analysis was performed on the cytoplasmic, secretory, and membrane fractions of *C. perfringens* cells using mouse anti-EntB, anti-TmpC, and anti-ArcB antisera, followed by HRP-conjugated goat anti-mouse IgG antibody to assess the subcellular localisation of the target antigens, as previously described.

Immunisation of chickens

To assess the immunoprotective effect of vaccine candidates, five groups of one-day-old chicks were randomly assigned (n = 10 per group). The group without vaccination and no challenge served as the blank control; the group without vaccination but challenged with *C. perfringens* served as the challenge control; the remaining groups were the EntB immunisation group, TmpC immune group, and ArcB immune group. For subunit vaccine candidate production, recombinant antigens were diluted with PBS to 2 mg/mL, combined with Montanide™ ISA 206 VG adjuvant in a 1:1 ratio, and thoroughly emulsified. Vaccination was performed on day 0 with 100 μg of each antigen protein in the vaccine formulation, administered via intramuscular injection (i.m.) into the leg muscles. Two booster immunisations were given on days 14 and 21 using the same dosage and method. The control group received PBS mixed with Montanide™ ISA 206 VG adjuvant. Blood samples were collected weekly from the wing vein of the control and vaccination groups, and serum was separated to measure antibody levels until week 8.

Challenge with C. perfringens

We used a necrotic enteritis challenge model induced by *Eimeria necatrix* (*E. necatrix*) with some modifications (Wang et al., 2022; Mohiuddin et al., 2023). At 23 days of age, all birds except for the blank control group were gavaged with 10,000 *E. necatrix* oocysts to induce NE. Starting on day 28, all birds (except the blank control) were given fresh *C. perfringens* bacterial culture, 1 mL per day by gavage, at a concentration of approximately 5×10^8 CFU/mL, for three consecutive days. No fasting was performed before gavage with *C. perfringens* bacteria.

The *C. perfringens* strain NE-1, stored at -70°C, was streaked onto sheep blood agar medium for revival (cultured at 37°C for 18-24 h). Ten single colonies were picked and inoculated into cooked meat medium and incubated anaerobically for 12 h. The bacteria were then inoculated at a 3 % (v/v) ratio into liquid thioglycolate medium supplemented with 5 % beef extract and grown at 37°C for 15 h as the challenge inoculum. Fresh broth culture was prepared and used daily (Jiang et al., 2015; Wang et al., 2022).

Intestinal lesion scoring and histopathological score

Seven chickens from each group were euthanised 4 h after the third *C. perfringens* challenge on day 30 to observe gross intestinal lesions. NE was scored using a 0-3 system: normal = 0, mild = 1 (mild mucus coating and loss of tension, thin or brittle wall), moderate = 2 (focal necrosis or ulceration), and severe = 3 (sloughed mucosa with blood in the lumen) (Hofacre et al., 1998; Richardson et al., 2017).

Duodenum tissues were collected for paraffin sectioning after dissection. H&E staining was performed for histopathological analysis. The following criteria were used for histopathological lesion scoring: 0, no inflammation, intact villi; 1, mild infiltration of inflammatory cells, relatively intact intestinal villi; 2, massive lymphocyte infiltration, villous swelling; 3, massive lymphocyte infiltration, villous necrosis or gland hyperplasia; 4, massive lymphocyte infiltration, diffuse villous necrosis, and shedding. The immune protection experiments for subunit candidate vaccines were conducted three times, and the experimental results showed the same trend; the data were pooled for analysis.

Lymphocyte proliferation analysis

Lymphocyte proliferation was assessed using the CCK-8 method following the manufacturer's instructions, with some modifications. The chicken peripheral blood lymphocyte isolation kit (Solarbio, Beijing, China) was used to isolate peripheral blood lymphocytes (PBL) from blood samples collected from birds one week after the final immunisation. PBL were seeded at a density of 2×10^5 cells per well in RPMI 1640 medium in a 96-well plate. The cells were incubated for 48 h at 37°C with 5% CO_2. Concanavalin A (ConA) or candidate antigen proteins were used to stimulate the cells during incubation. The candidate antigen proteins and conA were diluted to 20 μg/mL and 5 μg/mL,

respectively, in RPMI 1640 medium and then used to resuspend PBL. Cells treated with PBS served as a negative control. After 48 h of incubation, 10 μL of CCK-8 reagent (Solarbio, Beijing, China) was added to each well, and the plate was incubated for an additional 2 h at 37°C. The optical density (OD) at 450 nm was measured with an automated microplate reader. The experiment was conducted twice, and the data were pooled for analysis. Results are presented as a stimulation index (SI), where SI equals OD of antigen-stimulated cells/ PBS-stimulated cells.

Quantification of cytokine mRNA expression

Quantitative PCR was performed as previously described (Zhang et al., 2023a). PBL were cultured in 24-well plates at a density of 1×10^7 cells per well under the same conditions for 48 h. Cell samples were harvested for total RNA, which was then reverse-transcribed to cDNA. The mRNA expression of IFN-γ, IL-2, IL-4, and IL-6 in PBL was measured by qRT-PCR using the 7500 Fast Real-Time PCR System. The β-actin gene was used as an internal reference. The $2^{-\Delta\Delta CT}$ method was utilised for data analysis. The experiment was conducted twice, and the data were pooled for analysis. Primers are provided in Table 1.

Statistical analysis

All data are presented as means ± SD unless otherwise stated. Asterisks in the figures indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Differences among groups were analysed using the Mann-Whitney U test with GraphPad Prism 8 .0 software.

Results

Potential immunogenic proteins of C. perfringens were obtained by immunoprecipitation

To identify potential antigens with immunogenicity from *C. perfringens*, we employed immunoproteomics and reverse vaccinology approaches (Fig. 1). Initially, the whole bacterial proteins and secretory proteins of *C. perfringens* were extracted (Fig. 2A), and polyclonal sera against these proteins were prepared in rabbits. Subsequently, the antibodies were purified using Protein A/G affinity chromatography (Fig. 2B), and immunoprecipitation was performed separately with the whole bacterial proteins and secretory proteins of *C. perfringens* (Fig. 2C). The proteins pulled down by immunoprecipitation specifically reacted with rabbit polyclonal antibodies against whole cell proteins or secretory proteins of *C. perfringens* (Fig. 2D), indicating their potential immunogenicity. Ultimately, a total of 118 specific proteins with potential immunogenicity were identified from *C. perfringens* through immunoprecipitation and mass spectrometry analysis. Among these, 21 were secretory proteins, 107 were whole bacterial proteins, and 10 were present in both categories. With the exception of EF-TU (elongation factor Tu) and PFO (pyruvate ferredoxin oxidoreductase), all identified proteins have not been previously studied in the context of NE vaccine development.

