Immune response and protective efficacy of recombinant *Enterococcus faecalis* displaying dendritic cell–targeting peptide fused with *Eimeria tenella* 3-1E protein

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ABSTRACT Avian coccidiosis causes significant economic losses on the global poultry breeding industry. Exploration of new-concept vaccines against coccidiosis has gradually become a research hotspot. In this study, an *Enterococcus faecalis* strain (**MDXEF-1**) showing excellent performance isolated from chicken intestinal tract was used as a vector to deliver *Eimeria* target protein. The plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA harboring dendritic cell–targeting peptide (**DCpep**) fusion with *Eimeria tenella* NAΔ3-1E gene (3-1E protein–coding gene without start codon ATG and terminator codon TAA) was electrotransformed into MDXEF-1 to generate the recombinant bacteria MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA in which NAΔ3-1E protein was covalently anchored to the surface of bacteria cells by cell wall anchor (**CWA**) sequence. The expression of target fusion protein DCpep-NAΔ3-1E-CWA was detected by Western blot. Each chicken was immunized 3 times at 2-wk intervals with live *E. faecalis* expressing DCpep-NAΔ3-1E fusion protein (DCpep-NAΔ3-1E group), live *E. faecalis* expressing NAΔ3-1E protein (NAΔ3-1E group), and live *E. faecalis* containing empty vector only. The 3 immunized groups were then challenged with homologous *E. tenella* sporulated oocyst after immunizations, and the immune response and protective efficacy in each group were evaluated. The results showed that serum IgG levels, secretory IgA levels in cecal lavage, proportion of CD4+ and CD8α+ cells in peripheral blood, and mRNA expression levels of IL-2 and IFN-γ in the spleen were significantly higher in chickens in the DCpep-NAΔ3-1E group than in chickens of the NAΔ3-1E group ($P < 0.05$). Oral immunization to chickens with live *E. faecalis* expressing DCpep-NAΔ3-1E offered more protective efficacy against homologous challenge including significant improved body weight gain, increased oocyst decrease ratio, and reduced average lesion scores in cecum compared with chickens with live *E. faecalis* expressing NAΔ3-1E protein. These results suggest that recombinant *E. faecalis* expressing dendritic cell–targeting peptide fusion with *E. tenella* 3-1E protein could be a potential approach for prevention of *Eimeria* infection.

Key words: *Eimeria tenella*, DCpep-NAΔ3-1E, *Enterococcus faecalis*, immune response, protective efficacy

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INTRODUCTION

Avian coccidiosis, a widely spread intestinal protozoan disease caused by single or multiple *Eimeria* species, leads to severe economic losses on the global poultry breeding industry (Dalloul et al., 2006; Blake and Tomley, 2014). *Eimeria tenella* is one of the most prevalent *Eimeria* species in chicken, which mainly parasitized in the cecum and caused necrotic gut lesions, decreased feed utilization, and further impaired growth rate and even mortality. Nowadays, conventional strategies against coccidiosis primarily rely on prophylactic chemotherapy and application of live attenuated vaccines. However, emergence of drug resistant strains of *Eimeria* and drug residues in poultry products renders chemotherapy an unsustainable control method against coccidiosis (Shirley et al., 2007). Meanwhile, use of several known live *Eimeria* vaccines against coccidiosis sometimes may revert to virulence that causes immune failure and unexpected infection (Clarke et al., 2014). Recently, exploration of novel vaccines aiming at preventing avian coccidiosis has gradually become a research hot spot. It was reported that several novel delivery vectors could

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be used as tools to express *Eimeria* target proteins, mainly including tobacco, ginsenoside-based nanoparticles, *Mycobacterium bovis, Bacillus Calmette–Guerin*, *Bacillus subtilis*, and *Pichia pastoris* (Sathish et al., 2012; Zhang et al., 2012; Wang et al., 2014; Chen et al., 2015; Lin et al., 2015). Besides the aforementioned delivery tools, lactic acid bacteria are considered to be an ideal oral delivery vector with many advantages, including safety profile, convenient route of oral administration, weak immune response against itself, and establishing specific mucosal immunity (BaheyEI-Din et al., 2010). Effects of probiotics supplementation via feed or water on performance and response of chickens to *Eimeria* challenge were reported (Giannenas et al., 2014; Ritzi et al., 2014). In our laboratory, recombinant *Lactococcus lactis* NZ9000 expressing cytoplasmic, secreted, and anchored *E. tenella* 3-1E, *E. tenella* AMA1 protein, especially *Lactococci* expressing anchored target protein, was shown to provide partial protection against homologous challenge (Ma et al., 2013, 2017; Li et al., 2018). In recent years, studies of the intestinal bacteria flora have already become a hotspot. However, research using intestinal symbiotic bacteria as a delivery tool to express important *Eimeria* proteins was not reported until now. In our previous research, a strain of *Enterococcus faecalis* (named *MDXEF-1*) showing excellent performance was isolated from chicken ceca and characterized to be a delivery tool to express heterologous protein (data not published, Chinese patent ZL201410817717.5). We postulated that the recombinant live symbiotic *E. faecalis*-expressing anchored *Eimeria* 3-1E protein would provide immune protections against homologous challenge.

