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# Evaluation of an M cell-targeted FedA fusion protein as an oral vaccine candidate for post-weaning diarrhea

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**Abstract**

Post-weaning diarrhea (PWD), primarily caused by enterotoxigenic *Escherichia coli* (ETEC), remains a major constraint on swine production worldwide due to its impact on piglet health, growth performance, and farm economics. Fimbrial adhesins F4 and F18 play central roles in ETEC pathogenesis by mediating intestinal colonization. However, effective vaccines against $F18^+$ ETEC are still lacking, largely because of the weak immunogenicity and structural instability of F18 fimbriae. The major fimbrial subunit FedA has emerged as a promising antigen candidate, but successful oral vaccination requires efficient antigen delivery to mucosal immune sites. In this study, we aimed to enhance FedA immunogenicity by fusing it to PEP, an M cell-targeting peptide designed to improve antigen uptake in the intestinal mucosa. The *fedA* gene was cloned into the pET22b-*pep* vector to generate the pET22b-*pep-fedA* construct and expressed in *E. coli* BL21(DE3). The recombinant PEP-FedA fusion protein was purified to greater than 90% homogeneity using immobilized metal affinity chromatography. Oral immunization in mice elicited robust mucosal and systemic immune responses, as evidenced by significantly increased fecal secretory IgA and serum IgG levels compared with FedA alone. Moreover, fecal IgA specifically recognized $F18^+$ ETEC without cross-reactivity to $F4^+$ ETEC. These findings demonstrate that PEP-mediated M cell targeting substantially enhances FedA immunogenicity and highlight the potential of this strategy for developing effective oral vaccines against PWD.

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## Introduction

The swine industry plays a vital role in Vietnam's food supply chain. However, its development has been challenged by several infectious diseases, among which post-weaning diarrhea (PWD) represents one of the most serious threats both in Vietnam and worldwide. PWD typically occurs during the first two weeks after weaning and is characterized by sudden death, dehydration, diarrhea, and growth retardation (1). It can manifest not only as sporadic cases but also as outbreaks with mortality rates reaching approximately 30% (2). Multiple factors contribute to the onset of PWD, including environmental conditions, dietary changes, and microbial infections (3). Among pathogens, enterotoxigenic *Escherichia coli* (ETEC) is most frequently associated with the disease (4). The pathogenicity of ETEC is largely determined by two virulence factors: adhesins and enterotoxins. The main enterotoxins include heat-labile toxin (LT, detected in 31.9% of isolates) and two heat-stable toxins (STa in 38.1% and STb in 59.1%), which cause diarrhea by reducing intestinal absorption while increasing secretion of fluids and electrolytes (5). The two predominant ETEC

adhesins are F4 (45.1%) and F18 (33.9%) (5). These fimbrial adhesins facilitate bacterial colonization of the small intestine through receptor binding, enabling the secretion of enterotoxins that trigger diarrhea. Adhesion is receptor-dependent: $F4^+$ ETEC strains bind to the F4 receptor (F4R), while $F18^+$ ETEC strains bind to the F18 receptor (F18R). Importantly, receptor expression varies with piglet age. F4R is highly expressed in neonatal piglets, making $F4^+$ ETEC infections more common in this age group (6). Meanwhile, F18R expression increases from the third week of life onward, which explains why $F18^+$ ETEC is the predominant cause of PWD in post-weaning piglets (7).

Currently, antibiotics such as enrofloxacin, spectinomycin, and colistin are widely used to control PWD in piglets. However, their effectiveness is diminishing due to the rapid emergence of antibiotic-resistant bacteria through horizontal gene transfer (8). This issue is particularly critical for colistin, which is considered a last-resort treatment for multidrug-resistant Gram-negative infections in humans. Consequently, there is an urgent need to reduce antibiotic usage in livestock and to develop novel strategies for controlling PWD. Vaccination represents a powerful, cost-effective, and sustainable approach to protect pigs against ETEC. At present, commercially available oral vaccines provide intestinal protection against both $F4^+$ and $F18^+$ ETEC strains associated with PWD in piglets (Coliprotec F4/F18®) (9). As a live bacterial preparation, this vaccine is highly sensitive to antibiotics and disinfected water. Moreover, it carries a potential risk of environmental dissemination of the live strain, and its capacity for modification or expansion of antigenic components is relatively limited. These limitations could be effectively addressed through the development and application of recombinant vaccine platforms. The F18 fimbriae are composed of a major structural subunit, FedA, along with minor components FedB, FedC, FedE, and FedF (10). Among these, the major subunit FedA has been identified as a promising antigen candidate for vaccine development. As with other enteric diseases, oral vaccination is considered an attractive strategy for PWD prevention because it can stimulate mucosal immunity at the site of infection. However, oral delivery faces significant challenges, including the harsh gastrointestinal environment characterized by acidic pH and proteolytic enzymes as well as the risks of immune tolerance and antigen dispersion across the extensive intestinal surface.