Screening of potential candidate antigens

Antigens with high antigenicity, hydrophilicity, and stability are typically well-suited for efficient reactions and are relatively easy to produce and purify (Margaroni et al., 2023; Zhang et al., 2023c). The confidence score is used to evaluate and score candidate peptide sequences during peptide sequence identification by matching them with experimental tandem mass spectrometry (peptide spectrum matching). The higher the confidence score, the greater the accuracy of the identified protein (Ray et al., 2020). To screen for subunit candidate antigens from the proteins pulled down by immunoprecipitation, we predicted the antigenicity, hydrophilicity, stability, signal peptides, and transmembrane helices of the top 20 proteins with the highest confidence scores (Table 2). Antigenicity was predicted using VaxiJen 2.0 with a threshold of > 0.4, resulting in 16 proteins meeting the criteria. Signal peptides were predicted using the SignalP-5.0 online tool. Transmembrane helices were predicted using the TMHMM online tool, and hydrophilicity and stability were predicted using the Expasy online tool. Among the 16 proteins predicted to be antigenic, five were excluded due to hydrophobicity and instability. Proteins containing signal peptide structures without transmembrane structures are likely secretory proteins, which have advantages in immunity and production, such as TmpC and EntB. Although N-acetylglucosaminidase met the criteria, it was not selected due to its large molecular weight, which could complicate expression. Among the remaining proteins, ArcB was randomly selected. These three candidate proteins were subsequently tested as potential protective antigens. They were found to have over 97 % homology across the entire genome of 18 different subtypes of *C. perfringens* (Supplementary Table 1).

Homology analysis of candidate antigens in different species

To assess the amino acid sequence homology of candidate antigens across different species, homologous proteins of EntB, TmpC, and ArcB were randomly selected, and their amino acid sequences were aligned and analysed using DNAMAN software. As shown in Fig. 3, the amino

Table 1
The primers information.

Primer name	Sequences(5′–3′)	References
pET28a-EntB-EcoRI	ATGGGTCGCGGATCCGAATTCATGAATAGGAATAAGATAGCAGCTTTG	This study
pET28a-EntB-XhoI	GTGGTGGTGGTGGTGCTCGAGTTAAAGAACTCTTCTTGCTCTTCCTATT	
pET32a-TmpC-BamHI	GCCATGGCTGATATCGGATCCGGTGATACTGGTTCTAAGGGAGACA	
pET32a-TmpC-HindIII	CTCGAGTGCGGCCGCAAGCTTATTTATTAAGTCTCCTTGCTCATTAGAAA	
pET28a-ArcB-EcoR I	ATGGGTCGCGGATCCGAATTCATGGCAGTTAACTTAAAAGGAAGAAGC	
pET28a-ArcB-Xho I	GTGGTGGTGGTGGTGCTCGAGTTATCTTCCAGCAGTTGCTACCAT	
pET-28a-F	TAATACGACTCACTATAGGG	
pET-28a-R	GCTAGTTATTGCTCAGCGG	
IL-2-F	TCTGGGACCACTGTATGCTCT	Zhang et al. (2023a)
IL-2-R	ACACCAGTGGGAAACAGTATCA	
IFN-γ-F	AGCTGACGGTGGACCTATTATT	
IFN-γ-R	GGCTTTGCGCTGGATTC	
IL-4-F	TGAATGACATCCAGGGAGAG	
IL-4-R	GGCTTTGCATAAGAGCTCAG	
IL-6-F	CAAGGTGACGGAGGAGGAC	
IL-6-R	TGGCGAGGAGGGATTTCT	
β-actin-F	CAACACAGTGCTGTCTGGTGG	
β-actin-R	ATCGTACTCCTGCTTGCTGATCC	

Note: The underline represents the restriction enzyme site.

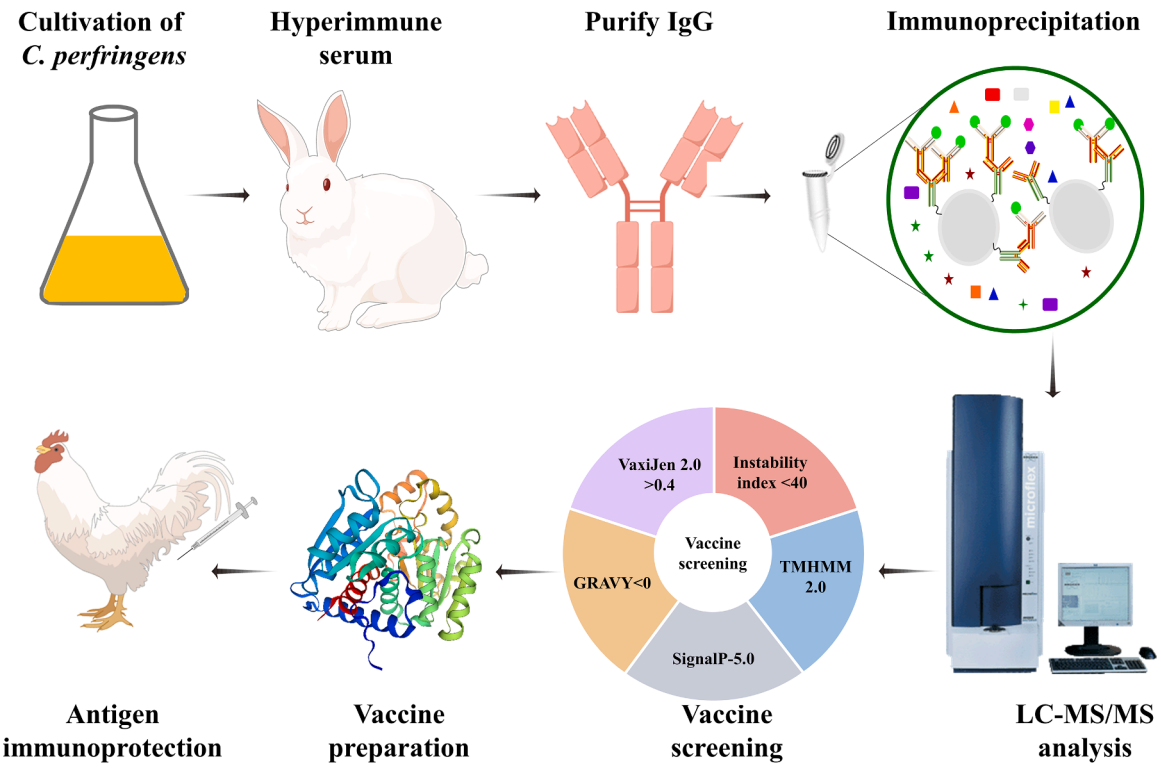


Fig. 1. Antigen screening flowchart for vaccine development. Initially, the whole bacterial proteins and secretory proteins of *C. perfringens* were extracted. High-titre sera were prepared by immunising rabbits, and the antibodies were purified. Immunoprecipitation was then performed separately with the secretory proteins and whole bacterial proteins of *C. perfringens*. Immunogenic proteins were identified via LC/MS-MS analysis. Candidate antigens were further evaluated based on antigenicity, instability index, hydrophilicity (GRAVY), and transmembrane signal peptide prediction. Finally, the immunogenicity and immune-protective efficacy of candidate antigens were determined through in vitro and in vivo experiments.