Dendritic cells (**DC**), a kind of professional antigen-presenting cells existed in the subepithelial lamina propria of the intestine, play a vital role in inducing humoral and cellular intestinal immune responses (Chirdo et al., 2005). Previous research demonstrated that fusion protein consists of DC-targeting peptide (**DCpep**), and protective antigen delivered by *Lactobacillus* effectively enhanced the antigen-induced systemic immune response (Mohamadzadeh et al., 2009). Will the reported DCpep fused with *E. tenella* protective antigen enhances the immunogenicity of the target protein? The main purpose of this study was to explore the immune response and protections against *Eimeria* challenge provided by the recombinant *E. faecalis*-expressing DCpep fused with the *E. tenella* 3-1E protein.

MATERIALS AND METHODS

Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1. *E. tenella* 3-1E polyclonal antibody was prepared and preserved in our laboratory (Ma et al., 2011). Probiotic strain of *E. faecalis* MDXEF-1 showing several excellent performances, including acid- and bile-resistant profile and antimicrobial property, isolated

Table 1. Strains and plasmids used in this study.

Bacterial strain orplasmids	Relevant characteristics	Source or references
Strain		
Escherichia coli DH5α	SupE44ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1, plasmid-free	TaKaRa
Lactococcus lacts subsp. cremoris NZ9000	Derivate strain of MG1363, with nisR and nisK genes for nisin induction, plasmid-free strain	NIZO
Enterococcus faecalis MDXEF-1	Isolated from a healthy chicken intestinal tract, with excellent properties, plasmid-free strain (data not published)	Stored in our laboratory
MDXEF-1/pTX8048-SP-Δ3-1E-CWA	With plasmid pTX8048-SP-3-1E-CWA in *E. faecalis* MDXEF-1	This study
MDXEF-1/pTX8048-SP-DCpep-Δ3-1E-CWA	With plasmid/pTX8048-SP-DCpep-3-1E-CWA in *E. faecalis* MDXEF-1	This study
MDXEF-1/pTX8048	With plasmid pTX8048 in *E. faecalis* MDXEF-1	Ma et al., 2013
plasmid		
pTX8048	pNZ8048 carrying 6 × his-tagged (His6) and thioredoxin (trxA) gene of *E. coli*; Cm	Douillard et al., 2011
pTX8048-SP-NAΔ3-1E-CWA	With fragment encoding signal peptide of secretion protein Usp45, NAΔ3-1E protein (without start codon ATG and terminator codon TAA) and cell wall anchor region with LPXTG-type anchoring motif in *P* TX8048	Ma et al., 2017
pTX8048-SP-DCpep-NAΔ3-1E-CWA	With fragment encoding signal peptide of secretion protein Usp45, dendritic cell–targeting peptides, NAΔ3-1E protein and cell wall anchor region with LPXTG-type anchoring motif in *P* TX8048	This study
pUC57-SP-DCpep-linker-TrxA-His6	With fragment SP-DCpep-linker-TrxA-His6, synthesized by Nanjing GenScript Biotechnology Co., Ltd. (Nanjing, China)	This study

from ceca of chicken was stored in our laboratory and was characterized to be a delivery tool to express target protein (data not published). *E. faecalis* MDXEF-1 cells were cultured at 30°C without shaking in M17 medium (Luqiao, Beijing) supplemented with 5 g/L glucose (GM17) and 10 μg/mL chloramphenicol.

Construction of the Recombinant E. faecalis Expressing DCpep-NAΔ3-1E Fusion Protein

Construction of the plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA is displayed in Figure 1. The fusion fragment SP-DCpep-linker-TrxA-His6 consists of a signal peptide (**SP**) of *Lactococcus lactis* secretion protein Usp45, DCpep (Curiel et al., 2004), linker sequence (G4S)2, coding sequence of trxA protein, and His6 amino acid was synthesized by Nanjing GenScript Biotechnology Co., Ltd. (Nanjing, China) and was then cloned into pUC57 vector to generate plasmid pUC57-SP-DCpep-linker-TrxA-His6. The purified fusion gene fragment SP-DCpep-linker-TrxA-His6 was subcloned into the *Nco* I/*Bam*H I sites of pTX8048-SP-NAΔ3-1E-CWA plasmid (Ma et al., 2017) to generate pTX8048-SP-DCpep-NAΔ3-1E-CWA. The plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA, pTX8048-SP-NAΔ3-1E-CWA, and empty vector pTX8048 (Douillard et al., 2011) were respectively transformed into *E. faecalis* MDXEF-1 by electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA). The subsequent screening and characterization of 3 recombinant-positive bacteria MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA, MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA, and MDXEF-1/pTX8048 were carried out as described in our previous report (Ma et al., 2017).