One promising strategy to enhance antigen uptake by immune-associated cells is to target a specific subset of intestinal epithelial cells known as microfold (M) cells. By accelerating antigen delivery to the target location, this strategy minimizes degradation caused by the harsh gastrointestinal environment. M cells are characterized by a thin glycocalyx and dome-like structures that overlay immune cells such as B cells and dendritic cells, thereby facilitating mucosal immune activation (11). M cells constitute a specialized epithelial subset within the follicle-associated epithelium of Peyer's patches and are estimated to account for approximately 20% of the epithelial cell population in this region (12). Owing to their relatively elevated expression compared with neighboring enterocytes, cytokeratin 18 is commonly employed as a histological marker for M cells identification (13). Because M cells are relatively scarce in the small intestine, efficient targeting requires interaction with specific surface receptors (14). Several receptor-ligand systems have been investigated, including lectins, monoclonal antibodies, and microbial proteins. Among these, microbial proteins represent an attractive option due to their ease of production and cost-effectiveness. Previous studies have shown that heat shock protein 60 (Hsp60) can interact with the prion protein receptor (PrPC) on the M cell surface (15). This receptor is a glycoprotein that is broadly expressed across mammalian species and is highly conserved between mice and swine. To improve vaccine delivery, ligands with small size and simple structure are preferred, as they can be readily fused to antigens without compromising antigenicity or eliciting undesired immune responses. Based on structural modeling of Hsp60, a peptide named PEP was predicted via bioinformatic analysis to interact with both murine PrPC (16) and swine counterpart (data not shown).

## Materials and methods

### Statement of Animal Ethics

Animals were maintained in the experimental animal facility, and experiments were performed in accordance with the EU Directive 2010/63/EU guideline approved by the Animal Care and Use Committee of the University of Science in Ho Chi Minh City (code: 915/GXN-KHTN).

### FedA antigen

In this study, the FedA antigen was fused with the PEP peptide to its C-terminus, cloned, and expressed (Figure 1).


Figure 1: Schematic representation of the PEP-FedA fusion protein.

### Strains and plasmids

*E. coli* DH5α and *E. coli* BL21(DE3) were used as host strains for DNA cloning and protein expression, respectively. The plasmids pET22b-*pep-gfp* and pET22b served as cloning vectors. $F4^+$ and $F18^+$ ETEC strains were kindly provided by A/Prof. Dr. Vu Khac Hung (Central

Vietnam Veterinary Institute, Khanh Hoa, Vietnam) (17). A polyclonal antibody against F18$^+$ ETEC was generously provided by Prof. Dr. Eric Cox (Ghent University, Belgium).

**Construction of pET22b-*pep-fedA* and pET22b-*fedA* vectors**

Both pET22b-*pep-fedA* and pET22b-*fedA* vectors were constructed using the All-In-One (AIO) method (18), based on the pET22b-*pep-gfp* and pET22b backbones, respectively. The *fedA* gene was amplified by PCR from the F18$^+$ ETEC strain using two sets of oligonucleotide primers: FedAFBamHI/FedARXhoI or FedAFNcoI/FedARXhoI (Table 1). The resulting PCR products and corresponding plasmids were digested with restriction enzymes (AIO products; Thermo Scientific), and ligated using T4 DNA ligase (Thermo Scientific). Recombinant plasmids were introduced into *E. coli* DH5α via chemical transformation, and transformants were selected on LB agar (Peptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L, pH 7.0) supplemented with ampicillin (Biobasic) at a final concentration of 100 µg/mL (LB-Amp). Candidate colonies were screened by PCR using the plasmid-specific primer T7pro and the gene-specific primer FedARXhoI (Table 1). Positive clones were verified by DNA sequencing to confirm in-frame cloning.

Table 1**:** List of enzymes and primers used in this study

| Plasmid | Enzyme | Primer name | Primer sequences (5' - 3') |
|---|---|---|---|
| pET22b-*pep-fedA* | *Bam*HI | FedAFBamHI | GGATCCGGTCAGCAAGGGGATGTTAAATT |
| | *Xho*I | FedARXhoI | CTCGAGCTTGTAAGTAACCGCGTAAGCC |
| pET22b-*fedA* | *Nco*I | FedAFNcoI | CCATGGGGTCAGCAAGGGGATGTTAAATT |
| | *Xho*I | FedARXhoI | CTCGAGCTTGTAAGTAACCGCGTAAGCC |
| - | - | T7pro | TAATACGACTCACTATAGGG |

Underlined letters denote restriction enzyme.