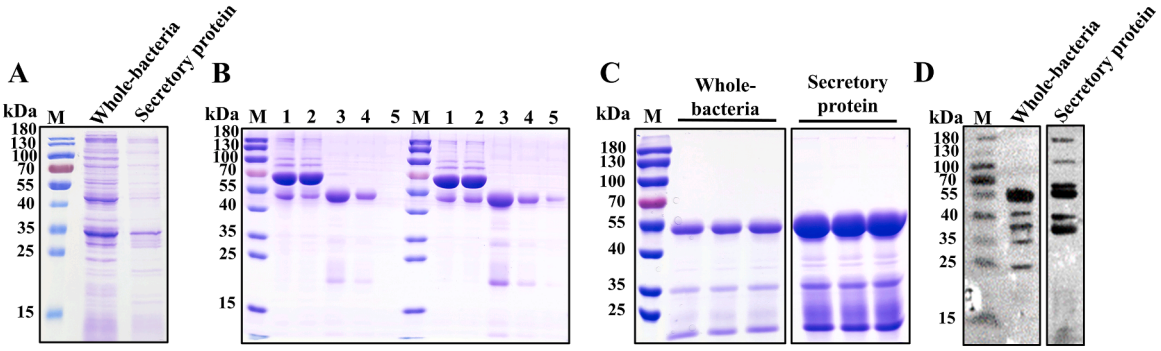


Fig. 2. Extraction of *C. perfringens* protein, purification of rabbit anti-*C. perfringens* antibodies, and analysis of immunoprecipitation. (A) Equal volumes (10 μL) of whole bacterial proteins and secretory proteins of *C. perfringens* were analysed by SDS-PAGE. (B) Purification of rabbit anti-*C. perfringens* antibodies by protein A/G affinity chromatography. Lane 1: rabbit serum before purification; Lane 2: the flow-through fraction; Lanes 3-5: purified rabbit anti-*C. perfringens* antibodies. (C) SDS-PAGE and (D) western blot analysis of *C. perfringens* protein immunoprecipitation eluate. The immunoprecipitation eluate (5 μL) was separated by SDS-PAGE and analysed by western blot with antibodies against *C. perfringens* whole bacterial proteins or secretory proteins.

acid homology of ArcB among different species is approximately 65.88 %. Regions of high homology include residues 44-64 aa, 84-140 aa, 221-237 aa, 269-277 aa, and 314-330 aa. The antigenic epitopes of the ArcB protein from *C. perfringens* were predicted using the SVMTriP online tool (Supplementary Table 2). The potential antigenic epitopes were found within the highly homologous regions of 84-140 aa, 221-237 aa, and 314-330 aa, suggesting that these regions are potential antigenic epitopes. The homology of EntB and TmpC proteins across different species is only 33.25 % and 44.03 %, respectively (Supplementary Figure 1).

The candidate antigens exhibit antigenicity and immunogenicity

To obtain highly purified antigen proteins, the candidate antigens were cloned into pET28a(+) or pET32a(+) vectors, named pET28a-EntB, pET32a-TmpC, and pET28a-ArcB, and transformed into *E. coli* BL21(DE3) Rosetta cells. The target proteins were induced under appropriate conditions to be expressed in soluble form, and the purified soluble proteins were obtained through Ni-NTA affinity chromatography (Fig. 4). The molecular weights of these three proteins were EntB

Table 2
Predicting the antigenicity, stability, and transmembrane signal of *C. perfringens* proteins.

No.	Accession No.	Proteins	Antigenicity score	Signal peptide	Instability index	GRAVY	TMHs
1	BAB82113.1	EF-Tu(Elongation factor Tu)	0.3218	Other	34.27	-0.204	0
2	BAB82237.1	AdhE(Aldehyde-alcohol dehydrogenase)	0.4548	Other	28.67	-0.080	0
3	BAB79875.1	ArcB(Ornithine carbamoyl transferase)	0.4492	Other	37.92	-0.362	0
4	BAB81406.1	RpsB(30S ribosomal protein S2)	0.3004	Other	38.92	-0.373	0
5	BAB81286.1	TmpC(Probable membrane lipoprotein TmpC)	0.5877	Sec/SPII	20.06	-0.299	0
6	BAB81764.1	Glutamate decarboxylase	0.5083	Other	34.15	-0.269	0
7	BAB81060.1	EntB(Probable enterotoxin)	0.9092	Sec/SPI	31.88	-0.580	0
8	BAB81855.1	PykA(Pyruvate kinase)	0.5216	Other	29.73	-0.017	0
9	BAB81037.1	RubY(Rubrerythrin)	0.4161	Other	33.01	-0.458	0
10	BAB80766.1	conserved hypothetical protein	0.4447	Other	43.38	0.050	1
11	BAB82049.1	probable maltose ABC transporter	0.3946	Sec/SPII	28.78	-0.250	1
12	BAB81973.1	Glucose-6-phosphate isomerase	0.4627	Other	39.51	-0.305	0
13	BAB81886.1	cell shape determining protein	0.3731	Other	35.24	0.059	0
14	BAB80937.1	N-acetylglucosaminidase	0.6137	Sec/SPI	28.07	-0.528	0
15	BAB81438.1	conserved hypothetical protein	0.4910	Other	40.25	-0.297	0
16	BAB81379.1	RecA	0.6260	Other	41.17	-0.198	0
17	BAB80774.1	FabH(3-oxoacyl-[acyl-carrier-protein] synthase 3)	0.5474	Other	30.31	-0.089	0
18	BAB80395.1	conserved hypothetical protein)	0.5320	Other	31.81	-0.358	0
19	BAB80003.1	TktC(Transketolase C-terminal section	0.5496	Other	29.40	0.066	0
20	BAB80527.1	PTS system	0.4248	Other	39.85	-0.310	0