Western Blot Analysis

The cell wall–anchored fusion protein DCpep-NAΔ3-1E was prepared as previously described (Ma et al., 2017). Briefly, bacteria pellets were washed and resuspended in TES (10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 25% sucrose). The buffer TES-LMR (TES containing 1 mg/mL lysozyme, 0.1 mg/mL mutanolysin, and 0.1 mg/mL RNase) was used to digest the cell wall. After centrifugation, the target cell wall–anchored protein existed in the supernatant was precipitated with 16% trichloroacetic acid. The harvested fusion protein DCpep-NAΔ3-1E was washed, dried, and then resuspended in 50 mM NaOH. The prepared cell wall–anchored DCpep-NAΔ3-1E protein samples were separated by 12% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. The membranes were probed with rabbit anti-3-1E specific antibodies (Ma et al., 2011) for 1.5 h. After incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Sigma, USA), the membranes were washed with TTBS (Tris-HCl 20 mmol/L, pH 7.5, NaCl 100 mmol/L, 0.1% Tween-20), and then, target bands were detected using ECL Chemiluminescence Detection Kit as per the manufacturer's instructions.

Oral Immunization and Challenge Experiment

One-day-old specific pathogen–free white Leghorn chickens were purchased from Harbin Veterinary Research Institute, Harbin, China. At 5 D of age, all chickens were weighed, and chickens with similar body weights were selected and randomly divided into 5 groups of 35 chickens each. Chickens were housed in individual cages (Table 2). Chickens in group 1 were immunized with MDXEF-1/pTX8048-SP-DCpep-3-1E-CWA; group 2 with MDXEF-1/pTX8048-SP-3-1E-CWA; and group 3 with MDXEF-1/pTX8048, orally with a 100 μL volume containing 5×10^9 CFU recombinant bacteria on 3 consecutive day at 2-wk intervals for a total of 3 immunizations. Chickens in the unchallenged control group 4 and challenged control group 5 were

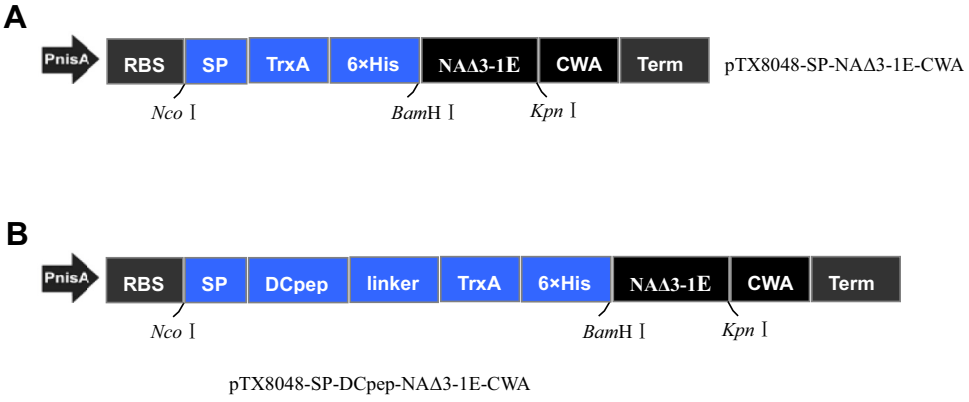

Figure 1. The schematic illustration of plasmids pTX8048-SP-NAΔ3-1E-CWA harboring synthesized fusion gene fragment SP-DCpep-linker-TrxA-His6. The fusion gene fragment SP-DCpep-linker-TrxA-His6 consists of signal peptide (SP) of *Lactococcus lactis* secretion protein Usp45, detritic cell-targeting peptides (DCpep) (Curiel et al., 2004), linker sequence (G4S)2, and coding sequence of trxA protein and His6 amino acid and was synthesized by Nanjing GenScript Biotechnology Co., Ltd. (Nanjing, China). The target gene fragment SP-DCpep-linker-TrxA-His6 was digested by *Nco I*/*BamH I*, and then subcloned into *Nco I*/*BamH I* sites of pTX8048-SP-NAΔ3-1E-CWA (A) (Ma et al., 2017) to construct plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA (B).

Table 2. Experimental design of immunizations and challenge.