**Expression of PEP-FedA and FedA proteins in *E. coli* BL21(DE3)**

The recombinant vectors pET22b-*pep-fedA* and pET22b-*fedA* were transformed into competent *E. coli* BL21(DE3) cells and selected on LB-Amp. Colonies were further screened by colony PCR using primers T7pro and FedARXhoI. Positive clones were inoculated into 5 mL LB-Amp broth and grown at 37°C for 16h with shaking, followed by 1:10 (v/v) subculture. When the cultures reached an $OD_{600}$ of 0.8–1.0, protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Biobasic). Cultures were incubated overnight at 16°C. Cell pellets were harvested and disrupted by sonication in PBS (phosphate buffered saline) on ice to obtain total, soluble, and insoluble fractions. Negative controls were prepared in parallel without IPTG induction. Expression was analyzed by 15% SDS-PAGE followed by Coomassie Brilliant Blue staining, and confirmed by Western blotting using a HRP-conjugated anti-polyHis antibody at a 1:10,000 dilution in PBS-T (phosphate buffered saline + 0.05% Tween-20; Proteintech). Signals were visualized using 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific).

**Purification of PEP-FedA and FedA proteins**

For purification, biomass from 100 mL induced cultures was sonicated on ice, and the soluble fraction was used as input material. Both PEP-FedA and FedA proteins were purified by immobilized metal affinity chromatography using an HP HiTrap column (GE Healthcare, USA). The column was equilibrated with binding buffer (20 mM phosphate, pH 7.4), loaded with the soluble protein solution, washed with washing buffer (20 mM phosphate, 75 mM imidazole, pH 7.4), and eluted with elution buffer (20 mM phosphate, 84 mM imidazole, pH 7.4). Purification efficiency was evaluated by SDS-PAGE followed by silver staining, and band intensities were quantified using GelAnalyzer software. The purified proteins were subsequently dialyzed to remove imidazole, concentrated via Amicon 10 kDa (Merck), and quantified by the Bradford assay.

***In vitro* evaluation of PEP-FedA/FedA and PrPC interaction by dot blotting**

Dot blotting was performed as described previously (19) to assess the interaction between the PEP peptide and PrPC. A total of 50 ng of PEP-FedA or FedA protein in 100 µL was spotted onto nitrocellulose membranes (dots 1 and 2). PrPC-GST protein and GST protein were spotted onto dots 3-4 and 5-6, respectively. Membranes were blocked with 3% bovine serum albumin (BSA) on PBS-T for 1h at room temperature. Subsequently, 100 µL of PEP-FedA was applied to dots 3 and 5, while FedA was applied to dots 4 and 6, followed by incubation for 1h. Membranes were washed five times with PBS-T before incubation with HRP-conjugated anti-polyHis antibody (Proteintech) for 1h at room temperature. After washing, signals were developed using TMB.

**Immunization with PEP-FedA and FedA proteins**

Animal experiments were approved by the Animal Care and Use Committee of the University of Science, Ho Chi Minh City (ACUCUS; approval no. 915/GXN-KHTN), in accordance with Directive 2010/63/EU. Nine male Swiss mice (20-25 g, 6-8 weeks old) were randomly divided into three groups (n = 3 per group): PBS, FedA, and PEP-FedA. Mice were housed individually. Blood samples were collected one week prior to immunization. Each group was orally gavaged once weekly for four weeks with either PBS, 100 µg of FedA in 100 µL PBS, or 100 µg of PEP-FedA in 100 µL PBS. Mice were fasted overnight prior to gavage and for 1h post-gavage. Fecal samples were collected daily and stored at −20°C. Blood was collected one week after the final immunization, and plasma was prepared by density-gradient centrifugation and stored at −20°C (20).

**Evaluation of fecal IgA and plasma IgG by indirect ELISA**

Fecal extracts were prepared by suspending 100 mg feces in 600 µL PBS, homogenizing, and centrifuging at 4°C. Microtiter plates were coated with 20 µg/mL FedA protein (100 µL/well) overnight at 4°C. Wells were blocked with 3% BSA for 1h at room temperature and washed three times with PBS-T. Primary antibodies from fecal extracts (1:5 dilution in PBS) and plasma (two-fold serial dilutions in PBS from 1:5 to 1:640; 1:640 was used) were prepared in 3% BSA and incubated for 1h. After washing, wells were incubated with HRP-conjugated goat anti-mouse IgA (Abcam) for fecal samples or HRP-conjugated goat anti-mouse IgG (Abcam) for plasma samples (1:10,000 dilutions in PBS) for 1h at room temperature. After further washing, wells were developed with TMB for 30 min, and reactions were stopped with 2 N HCl. Optical density was measured at 450 nm. Negative controls (without protein coating or primary antibody) were included, and cutoff values were defined as the mean of negative controls plus three standard deviations. Values exceeding the cutoff were considered positive.