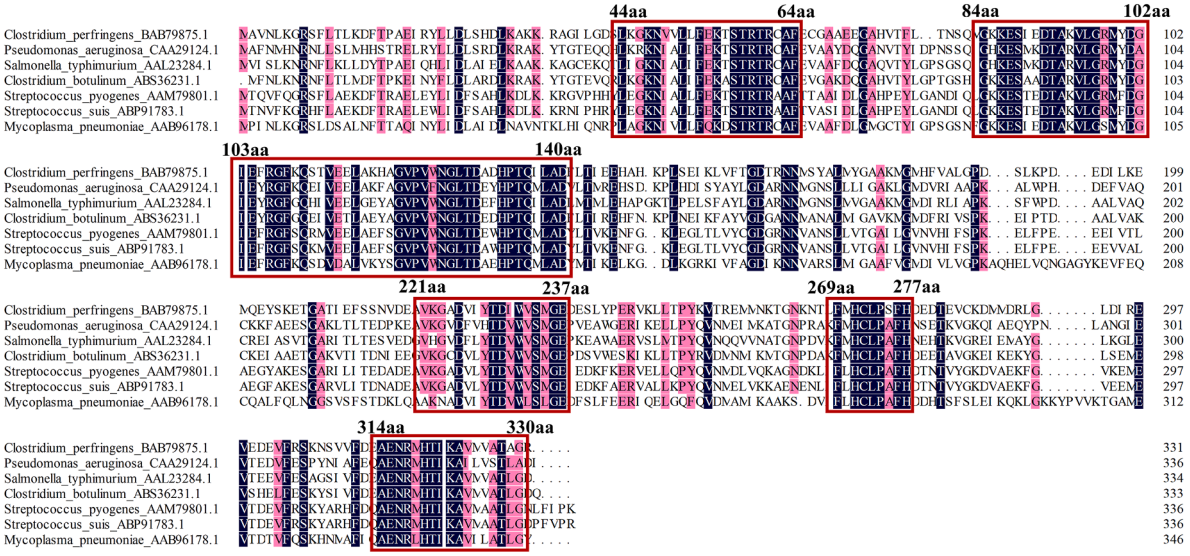


Fig. 3. Analysis of ArcB protein amino acid sequence homology from different species. The Amino acid homology of ArcB was analysed using DNAMAN (v6.0) software. Black shading represents 100 % homology, while pink shading represents homology greater than 75 %. The red box highlights regions of high homology.

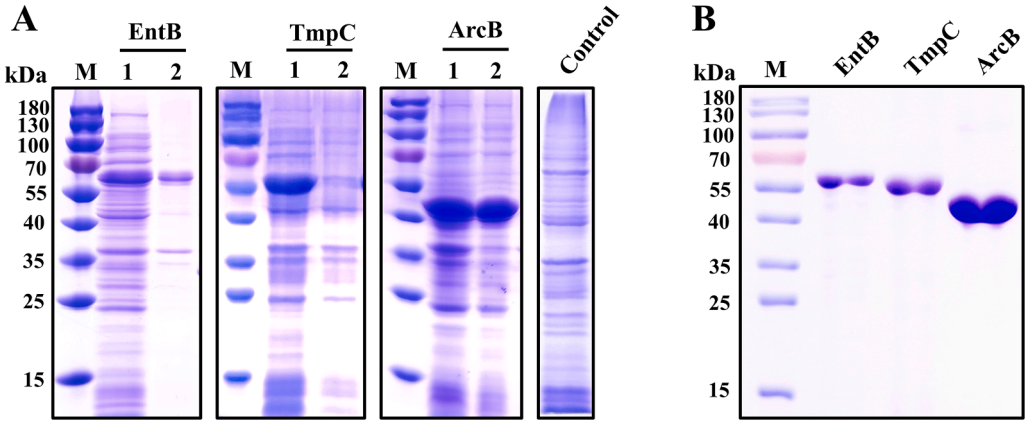


Fig. 4. SDS-PAGE analysis of recombinant protein expression and purification. Recombinant proteins were expressed in *E. coli* and purified using high-affinity Ni-NTA resin. **(A)** SDS-PAGE analysis of recombinant protein expression. Lane 1: lysis supernatant of expression strain; Lane 2: lysis precipitate of expression strain. **(B)** SDS-PAGE analysis of purified recombinant proteins.

(~59.5 kDa), TmpC (~55.3 kDa), and ArcB (~37.5 kDa).

To evaluate the immunogenicity of the antigens, polyclonal antisera against EntB, TmpC, and ArcB were prepared in mice. Western blot analysis was performed using recombinant protein antisera on subcellular fractions of *C. perfringens*. The antisera for all antigens exhibited specific reactions with subcellular fractions of *C. perfringens*. Consistent with the immunoprecipitation results, EntB was primarily expressed in the secretory proteins of *C. perfringens*, with minimal expression in the cytoplasm and membrane proteins, while TmpC and ArcB were mainly expressed in the cytoplasm (Fig. 5A).

To determine the antigenicity of the recombinant proteins, western blot analysis was performed using rabbit polyclonal antibodies against whole cell proteins or secretory proteins of *C. perfringens* with EntB, TmpC, and ArcB recombinant proteins. All three proteins reacted specifically with rabbit anti-*C. perfringens* antibodies, while the corresponding negative control showed no significant reaction (Fig. 5B and C). EntB exhibited specific reactions with both antibodies against whole bacterial proteins and secretory proteins of *C. perfringens*, while TmpC and ArcB showed specific reactions with antibodies against whole bacterial proteins only, confirming the results of the antigen immunogenicity tests.

Candidate antigens induce robust adaptive immune response

To assess the serum specific-IgY antibody response levels of the three subunit antigens in chickens, serum was collected weekly from two weeks after the initial immunisation, and indirect ELISA was used to detect serum antibody titres (Supplementary Figure 2). The specific-IgY titres of EntB, TmpC, and ArcB in chickens were $1 \times 10^{4.2}$, $1 \times 10^{3.7}$, and $1 \times 10^{4.1}$, respectively, one week after the third immunisation (Fig. 6B). The specific humoral immune responses induced by all three candidate antigens in chickens were significantly higher than those in the PBS immune group ($P < 0.01$), indicating that these antigens possess excellent immunogenicity.

Peripheral blood lymphocytes (PBL) were used to evaluate the cellular immune response elicited by candidate subunit vaccines in chickens. The proliferation level of lymphocytes induced by EntB, TmpC, and ArcB was significantly higher than that of PBL stimulated by PBS ($P < 0.01$; Fig. 6C). PBL stimulated with EntB, TmpC, and ArcB recombinant proteins generated substantially greater mRNA levels of IFN-γ, IL-2, IL-4, and IL-6 compared to PBS-treated cells ($P < 0.01$; Fig. 6D-G).

Vaccine candidates protect chickens against C. perfringens infection

Pathogenic *C. perfringens* strains were gavage-administered to experimental chickens daily from day 28 to day 30 after the third immunisation. The protective effect against NE was assessed by scoring intestinal lesions 4 h after the final infection. All three subunit vaccine candidates significantly reduced the intestinal lesion score compared with the challenge control ($P < 0.01$). Specifically, the challenge control showed blood clots or tightly attached cellulose exudation in the intestine lumen. When these exudates were removed, extensive punctate necrosis appeared in the intestinal mucosa. In contrast, all three subunit vaccine candidate immunisation groups markedly reduced *C. perfringens*-induced damage to the gut (Fig. 7A and B).