Groups	Number	Immunizations[1]			Challenge[2]
		Primary immunization at day of 5, 6, and 7	Secondary immunization at day of 21, 22, and 23	Third immunization at day of 37, 38, and 39	Challenge at day of 54
1	35	MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA (100 μL)	MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA (100 μL)	MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA (100 μL)	Challenge
2	35	MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA (100 μL)	MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA (100 μL)	MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA (100 μμL)	Challenge
3	35	MDXEF-1/pTX8048 (100 μμL)	MDXEF-1/pTX8048 (100 μL)	MDXEF-1/pTX8048 (100 μL)	Challenge
4	35	PBS (pH7.2)	PBS (pH7.2)	PBS (pH7.2)	Unchallenge
5	35	PBS (pH7.2)	PBS (pH7.2)	PBS (pH7.2)	Challenge

[1]Oral immunizations with recombinant *Enterococcus faecalis* MDXEF-1 (100 μL containing 5×10^9 CFU per chicken) for 3 times, each for 3 consecutive day at intervals of 2 wk.
[2]At 54 day of age, chickens except for group 4 were challenged orally with 2×10^4 sporulated oocysts of *Eimeria tenella*.

both sham inoculated, orally with 100 μL PBS (pH 7.2). At 54 D of age, all chickens (except those in group 4) were challenged orally with 2×10^4 *E. tenella* sporulated oocysts. Animal experiments were performed in accordance with the regulations of the Animal Experiment Ethics Committee of Northeast Agricultural University, Harbin, Heilongjiang Province, China.

Humoral Immune Responses Induced by 3-1E-expressing E. faecalis

On day 14 after each immunization, serum and cecal lavage fluid were prepared from chickens (n = 5) in each group. Specific serum IgG levels and secreted IgA (**sIgA**) levels in cecal lavage fluid were determined by indirect enzyme-linked immunosorbent assay as per the protocol as previously described (Ma et al., 2017). Briefly, 96-well plates were coated with 100 μL (1.0 μg) *E. tenella* 3-1E proteins per well and incubated overnight at 4°C. Each well was then blocked with 5% skim milk in PBS containing 0.05% Tween-20. Then, 100 μL of diluted serum (1:128) and cecal lavage fluid (1:100) were added to each well to incubate at 37°C. The secondary antibody horseradish peroxidase–conjugated goat anti-chicken IgG and goat anti-chicken IgA (Sigma-Aldrich, USA) diluted at a ratio of 1:1,500 was added to incubate at 37°C. The substrate solution (0.01% H_2O_2 and 1 mg/mL o-phenylenediamine) was added to stop the reaction. Each sample was tested in triplicate.

Cellular Immune Responses Induced by 3-1E-expressing E. faecalis

On day 14 after third oral immunization, lymphocytes in peripheral blood from 5 chickens in each group were isolated using Lymphocyte Separation Medium (1.077 g/mL) (Tianjin Haoyang Biological Manufacture, China) in accordance with the manufacturer's instruction. The isolated cells were adjusted to a final concentration of 1×10^7 cells/mL. A volume of100 μL of lymphocytes containing 1×10^6 cells was incubated with fluorescein isothiocyanate–conjugated mouse anti-chicken CD4+ antibody (0.5 mg/mL) and phycoerythrin-conjugated mouse anti-chicken CD8α+ antibody (0.5 mg/mL) (Southern Biotech) for 20 min at 4°C in the dark. After centrifuging the mixture, the cell pellets were washed with PBS (pH7.4) twice and then resuspended in 500 μL of PBS (pH7.4). The relative quantification of T lymphocytes subtypes was measured by flow cytometry (Epics XL MCL, Beckman Coulter).

Expression of Chicken IL-2 and Chicken IFN- γ in the Spleen

On day 14 after third oral immunization, the spleens from 5 chickens in each group were sampled. The mRNA expression levels of chicken interleukin-2 (**ChIL-2**) and chicken gamma interferon (**ChIFN-γ**) in the spleen were quantified using real-time PCR as described in a previous

Table 3. Primers used in the experiment.

Primers	Primers sequences	PCR product (base pairs)
ChIL-2-F	5′GTGGCTAACTAATCTGCTGTCC3′	105 bp
ChIL-2-R	5′GTAGGGCTTACAGAAAGGATCAA3′	
ChIFN-γ-F	5′CAAAGCCGCACATCAAACA3′	80 bp
ChIFN-γ-R	5′TTTCACCTTCTTCACGCCATC3′	
GAPDH-F	5′AGAACATCATCCCAGCGT3′	133 bp
GAPDH-R	5′CGGCAGGTCAGGTCAACA3′	