**Evaluation of fecal IgA reactivity with ETEC strains by dot blotting**

The interaction of fecal IgA with ETEC strains was assessed by dot blotting (21). Nitrocellulose membranes were blocked with 3% BSA for 1h at room temperature. Five microliters of $F18^+$ ETEC were spotted on dots 1-4, $F4^+$ ETEC on dots 5-7, and *E. coli* DH5α on dots 8-9. Dot 1 received antisera containing anti-F18 antibodies from infected pigs, used at a 1:1000 dilution in PBS. Dots 2, 5, and 8 were incubated with 1:5 diluted fecal extract from the PEP-FedA group; dots 3, 6, and 9 with extract from the FedA group; and dots 4, 7, and 10 with extract from the PBS group. After 1h incubation, membranes were washed five times with PBS-T. Dot 1 was probed with HRP-conjugated goat anti-swine IgG (1:10,000 dilution in PBS), while the remaining dots were probed with HRP-conjugated goat anti-mouse IgA (1:10,000 dilution in PBS). Following 1h incubation and washing, signals were visualized with TMB.

**Statistical analysis**

One-way ANOVA followed Tukey's multiple comparison test was used to determine the statistical significance. Data from three independent experiments are presented as mean ± SD by using GraphPad Prism 9. Statistical significance was defined as $P<0.01$ (**), and $P<0.001$ (***) (22).

**Results**

**Construction of pET22b-*pep-fedA* and pET22b-*fedA* vectors**

The plasmids pET22b-*pep-gfp* and pET22b were used as cloning backbones, while the *fedA* gene was amplified from the $F18^+$ ETEC strain by PCR. Both the amplified gene and plasmid backbones were digested with the appropriate restriction enzymes, and the products were confirmed by agarose gel electrophoresis. The results showed that pET22b-*pep-gfp* and pET22b plasmids were successfully isolated, appearing as large DNA bands (Figure 2a,b, lane 1), and were linearized after restriction digestion (Figure 2a,b, lane 2). A DNA fragment of approximately 501 bp, corresponding to the *fedA* gene, was also obtained (Figure 2a,b, lane 2).

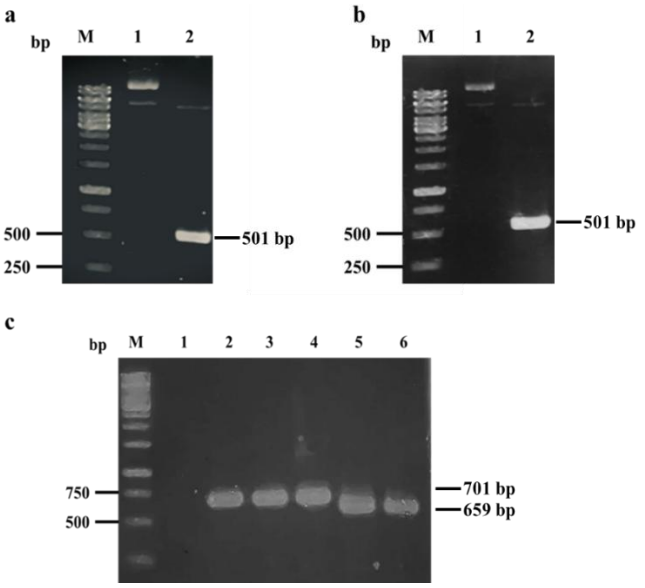



Figure 2: Isolation and screening of recombinant vectors: Isolation of plasmids and AIO products for construction of pET22b-*pep-fedA* (a) and pET22b-*fedA* vectors (b); PCR screening of positive colonies (c).

The recombinant vector pET22b-*pep-fedA* was constructed by inserting the *fedA* gene downstream of the *pep* sequence. Specifically, the *gfp* gene was excised from the pET22b-*pep-gfp* vector using *Bam*HI and *Xho*I, and replaced with the *fedA* gene. The ligation products were transformed into *E. coli* DH5α, and transformants were selected on LB-Amp. Candidate colonies were screened by PCR using primers T7pro and FedARXhoI. Agarose gel analysis revealed PCR products of 701 bp (Figure 2c, lanes 2-4), confirming successful replacement of *gfp* with *fedA* in the pET22b-*pep-fedA* construct. Similarly, the pET22b-*fedA* vector was generated by cloning the *fedA* gene into the multiple cloning site of pET22b between the *Nco*I and *Xho*I sites. Agarose gel electrophoresis of PCR-amplified colonies showed a fragment of 659 bp (Figure 2c, lanes 5, 6), consistent with the expected size of *fedA*. Sequencing analysis further confirmed that the cloned *fedA* gene was 100% identical to the published sequence in GenBank (Accession WP_122986601).