Histopathological observations revealed that the challenge control group exhibited intestinal gland hyperplasia (red arrows), villous necrosis and dissolution (blue arrows), with the surface covered by necrotic tissue and a large number of lymphocytes, compared to normal intestinal tissues. The EntB, TmpC, and ArcB immune groups showed relatively intact mucosal structures, with only villous swelling, a small amount of epithelial cell shedding, and minor lymphocyte infiltration (Fig. 8A). The challenge of *C. perfringens* resulted in significantly reduced inflammatory response and histopathological scores in the three subunit vaccine candidate immunised groups compared to the challenge control group ($P < 0.01$; Fig. 8B).

Discussion

NE caused by *C. perfringens* is a serious intestinal disease in poultry production, and there is currently no effective approach to prevent the onset of NE (Alizadeh et al., 2021). In this study, we examined three potential protective antigens that demonstrated considerable immunogenicity and could offer excellent protection against *C. perfringens* infection in chickens.

The immunogenicity and antigenicity of antigens are critical for the development of subunit vaccines, as they determine whether the vaccine can induce sufficient and long-lasting protective immunity after immunisation (Zhang et al., 2023b). In this study, the three selected candidate antigens exhibited excellent antigenicity and immunogenicity, indicating their potential as attractive antigen targets. The capacity of subunit antigens to trigger early specific immune responses in chickens is an important indicator of their vaccine potential, as it provides immune protection for chickens before they become susceptible to NE (Cooper and Songer, 2009). Studies have shown that chickens have depleted maternal antibodies at three weeks of age, a critical period for the prevalence of NE (Keyburn et al., 2013). In our research, the specific-IgY titres of EntB, TmpC, and ArcB in chickens were all significantly higher than those in the PBS immune group at three weeks of age, indicating that these candidate antigens induced strong specific humoral immune responses in chickens when antibodies were most needed.

Classically, antigens secreted into the extracellular space are more

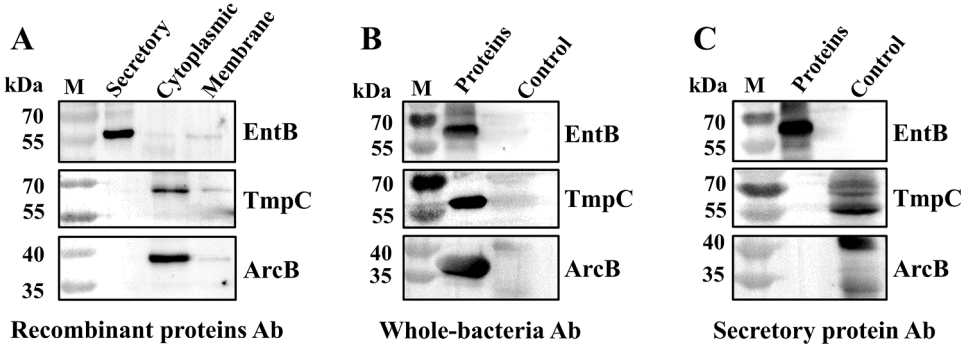


Fig. 5. Immunogenicity and antigenicity analysis of candidate antigens. (A) Immunogenicity and subcellular localisation of candidate antigens were investigated using western blot analysis. Secretory, cytoplasmic, and membrane proteins (10 μg each) were separated by SDS-PAGE. The membrane was incubated with anti-EntB, anti-TmpC, and anti-ArcB antisera. To evaluate antigenicity, western blot analysis was performed by incubating the recombinant proteins (10 μg each) with antibodies against the whole bacterial protein **(B)** or secretory protein **(C)** of *C. perfringens*.