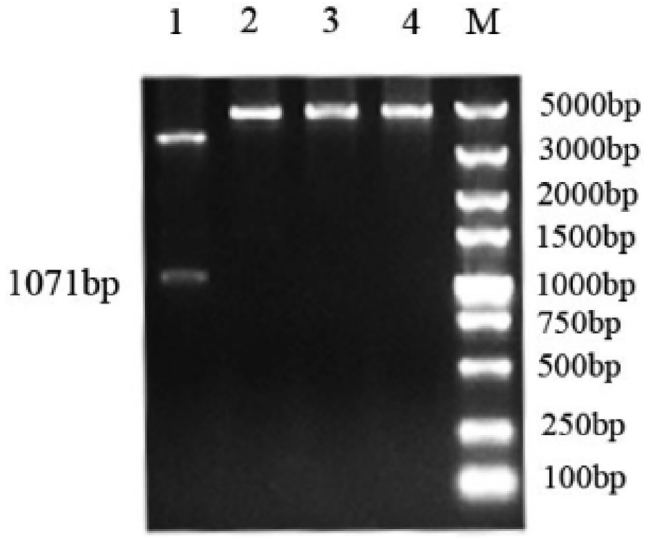

Figure 2. Identification of plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA. The recombinant positive plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA was identified by digestion with *NcoI* and *Kpn I*, and an expected 1,071 bp fragment was observed. (M) DNA Marker (DL 5000). Lane (1) Fragment of pTX8048-SP-DCpep-NAΔ3-1E-CWA disgested by *Nco I* and *Kpn I*. Lane (2), (3), and (4) Fragment of pTX8048-SP-DCpep-NAΔ3-1E-CWA digested by *Nco I*, *BamH I*, and *Kpn I*, respectively.

report (Wang et al., 2018). Briefly, total RNA extracted from spleens was reverse transcribed, and the real-time PCR was performed using the SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Biotech Corp., Dalian, China) in accordance with the manufacturer's instructions. The primers pairs ChIL-2-F/ChIL-2-R and ChIFN-γ-F/ChIFN-γ-R used for real-time PCR are listed in Table 3. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference. The mRNA expression levels of ChIL-2 and ChIFN-γ were analyzed as per the method reported by Livak et al. (2001).

Evaluation of Immune Protective Efficacy

The protective efficacies were evaluated based on calculating the body weight gain (**BWG**), lesion scores in ceca, and oocyst decrease ratio from chickens in each group. Chickens randomly selected from each group (n = 10) were weighed both before challenge and on day 7 post infection (**PI**) to record the BWG. Ten chickens from each group were selected to assess lesions in the ceca on day 7 PI as per the method reported by Johnson and Reid (1970). Feces from 10 chickens housed in 10 individual cages in each group were collected between day 7 and 11 PI and weighed, and oocyst counting and calculation of the oocyst decrease ratio were carried out as described in a previous report (Ma et al., 2011).

Statistics Analysis

Data were expressed as means ± SD and subjected to one-way analysis of variance. Differences between mean values were compared by analysis of variance Tukey multiple comparison procedures. Results were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

RESULTS

Construction of Recombinant Plasmid pTX8048-SP-DCpep-NA3-1E-CWA

The objective 1,071 bp fragment approximately equal to target fusion protein and NA3-1E (Figure 1)

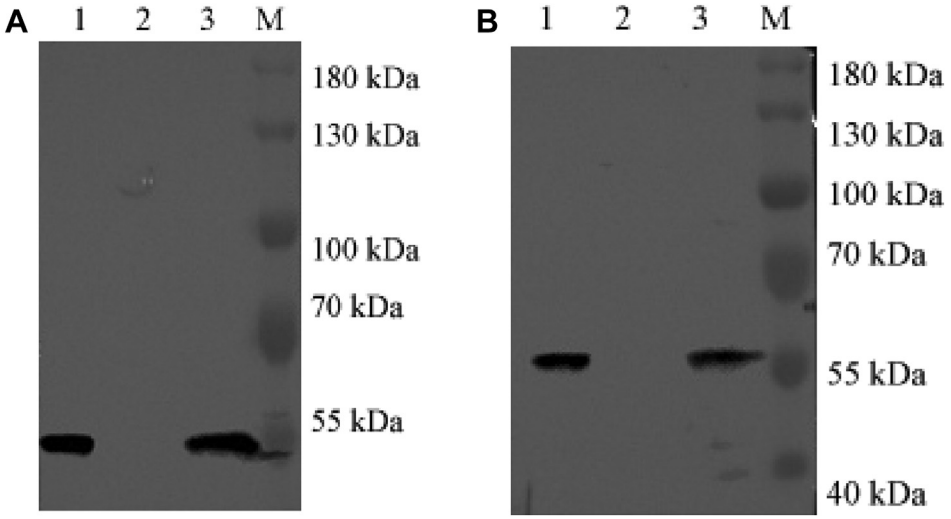

Figure 3. Western blot detection of cell wall–anchored fusion protein SP-TrxA-His6-NAΔ3-1E and SP-DCpep-linker-TrxA-His6-NAΔ3-1E. Target fusion protein SP-TrxA-His6-NAΔ3-1E and SP-DCpep-linker-TrxA-His6-NAΔ3-1E were separated by 12% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with rabbit anti-3-1E polyclonal antibodies (Ma et al., 2011). The expected band corresponding to SP-TrxA-His6-NAΔ3-1E (53 kDa, A) and SP-DCpep-linker-TrxA-His6-NAΔ3-1E (55 kDa, B) was observed. (A) Lane (1) and (3) cell wall–anchored fusion protein SP-TrxA-His6-NAΔ3-1E from nisin-induced MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA. Lane (2) cell wall–anchored fusion protein from nisin-induced MDXEF-1/pTX8048 (negative control). (B) Lane (1) and (3) cell wall–anchored fusion protein SP-DCpep-linker-TrxA-His6-NAΔ3-1E from nisin-induced MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA. Lane (2), cell wall-anchored fusion protein from nisin-induced MDXEF-1/pTX8048 (negative control).