**Expression of PEP-FedA and FedA proteins in *E. coli* BL21(DE3)**

Recombinant plasmids pET22b-*pep-fedA* and pET22b-*fedA* were transformed into competent *E. coli* BL21(DE3) cells. Following PCR confirmation, positive clones were cultured in LB-Amp medium and induced with IPTG. SDS-PAGE analysis with Coomassie Brilliant Blue staining revealed distinct protein bands at ~18 kDa, consistent with the predicted size of FedA (Figure 3a, lanes 4 and 7). These proteins were present in both soluble (Figure 3a, lanes 5 and 8) and insoluble fractions (Figure 3a, lanes 6 and 9). Negative controls include *E. coli* BL21(DE3) (+IPTG), *E. coli* BL21(DE3)/pET22b-*pep-fedA* (–IPTG), and *E. coli* BL21(DE3)/pET22b-*fedA* (–IPTG) showed no detectable expression (Figure 3a, lanes 1-3). Western blotting using HRP-conjugated anti-polyHis antibody confirmed the identity of the expressed proteins (Figure 3b). Together, these results demonstrate that both PEP-FedA and FedA were successfully expressed in soluble form at high levels.

**Purification of PEP-FedA and FedA proteins and *in-vitro* evaluation of their interaction with PrPC by dot blotting**

PEP-FedA and FedA proteins, each fused with a C-terminal polyHis-tag, were purified by immobilized metal affinity chromatography using a HisTrap column. Both proteins were present in the loading solution (Figure 4a,b, lane 1) and efficiently retained via polyHis-$Ni^{2+}$interactions, with no detectable target proteins in the flow-through fraction (Figure 4a,b, lane 2). The washing step removed the majority of contaminating proteins, with only minor loss of target proteins (Figure 4a,b, lane 3). Elution yielded highly purified proteins, with purity levels of 94.5% for PEP-FedA and 93.9% for FedA, as determined by GelAnalyzer software (Figure 4a,b, lane 4). These results demonstrate successful purification of both recombinant proteins, suitable for subsequent functional assays.

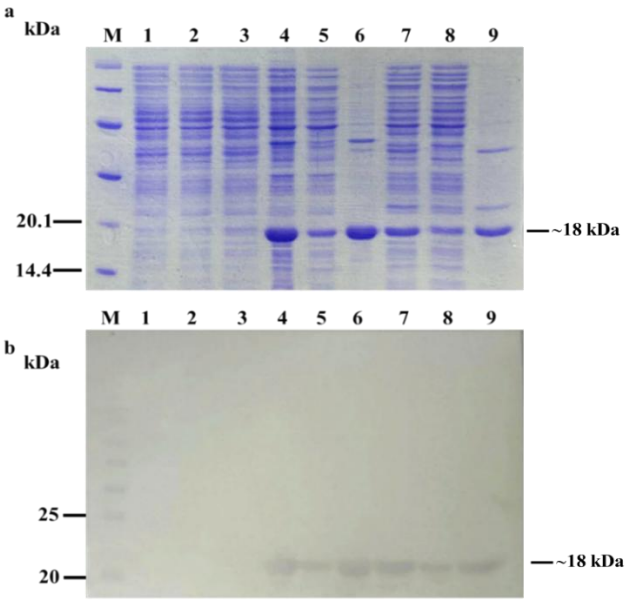
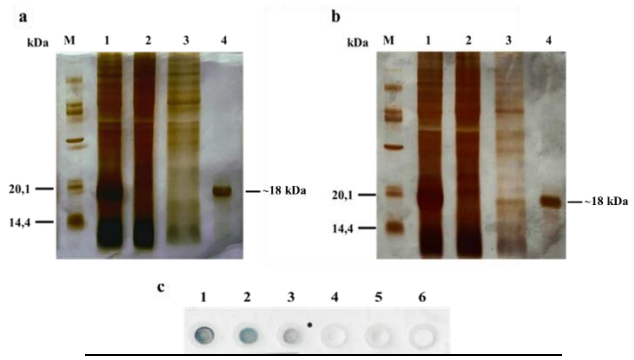



Figure 3: Validation of recombinant protein expression**:** Expression of PEP-FedA and FedA proteins analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (a); Western blotting with HRP-conjugated anti-polyHis antibody (b).




| Dot | Protein | |
|---|---|---|
| | 1st | 2nd |
| 1 | PEP-FedA | ( - ) |
| 2 | FedA | ( - ) |
| 3 | PrPC-GST | PEP-FedA |
| 4 | PrPC-GST | FedA |
| 5 | GST | PEP- FedA |
| 6 | GST | FedA |

Figure 4: Purification of PEP-FedA (a) and FedA (b) proteins analyzed by SDS-PAGE and silver staining and interaction of purified protein with PrPC by Dot blot assay (c).

The ability of PEP-FedA to interact with PrPC was further assessed by dot blotting. Detection was performed using an anti-polyHis antibody conjugated with HRP, with signals visualized via TMB substrate. Positive controls (PEP-FedA and FedA proteins) generated strong signals (Figure 4c, dots 1 and 2). Importantly, a distinct, though weaker, signal was observed only in the spot containing PrPC-GST incubated with PEP-FedA (Figure 4c, dot 3), confirming a specific interaction between PEP and the PrPC receptor. No signals were detected in other conditions (Figure 4c, dots 4-6). Taken together, these findings provide experimental evidence that the PEP peptide can mediate binding between FedA and PrPC.