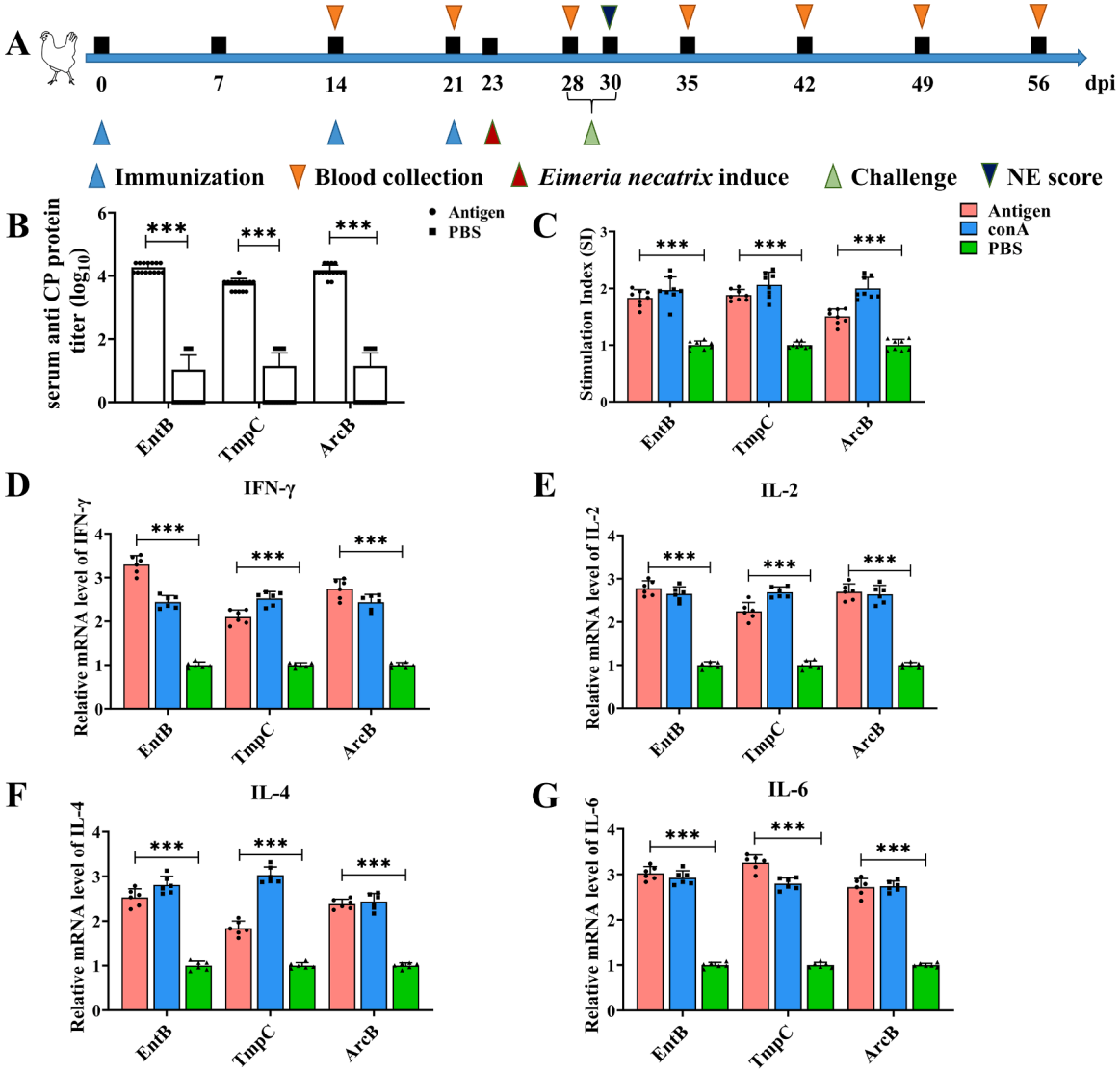


Fig. 6. Specific immune response induced by candidate antigens in chickens. (A) Immunisation and challenge procedures for subunit vaccine candidates. The experiment was conducted in triplicate. **(B)** Antigen specific-IgY titres in serum were measured by ELISA one week after the third vaccination. **(C)** Proliferative response of peripheral blood lymphocytes in chickens measured using the CCK-8 method. **(D-G)** Quantification of IFN-γ, IL-2, IL-4, and IL-6 mRNA transcript levels in lymphocytes using RT-qPCR. *P* values were calculated by Mann-Whitney U test (ns, not significant; $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). Data are expressed as mean ± S.D.

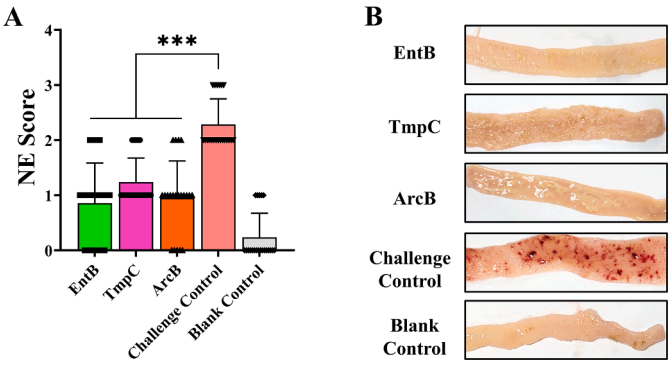


Fig. 7. Intestinal lesion score. Four hours after the final challenge, all birds were euthanised and dissected for observation of intestinal lesions **(A)** and NE lesion scores **(B)**. The experiment was conducted in triplicate ($n = 7$). *P* values were calculated by Mann-Whitney U test (ns, not significant; $^{***}P < 0.001$). Data are expressed as mean ± S.D.

easily recognised by antigen-presenting cells (APCs), thereby activating a more significant immune response (Xin et al., 2008). In this study, among the three proteins predicted to have high antigenicity, EntB was identified as a secretory protein. Consistent with the research of Xin et al., the cellular responses and antibody titres induced by EntB were the highest among the three proteins (Xin et al., 2008), indicating that the secretory nature of EntB confers better antigen accessibility and immunogenicity. Heidarpanah et al. immunised broiler chickens with five surface or secreted proteins from virulent *C. perfringens*, producing high-specificity serum IgY titres and providing partial protection against necrotic enteritis in broiler chickens (Heidarpanah et al., 2023a). Our results demonstrate a feasible approach to screening antigens, prioritising those with secretory properties or surface localisation.

Activated innate immunity promotes the maturation of APCs and the activation of T cells, which are critical for the protective immune response elicited by vaccines (Iwasaki and Medzhitov, 2010; Ko et al., 2022). In this study, all three proteins induced a significant upregulation of IL-6, with TmpC inducing the highest expression level of IL-6. These

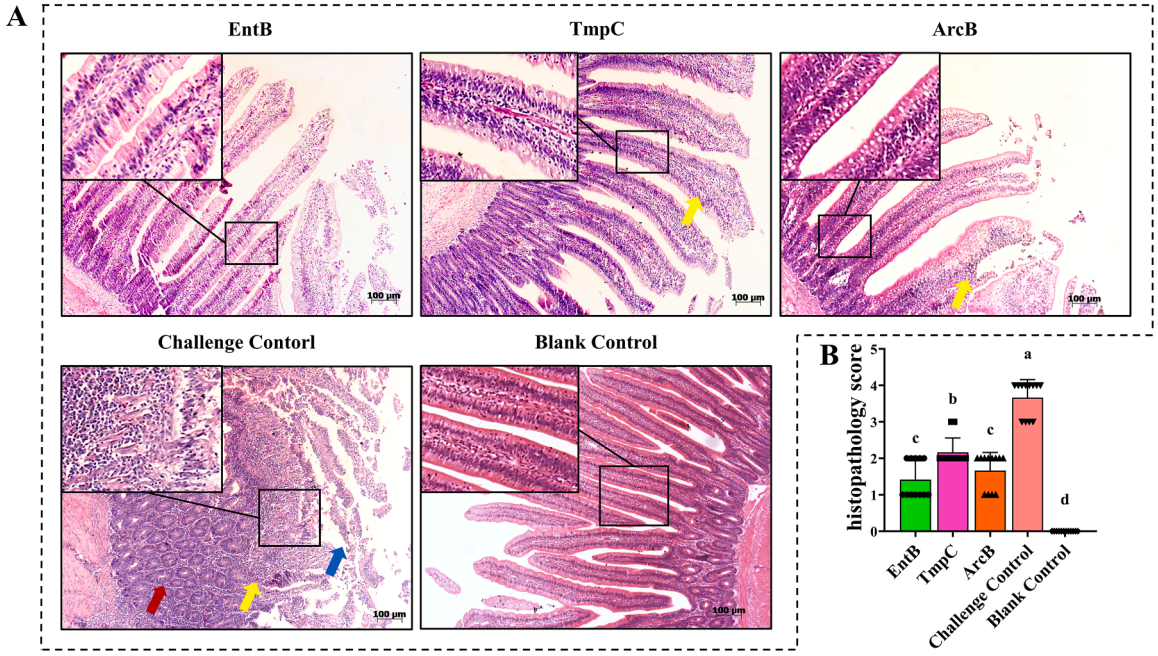


Fig. 8. Duodenum histopathology. During the observation of gross lesions, the duodenum was collected, processed into paraffin sections, and histopathological examination was performed using H&E staining on day 30. **(A)** Representative histopathology images of intestinal tissue stained with H&E. The red arrow indicates glandular hyperplasia, the yellow arrow indicates lymphocyte infiltration, and the blue arrow indicates villous shedding. **(B)** Histopathology lesion score. Scale bar = 100 μm. Distinct letters indicate significant differences between groups ($P < 0.05$). *P* values were calculated by Mann-Whitney U test. Data are expressed as mean ± S.D.

findings suggest that TmpC induces innate immunity while promoting the elevation of acquired immunity, thereby facilitating an immune response to *C. perfringens* infection through a systemic approach.