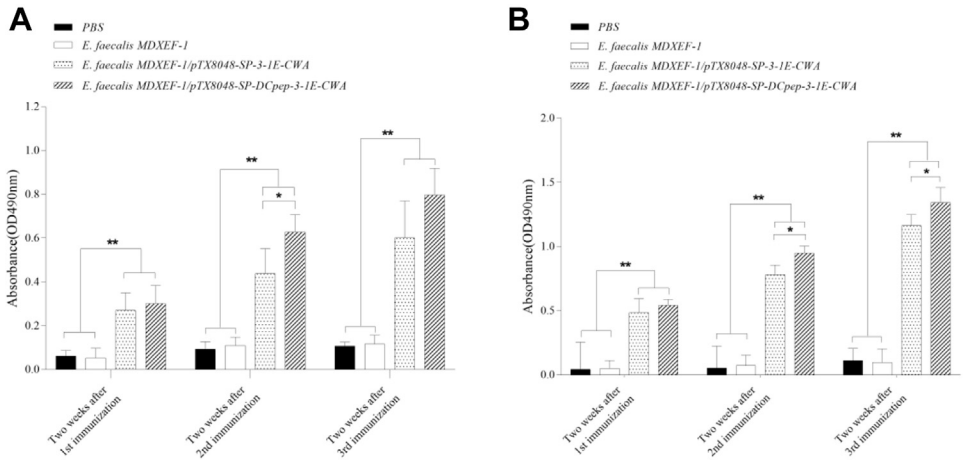

Figure 4. (A)The IgG levels in serum and (B) secreted IgA levels in cecal lavage fluid from chickens immunized with 3-1-expressing *Enterococcus faecalis* MDXEF-1. On day 5 to 7, 21 to 23, and 37 to 39, chickens in each group were orally inoculated with 5×10^9 CFU live bacteria of MDXEF-1/pTX8048-SP-DCpep-3-1E-CWA, MDXEF-1/pTX8048-SP-3-1E-CWA, and MDXEF-1/pTX8048 and PBS (pH7.2), respectively. The sera and cecal lavage fluid were prepared from chickens (n = 5) in each group after 3 immunizations. Specific IgG levels in serum and secreted IgA levels in cecal lavage fluid were determined by indirect ELISA. Data are expressed as mean ± SD (n = 5). $^*P < 0.05$, $^{**}P < 0.01$.

was generated from plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA after digestion with *Nco* I and *Kpn* I (Figure 2). The plasmid pTX8048-SP-DCpep-NAΔ3-1E-CWA was electroporated into *E. faecalis* MDXEF-1, and the positive bacteria MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA was screened and characterized.

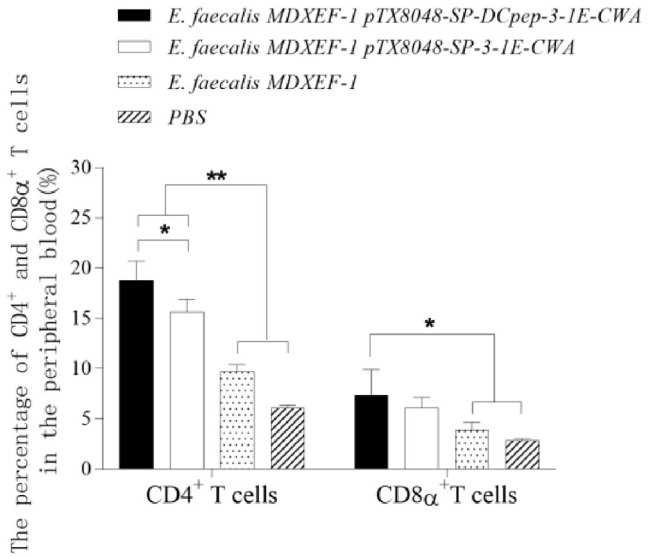

Figure 5. The percentage of CD4+ and CD8α+ T cells in peripheral blood from chickens immunized with 3-1-expressing *Enterococcus faecalis* MDXEF-1. After 3 oral immunizations with 5×10^9 CFU live bacteria of MDXEF-1/pTX8048-SP-DCpep-3-1E-CWA, MDXEF-1/pTX8048-SP-3-1E-CWA, and MDXEF-1/pTX8048 and PBS (pH7.2) (control group), lymphocytes in peripheral blood from chickens (n = 5) in each immunized group were isolated by lymphocyte separation medium as per the manufacturer's instruction. The isolated cells were incubated with fluorescein isothiocyanate–conjugated mouse anti-chicken CD4+ antibody (0.5 mg/mL) and phycoerythrin-conjugated mouse anti-chicken CD8α+ antibody (0.5 mg/mL) (Southern Biotech). The relative quantification of T lymphocytes subtypes were measured by flow cytometry (Epics XL MCL , Beckman Coulter). Data are expressed as mean ± SD (n = 5). $^*P < 0.05$, $^{**}P < 0.01$.