**Evaluation of fecal IgA and plasma IgG by indirect ELISA**

Mice were orally administered 100 µL of PBS, FedA protein, or PEP-FedA protein, and immune responses were evaluated by ELISA using fecal extracts (1:5 dilution) and plasma samples (1:640 dilution).

Antigen-specific mucosal responses differed significantly among the groups (Figure 5a). In the PBS group, $OD_{450}$ values remained consistently low (0.102-0.186) throughout the experiment, indicating no detectable response. In contrast, both the FedA and PEP-FedA groups exhibited progressive increases in $OD_{450}$ values, with statistically significant differences compared to the PBS group. In the FedA group, fecal IgA responses became detectable by day 5 and peaked at 1.110 ± 0.016 on day 17. The PEP-FedA group displayed an earlier onset of immune response, with $OD_{450}$ values significantly higher than those of both the PBS and FedA groups from day 3 onward. The maximum $OD_{450}$ value, 3.092 ± 0.023, was recorded on day 17. In both antigen-treated groups, $OD_{450}$ values followed a cyclical pattern across the oral administration schedule, increasing and peaking 3-5 days after each immunization before declining until the next dose. The highest responses in both groups were observed on day 17. These results indicate that repeated stimulation enhanced both the magnitude and the durability of mucosal responses. The stronger and earlier responses in the PEP-FedA group suggest that fusion with the M cell targeting peptide significantly improved immunogenicity (23).

Systemic immune responses were assessed by measuring serum IgG levels after four weeks (Figure 5b). In the PBS group, $OD_{450}$ values remained low (0.168 ± 0.006). Both FedA and PEP-FedA groups showed higher IgG responses (0.369 ± 0.033 and 0.566 ± 0.076, respectively), with statistically significant differences among all groups. The highest systemic response was observed in the PEP-FedA group, supporting the role of PEP in enhancing M cell targeting and antigen uptake.

Together, these findings demonstrate that fusion of FedA with the PEP peptide substantially improved both mucosal and systemic immune responses. By targeting M cells, PEP facilitated antigen transport across the follicle-associated epithelium, thereby enhancing immune activation. Concurrent induction of fecal IgA and serum IgG underscores the potential of PEP-FedA as an oral vaccine candidate against $F18^+$ ETEC.

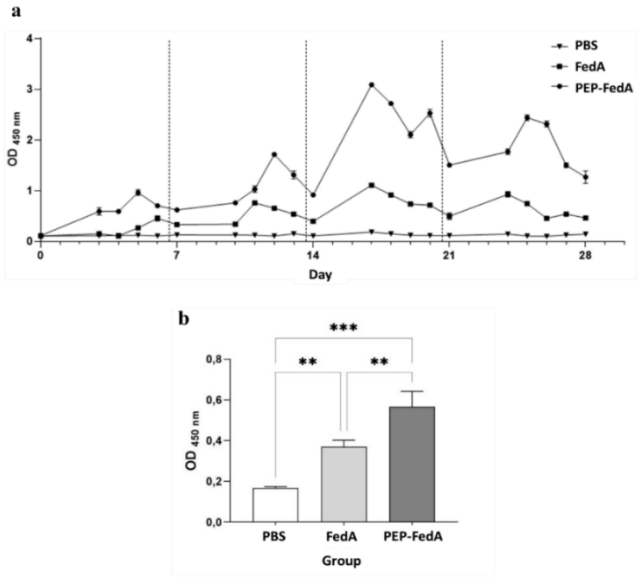



Figure 5: Measurement of antibody responses: Daily $OD_{450}$ values of fecal IgA antibodies (a); Serum IgG antibodies measured at 1:640 dilution (b). **, P<0.01; ***, P<0.001.

**Evaluation of fecal IgA reactivity with ETEC strains by dot blotting**

To evaluate the ability of mucosal antibodies to recognize ETEC, bacterial suspensions - including $F18^+$ ETEC (Dots 2-4), $F4^+$ ETEC (Dots 5-7), and *E. coli* DH5α (Dots 8-10) - were incubated with fecal extracts (day 17, 1:5 dilution) from the PEP-FedA (Dots 2, 5, 8), FedA (Dots 3, 6, 9), and PBS groups (Dots 4, 7, 10) (Figure 6). $F18^+$ ETEC incubated with anti-F18 antibodies from $F18^+$ ETEC-infected pigs served as a positive control. Dot blotting results showed a strong signal in the positive control (Figure 6, dot 1). Both the PEP-FedA and FedA groups generated positive signals against $F18^+$ ETEC, with the PEP-FedA group exhibiting a stronger response than the FedA group (Figure. 6, dots 2 and 3), no or only faint signals were observed in the PBS group (Figure 6, dot 4). However, incubation with the $F4^+$ ETEC strain yielded only weak or background-level signals (Figure 6, dots 5-7), demonstrating that anti-F18 IgA antibodies did not cross-react with this strain. Some background IgA activity was detected against *E. coli* DH5α, likely reflecting natural antibody responses to environmental antigens encountered during rearing (Figure 6, dots 8-10).