The *ArcB* gene encodes ornithine carbamoyltransferase, an enzyme widely distributed in bacteria (Oosthuizen et al., 2002; Yi et al., 2020). In our research, the amino acid homology of ArcB in different bacteria is approximately 65.88 %. Regions with high homology, such as 84-140 aa, 221-237 aa, and 314-330 aa, are potential epitope enrichment regions, suggesting that when considering the immunogenicity of ArcB proteins in other bacterial species, focus should be placed on the aforementioned amino acid residue sites.

NE is a multifactorial disease, and previous NE models have been unstable (Fries-Craft et al., 2023). In this study, we induced NE by *E. necatrix* inoculation for five days prior to the *C. perfringens* challenge, resulting in moderate to severe NE lesions in all birds in the challenge control group. All three candidate antigens significantly reduced NE lesion scores and histopathological lesion scores compared to the challenge control group, indicating that these subunit vaccine candidates provide significant immune protection against *C. perfringens* infection in chickens.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Huoying Shi reports financial support was provided by National Natural Science Foundation of China. Huoying Shi reports financial support was provided by Natural Science Foundation of Jiangsu Province. Huoying Shi reports financial support was provided by Jiangsu Province Science and Technology Program Special Fund Project. Huoying Shi reports financial support was provided by National Key R&D Program of China. Huoying Shi reports financial support was provided by Postgraduate Research & Practice Innovation Program of Jiangsu Province. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by Jiangsu Province Science and Technology Program Special Fund Project (BZ2022042); National Key R&D Program of China (2023YFE0123500); the National Natural Science Foundation of China (32302823, 32172802, 32002301, 31672516, 31172300, 30670079); Natural Science Foundation of Jiangsu Province (BK20230576); Postgraduate Research & Practice Innovation Program of Jiangsu Province (Yangzhou University; No. SJCX21_1641); Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and supported by the 111 Project D18007.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104436.

References

Alizadeh, M., Shojadoost, B., Boodhoo, N., Astill, J., Taha-Abdelaziz, K., Hodgins, D.C., Kulkarni, R.R., Sharif, S., 2021. Necrotic enteritis in chickens: a review of pathogenesis, immune responses and prevention, focusing on probiotics and vaccination. Anim. Health Res. Rev. 22, 147–162.

Almagro Armenteros, J.J., Tsirigos, K.D., Sønderby, C.K., Petersen, T.N., Winther, O., Brunak, S., von Heijne, G., Nielsen, H., 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 37, 420–423.

Burman, C., Findlow, J., Marshall, H.S., Safadi, M.A.P., 2023. National and regional differences in meningococcal vaccine recommendations for individuals at an increased risk of meningococcal disease. Expert Rev. Vaccines 22, 839–848.

Cooper, K.K., Songer, J.G., 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe 15, 55–60.

Fatoba, A.J., Adeleke, V.T., Maharaj, L., Okpeku, M., Adeniyi, A.A., Adeleke, M.A., 2022. Design of a multiepitope vaccine against chicken anemia virus disease. Viruses 14, 1456.

Fernandes Da Costa, S.P., Mot, D., Bokori-Brown, M., Savva, C.G., Basak, A.K., Van Immerseel, F., Titball, R.W., 2013. Protection against avian necrotic enteritis after immunisation with NetB genetic or formaldehyde toxoids. Vaccine 31, 4003–4008.

Fries-Craft, K., Graham, D., Hargis, B.M., Bobeck, E.A., 2023. Evaluating a *Salmonella* Typhimurium, *Eimeria maxima*, and *Clostridium perfringens* coinfection necrotic enteritis model in broiler chickens: repeatability, dosing, and immune outcomes. Poult. Sci. 102, 103018.

Gaucher, M.-L., Quessy, S., Letellier, A., Arsenault, J., Boulianne, M., 2015. Impact of a drug-free program on broiler chicken growth performances, gut health, *Clostridium perfringens* and *Campylobacter jejuni* occurrences at the farm level. Poult. Sci. 94, 1791–1801.

Gharib-Naseri, K., Dorigam, J.C.P., Doranalli, K., Morgan, N., Swick, R.A., Choct, M., Wu, S.-B., 2021. Bacillus amyloliquefaciens CECT 5940 improves performance and gut function in broilers fed different levels of protein and/or under necrotic enteritis challenge. Anim. Nutr. 7, 185–197.

Heidarpanah, S., Thibodeau, A., Parreira, V.R., Quessy, S., Segura, M., Gottschalk, M., Gaudreau, A., Juette, T., Gaucher, M.-L., 2023a. Evaluation of the immunoprotective capacity of five vaccine candidate proteins against avian necrotic enteritis and impact on the caecal microbiota of vaccinated birds. Animals (Basel) 13, 3323.

Heidarpanah, S., Thibodeau, A., Parreira, V.R., Quessy, S., Segura, M., Meniaï, I., Gottschalk, M., Gaudreau, A., Juette, T., Gaucher, M.-L., 2023b. Immunization of broiler chickens with five newly identified surface-exposed proteins unique to *Clostridium perfringens* causing necrotic enteritis. Sci. Rep. 13, 5254.

Hofacre, C.L., Froyman, R., Gautrias, B., George, B., Goodwin, M.A., Brown, J., 1998. Use of Aviguard and other intestinal bioproducts in experimental *Clostridium perfringens*-associated necrotizing enteritis in broiler chickens. Avian Dis. 42, 579–584.

Innes, G.K., Randad, P.R., Korinek, A., Davis, M.F., Price, L.B., So, A.D., Heaney, C.D., 2020. External societal costs of antimicrobial resistance in humans attributable to antimicrobial use in livestock. Annu. Rev. Public Health 41, 141–157.

Iwasaki, A., Medzhitov, R., 2010. Regulation of adaptive immunity by the innate immune system. Science 327, 291–295.

Jiang, F., Han, Y., Liu, Y., Xue, Y., Cheng, P., Xiao, L., Gong, W., 2023. A comprehensive approach to developing a multi-epitope vaccine against *Mycobacterium tuberculosis*: from in silico design to in vitro immunization evaluation. Front. Immunol. 14, 1280299.

Jiang, Y., Mo, H., Willingham, C., Wang, S., Park, J., Kong, W., Roland, K.L., Curtiss, R., 2015. Protection against necrotic enteritis in broiler chickens by regulated delayed lysis *Salmonella* vaccines. Avian Dis. 59, 475–485.

Keyburn, A.L., Portela, R.W., Ford, M.E., Bannam, T.L., Yan, X.X., Rood, J.I., Moore, R.J., 2013. Maternal immunization with vaccines containing recombinant NetB toxin partially protects progeny chickens from necrotic enteritis. Vet. Res. 44, 108.

Ko, C.-N., Zang, S., Zhou, Y., Zhong, Z., Yang, C., 2022. Nanocarriers for effective delivery: modulation of innate immunity for the management of infections and the associated complications. J. Nanobiotechnology 20, 380.

Lau, Y.T., Tan, H.S., 2023. *Acinetobacter baumannii* subunit vaccines: recent progress and challenges. Crit. Rev. Microbiol. 0, 1–16.