Protein Expression Assays and Immunoblotting Analysis

The cell wall–anchored fusion protein was separated by 12% SDS-PAGE, transferred onto nitrocellulose membranes, and the expression of target protein was detected by Western blot. The band corresponding to SP-TrxA-His6-NAΔ3-1E and SP-DCpep-linker-TrxA-His6-NAΔ3-1E was observed (Figure 3).

Humoral Immune Responses Induced by E.faecalis Expressing DCpep-NAΔ3-1E Protein

On day 14 after each immunization, the specific IgG levels in serum (Figure 4A) and secreted IgA (sIgA) levels in cecal lavage fluid (Figure 4B) from chickens in the groups immunized with 3-1-expressing *E. faecalis* MDXEF-1 were all highly significantly higher than that in the PBS and MDXEF-1/pTX8048 group ($P < 0.01$). On day 14 after secondary and third immunization, sIgA levels in cecal lavage fluid from chickens immunized with MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA were higher than in chickens immunized with MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA ($P < 0.05$).

Cellular Immune Responses Induced by E. faecalis Expressing DCpep-NAΔ3-1E Protein

On day 14 after tertiary oral immunization, the percentage of CD4+ T cells in peripheral blood from chickens immunized with MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA was significantly higher than that of chickens immunized with MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA ($P < 0.05$), and both of the aforementioned 2 groups were highly significantly higher than the group immunized with MDXEF-1/pTX8048

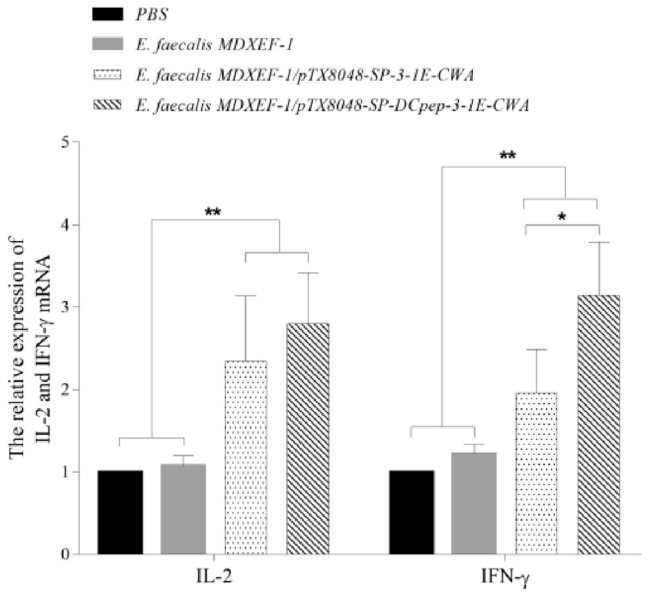

Figure 6. The mRNA expression levels of ChIFN-γ and ChIL-2 in spleen of chickens. On day 5 to 7, 21 to 23, and 37 to 39, chickens in each group were orally immunized with 5 × 109 CFU live bacteria of MDXEF-1/pTX8048-SP-DCpep-3-1E-CWA, MDXEF-1/pTX8048-SP-3-1E-CWA, and MDXEF-1/pTX8048 and PBS (pH7.2), respectively. After 3 immunizations, the mRNA expression levels of ChIFN-γ and ChIL-2 in spleen of chickens (n = 5) was assayed by real-time PCR. The cytokine mRNA levels of individual chicken in each group were divided by mRNA levels of glyceraldehyde-3-phosphate dehydrogenase of the same chicken to normalize the relative mRNA levels of ChIL-2 and ChIFN-γ. Data are expressed as mean ± SD (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$.

and PBS (P < 0.01). Significant change was not observed between the MDXEF-1/pTX8048 and PBS group (Figure 5). On day 14 after tertiary oral immunization, the percentage of CD8α+ T cells in peripheral blood from chickens immunized with MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA was significantly higher than that in the group immunized with MDXEF-1/pTX8048 and PBS (P < 0.05), and the MDXEF-1/pTX8048 and PBS groups did not show significant change (Figure 5).

Cytokine mRNA Expression in the Spleen

On day 14 after tertiary oral immunization, the mRNA expression levels of ChIL-2 and ChIFN-γ in the spleen of chickens in the groups immunized with 3-1E-expressing *E. faecalis* MDXEF-1 were highly significantly higher than those of chickens in the MDXEF-1/pTX8048 and PBS groups ($P < 0.01$). The mRNA expression levels of ChIFN-γ in the spleen of chickens immunized with MDXEF-1/pTX8048-SP-DCpep-NAΔ3-1E-CWA were significantly higher than those in chickens immunized with MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA (P < 0.05) (Figure 6).