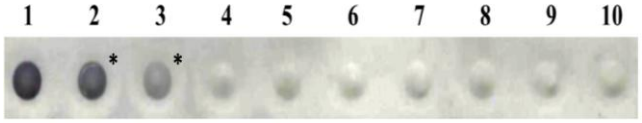



Figure 6: Evaluation of fecal IgA recognition and bacterial interaction by dot blotting.

Taken together, these results indicate that fecal IgA antibodies elicited by both FedA and PEP-FedA immunization specifically recognized the $F18^+$ ETEC strain, with the PEP-FedA group showing the strongest reactivity, while no cross-reactivity was observed with $F4^+$ ETEC strain.

**Discussion**

Anti-FedF antibodies can block the interaction between ETEC and host cells. However, FedF exhibits low immunogenicity and typically requires a carrier protein or adjuvant to enhance its immune response (24). Research by Ruan also demonstrated the effectiveness of FedF when incorporated into multivalent vaccine complexes (25). However, no studies have yet developed a system target M cells using the FedF antigen alone/or in fusion with a carrier protein. Although FedA does not serve as the adhesin subunit, inducing robust mucosal secretory IgA (sIgA) responses against this antigen may still contribute meaningfully to protection through several non-neutralizing mechanisms. Anti-FedA sIgA can extensively coat the bacterial surface, thereby reducing effective interactions between ETEC and the intestinal epithelium, promoting bacterial agglutination, limiting motility, and facilitating mechanical clearance by peristalsis. FedA-specific antibodies were generated, they failed to confer effective protection (26). Compared with the study by Verdonck, which used the entire F18 fimbriae in piglets, the present study focused on the FedA subunit and employed a mouse model (26). Both studies, however, required relatively high antigen doses compared with other fimbrial vaccines. For example, Verdonck used 10-30 mg of F18 antigen, whereas Van den Broeck reported protective responses with only 2 mg of F4 antigen (26,27). Similarly, Sanchez-Alvarez achieved immunogenicity using 5 µg of antigen, in contrast to the 100 µg FedA dose used here (28). These findings highlight the inherently low immunogenicity of FedA, and low antibody levels may limit the ability to protect pigs against ETEC infection. Incorporation of the PEP peptide markedly enhanced immune responses, eliciting stronger and more rapid mucosal and systemic immunity than FedA alone, which may contribute to improved protective efficacy. While FedA alone may not provide high protective efficacy, FedF requires a carrier protein and has not yet been applied in M cell targeted systems. Therefore, the PEP-FedA-FedF fusion represents a promising strategy that may lead to the development of a more effective vaccine platform.

The fusion of the PEP peptide with the model antigen (Green Fluorescent Protein) served as the basis for this study. Building upon these findings, the present research provides deeper insights into immune response kinetics and demonstrates that antigen-specific antibodies can effectively recognize and interact with biologically relevant antigens (16). Despite differences in target antigens (VP1 vs. FedA), targeting peptides (Co1 vs. PEP), and protein properties (refolded vs. soluble), the findings of this study are consistent with those of Ngo-My (20). Studies fused antigens with an M cell targeting peptide, demonstrated enhanced immune responses in mouse models. Although employing a different targeting strategy (antibody vs. peptide), Shima's study similarly demonstrated enhanced mucosal immune responses and increased protection against *Salmonella* infection in mice (29). Notably, peptides offer practical advantages over antibody-based approaches in terms of production simplicity and feasibility. To overcome the limitations imposed by the gastrointestinal environment, M cell–targeted delivery systems have been developed. Specifically, antigen complexes can be encapsulated in thiolated Eudragit-based carriers to prolong antigen retention time and enable controlled release at sites with a high density of M cells (a chemical-based strategy) (30). Alternatively, antigen complexes may be displayed on the surface of probiotic microorganisms such as *Lactobacillus casei* (31,32). In addition, studies on the surface display of heterologous proteins in *Pichia pastoris* (21) and *Saccharomyces cerevisiae* (33,34) indicate that yeast-based platforms may serve as promising vehicles. These approaches utilize naturally derived excipients, enhance mucosal immune targeting, and offer potential cost-effectiveness and improved biosafety. Together, these findings support the value of M cell targeting as a strategy to improve mucosal immunity.