Margaroni, M., Agallou, M., Tsanaktsidou, E., Kammona, O., Kiparissides, C., Karagouni, E., 2023. Immunoinformatics approach to design a multi-epitope nanovaccine against *Leishmania Parasite*: elicitation of cellular immune responses. Vaccines (Basel) 11, 304.

Meniaï, I., Thibodeau, A., Quessy, S., Parreira, V.R., Fravalo, P., Beauchamp, G., Gaucher, M.-L., 2021. Putative antigenic proteins identified by comparative and subtractive reverse vaccinology in necrotic enteritis-causing *Clostridium perfringens* isolated from broiler chickens. Bmc Genomics [Electronic Resource] 22, 890.

Mishra, N., Smyth, J.A., 2017. Oral vaccination of broiler chickens against necrotic enteritis using a non-virulent NetB positive strain of *Clostridium perfringens* type A. Vaccine 35, 6858–6865.

Mohiuddin, M., Song, Z., Liao, S., Qi, N., Li, J., Lv, M., Lin, X., Cai, H., Hu, J., Liu, S., Zhang, J., Gu, Y., Sun, M., 2023. Animal model studies, antibiotic resistance and toxin gene profile of NE reproducing *Clostridium perfringens* Type A and Type G strains isolated from commercial poultry farms in China. Microorganisms 11, 622.

Moore, R.J., 2024. Necrotic enteritis and antibiotic-free production of broiler chickens: challenges in testing and using alternative products. Anim. Nutr. 16, 288–298.

Mot, D., Timbermont, L., Delezie, E., Haesebrouck, F., Ducatelle, R., Van Immerseel, F., 2013. Day-of-hatch vaccination is not protective against necrotic enteritis in broiler chickens. Avian Pathol. 42, 179–184.

Oosthuizen, M.C., Steyn, B., Theron, J., Cosette, P., Lindsay, D., Von Holy, A., Brözel, V. S., 2002. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl. Environ. Microbiol. 68, 2770–2780.

Qiao, Q., Song, M., Song, C., Zhang, Y., Wang, X., Huang, Q., Wang, B., Yang, P., Zhao, S., Li, Y., Wang, Z., Zhao, J., 2021. Single-dose vaccination of recombinant chimeric Newcastle Disease Virus (NDV) LaSota vaccine strain expressing Infectious Bursal Disease Virus (IBDV) VP2 Gene provides full protection against genotype VII NDV and IBDV challenge. Vaccines (Basel) 9, 1483.

Ray, T.A., Cochran, K., Kozlowski, C., Wang, J., Alexander, G., Cady, M.A., Spencer, W.J., Ruzycki, P.A., Clark, B.S., Laeremans, A., He, M.-X., Wang, X., Park, E., Hao, Y., Iannaccone, A., Hu, G., Fedrigo, O., Skiba, N.P., Arshavsky, V.Y., Kay, J.N., 2020. Comprehensive identification of mRNA isoforms reveals the diversity of neural cell-surface molecules with roles in retinal development and disease. Nat. Commun. 11, 3328.

Richardson, K., Hofacre, C., Mathis, G., Lumpkins, B., Phillips, R., 2017. Impact of controlling bacteria in feed on broiler performance during a Clostridial challenge. Avian Dis. 61, 453–456.

Safi, A.U.R., Mansour Salih, M., Rahman, H., Khattak, B., El Askary, A., Hussain Khalifa, E., Qasim, M., 2023. Immunoaffinity-based mass spectrometric characterization of immunoreactive proteins of *Salmonella* Typhi. Saudi J. Biol. Sci. 30, 103502.

Santos Junior, M.N., Santos, R.S., Neves, W.S., Fernandes, J.M., de Brito Guimarães, B.C., Barbosa, M.S., Silva, L.S.C., Gomes, C.P., Rezende, I.S., Oliveira, C.N.T., de Macêdo Neres, N.S., Campos, G.B., Bastos, B.L., Timenetsky, J., Marques, L.M., 2020. Immunoinformatics and analysis of antigen distribution of *Ureaplasma diversum* strains isolated from different Brazilian states. BMC Vet Res 16, 379.

Teng, W.L., Bannam, T.L., Parsons, J.A., Rood, J.I., 2008. Functional characterization and localization of the TcpH conjugation protein from *Clostridium perfringens*. J. Bacteriol. 190, 5075–5086.

To, H., Suzuki, T., Kawahara, F., Uetsuka, K., Nagai, S., Nunoya, T., 2017. Experimental induction of necrotic enteritis in chickens by a netB-positive Japanese isolate of *Clostridium perfringens*. J. Vet. Med. Sci. 79, 350–358.

Wang, S., Hofacre, C.L., Wanda, S.-Y., Zhou, J., Callum, R.A., Nordgren, B., Curtiss, R., 2022. A triple-sugar regulated *Salmonella* vaccine protects against *Clostridium perfringens*-induced necrotic enteritis in broiler chickens. Poult. Sci. 101, 101592.

Wang, N., Qiu, C., Chen, M., Liu, T., Wang, T., 2019. Covering aluminum oxide nanoparticles with biocompatible materials to efficiently deliver subunit vaccines. Vaccines (Basel) 7, 52.

Xin, W., Wanda, S.-Y., Li, Y., Wang, S., Mo, H., Curtiss, R., 2008. Analysis of Type II secretion of recombinant *Pneumococcal* PspA and PspC in a *Salmonella* enterica Serovar Typhimurium vaccine with regulated delayed antigen synthesis. Infect. Immun. 76, 3241–3254.

Yao, B., Zhang, L., Liang, S., Zhang, C., 2012. SVMTriP: A method to predict antigenic epitopes using support vector machine to integrate tri-peptide similarity and propensity (A Porollo, Ed.). PLoS One 7, e45152.

Yi, L., Li, J., Fan, Q., Mao, C., Jin, M., Liu, Y., Sun, L., Grenier, D., Wang, Y., 2020. The otc gene of *Streptococcus suis* plays an important role in biofilm formation, adhesion, and virulence in a murine model. Vet. Microbiol. 251, 108925.

Zhang, G., Han, L., Li, Z., Chen, Y., Li, Q., Wang, S., Shi, H., 2023a. Screening of immunogenic proteins and evaluation of vaccine candidates against *Mycoplasma synoviae*. npj Vaccines 8, 121.

Zhang, Y., Xu, J., Hu, Z., Fan, X., 2023b. Advances in protein subunit vaccines against tuberculosis. Front. Immunol. 14, 1238586.

Zhang, T., Zhang, M., Xu, Z., He, Y., Zhao, X., Cheng, H., Chen, X., Xu, J., Ding, Z., 2023c. The screening of the protective antigens of *Aeromonas hydrophila* using the reverse vaccinology approach: potential candidates for subunit vaccine development. Vaccines (Basel) 11, 1266.