Assessment of antibody protective capacity remains essential. In the study by Verdonck, antibodies induced by F18 fimbriae failed to protect piglets against $F18^+$ ETEC challenge (26). Our dot blotting results demonstrated that IgA antibodies from PEP-FedA-immunized mice specifically recognized $F18^+$ ETEC, but challenge studies *in vivo* will be required to confirm protective efficacy. Given the host specificity of $F18^+$ ETEC for pigs, *in vivo* trials will be optimized and performed in piglet models to ensure biological relevance. Although direct evidence for PrPC expression on porcine M cells is lacking, several indirect findings support its potential presence. PrPC is a highly conserved glycoprotein broadly expressed across mammalian species, and murine studies have demonstrated its expression on intestinal M cells, where it may participate in antigen sampling and mucosal immune interactions (35,36). Given the evolutionary conservation of both PrPC

and M cells function, porcine M cells are likely to express this protein. This hypothesis requires experimental validation before assessing the efficacy of PEP-F18 in piglet models.

The resulting recombinant protein was evaluated for its ability to elicit secretory IgA and systemic IgG responses against the FedA antigen and to determine its specificity toward F18$^+$ ETEC strains. In future studies, further immunological evaluations and challenge experiments in orally immunized piglets will be conducted to determine the protective efficacy of the vaccine candidate. If successful, fusion of antigens with the PEP peptide could serve as a broadly applicable platform for the development of oral vaccines against ETEC and other enteric pathogens.

## Conclusion

In this study, pET22b-*pep-fedA* and pET22b-*fedA* vectors were successfully constructed, and the corresponding PEP-FedA and FedA proteins were expressed and purified at > 90% purity. The PEP peptide demonstrated binding to the PrPC receptor *in vitro* and conferred enhanced immunogenicity *in vivo*. Oral immunization of mice with PEP-FedA elicited both mucosal and systemic immune responses, and the induced IgA antibodies specifically recognized F18$^+$ ETEC. These results provide strong evidence that the PEP peptide enables effective M cell targeting.

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## Author contributions

Quoc-Gia Mai: Data curation, Formal analysis, Methodology, Writing - review & editing. Khoi-Nguyen Le-Hoang: Data curation, Formal analysis. Hieu Tran-Van: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

## Conflict of interest statement

The authors have no relevant financial or non-financial interests to disclose.

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# تقييم خلاي M مستهدفة بروتين الاندماج FedA كلقاح عن طريق الفم للإسهال بعد الفطام

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**الخلاصة**

لا يزال إسهال ما بعد الفطام، الناجم بشكل أساسي عن الإشريكية القولونية المعوية، يشكل عائقا رئيسيا على إنتاج الخنازير في جميع أنحاء العالم بسبب تأثيره على صحة الخنازير وأداء النمو واقتصاديات المزرعة. تلعب المواد اللاصقة الفيمبريالية إف ٤ و إف ١٨ أدوارا مركزية في التسبب في المرض عن طريق التوسط في استعمار الأمعاء. ومع ذلك، لا تزال اللقاحات الفعالة ضد الفورمولا ١٨-إيتيك غير متوفرة، ويرجع ذلك إلى حد كبير إلى ضعف المناعة وعدم الاستقرار الهيكلي للفورمولا ١٨ فيمبريا. وقد برزت الوحدة الفرعية فيمبريال الرئيسية فدا كمرشح مستضد واعدة، ولكن التطعيم عن طريق الفم الناجح يتطلب تسليم مستضد فعالة لمواقع المناعة المخاطية. في هذه الدراسة، ونحن تهدف إلى تعزيز المناعة فدا عن طريق دمجها ل بيب، خلية م تستهدف الببتيد تهدف إلى تحسين امتصاص المستضد في الغشاء المخاطي في الأمعاء. تم استنساخ الجين فدا في بيت٢٢ب بيب ناقلات لتوليد بيت٢٢ب بيب فدا بناء وأعرب في كولاي بل٢١ (دي٣). تم تنقية بروتين الانصهار بيب فدا المؤتلف إلى تجانس أكبر من ٩٠ ٪ باستخدام اللوني تقارب المعادن يجمد. التحصين عن طريق الفم في الفئران أثارت الاستجابات المناعية المخاطية والجهازية قوية، كما يتضح من زيادة كبيرة في إفراز البراز إيغا ومستويات مفتش المصل مقارنة مع فدا وحدها. وعلاوة على ذلك، برازي إيغا المعترف بها على وجه التحديد إف ١٨-إيتيك دون تفاعل عبر إلى إف ٤-إيتيك. تظهر هذه النتائج أن استهداف الخلايا المميزة بوساطة بيب يعزز بشكل كبير مناعة الاتحاد الفيدرالي ويسلط الضوء على إمكانات هذه الاستراتيجية لتطوير لقاحات فموية فعالة ضد الأشخاص ذوي الإعاقة.