Protection Offered by Oral Immunization With 3-1E-expressing E. faecalis

No chickens died from *E. tenella* challenge in each group. Body weight gain, average lesion scores in cecum, and oocyst decrease ratio are shown in Table 4. Body weight gain of chickens (group 1 and 2) immunized with 3-1E-expressing *E. faecalis* MDXEF-1 was highly significantly higher than that of chickens in the MDXEF-1/pTX8048 and PBS groups (P < 0.01). Chickens in group 1 and 2 displayed lower cecal lesion scores, lower oocyst shedding, and higher oocyst decrease ratio compared with chickens in the MDXEF-1/pTX8048 and PBS groups (P < 0.01). Statistical differences for immune protections were not observed between the two 3-1E-expressing *E. faecalis* MDXEF-1 groups.

DISCUSSION

Avian coccidiosis is responsible for serious annual economic losses in the world poultry industries (Chapman et al., 2013). Currently, the defects such as emergence of drug-resistant *Eimeria* species, drug residues, and virulence reversion of commercial vaccines accompanied by application of traditional measures including anticoccidial drugs and anticoccidia attenuated live vaccine

Table 4. Evaluation of protective effects against coccidiosis after immunization with recombinant live *Enterococcus faecalis* expressing target protein.

Groups	Body weight before challenge (g)	Body weight on day 7 after infection (g)	Body weight gain (g)	Relative body weight gain (%)	Average lesion score in cecum	Oocyst decrease ratio (%)
1	564.02 ± 8.27	670.77 ± 9.52^{B}	106.75 ± 7.17^{B}	83.21%	1.6 ± 0.54^{C}	38.52%
2	561.79 ± 7.83	660.82 ± 9.88^{B}	99.03 ± 4.07^{B}	80.57%	2.4 ± 0.54^{C}	32.09%
3	565.03 ± 8.45	619.84 ± 11.35^{C}	54.81 ± 4.33^{C}	57.73%	3.0 ± 0.71^{B}	11.38%
4	562.84 ± 9.72	696.42 ± 16.12^{A}	133.58 ± 10.75^{A}	100%	/	/
5	566.09 ± 7.95	613.26 ± 8.09^{C}	47.17 ± 3.62^{C}	45.34%	3.6 ± 0.54^{A}	0

$^{A-C}$The values represent mean ± SD. Highly significant difference ($P < 0.01$) between numbers are represented with different capital letters.

Note: Chickens randomly selected from each group (n = 10) were weighed both before challenge and on day 7 post infection (PI) to record body weight gain (BWG). Relative BWG was calculated using the following equation: the relative body weight gain = the average BWG of chickens in each group/the average BWG of chickens in unchallenged control group. Ten chickens from each group were selected to assess lesions in ceca on day 7 PI as per the method reported by Johnson and Reid (1970). Feces from 10 chickens housed in 10 individual cages in each group were collected between day 7 and 11 PI and weighed, and oocyst counting and calculation of oocyst decrease ratio were carried on as described in a previous report (Ma et al., 2011). The oocyst decrease ratio was calculated as follows: (the number of oocysts from challenged control chickens − vaccinated chickens)/challenged control chickens × 100%. "/" showed no pathologic lesions in cecum, and no oocyst was observed.

No significant difference ($P > 0.05$) between numbers are represented with the same letter. Group 1 were immunized with MDXEF-1/pTX8048-SP-DCpep-NA3-1E-CWA, group 2 with MDXEF-1/pTX8048-SP-NA3-1E-CWA, and group 3 with MDXEF-1/pTX8048, orally with a 100 μL volume containing 5 × 10^{9} CFU recombinant bacteria on 3 consecutive day at 2-wk intervals for a total of 3 immunizations. Chickens in the unchallenged control group 4 and challenged control group 5 were both sham inoculated orally with 100 μL PBS (pH 7.2).

against coccidiosis could not be avoided. Novel commercial vaccines without obvious drawbacks aiming at effectively preventing and controlling coccidiosis are still at the exploring stage (Blake et al., 2014). We previously constructed 3 kinds of live *Lactococci* expressing *E. tenella* 3-1E protein (Ma et al., 2013; 2017) and *E. tenella* AMA1 protein (Li et al., 2018), and the results showed that recombinant live *Lactococci* expressing target protein provided partial immune protections against *E. tenella* challenge. Furthermore, our previous research also proved that the surface-anchored *Eimeria* 3-1E protein delivered by *L. lacits* can induce more protective immunity and protection than the secreted or cytoplasmic 3-1E protein (Ma et al., 2017).

In the present study, we focus on the symbiotic bacteria *E. faecalis* strain (**MDXEF-1**), which was isolated from the chicken intestinal tract and characterized to be a delivery tool to express *E. tenella* 3-1E protein (data not published). Will the surface anchored *E. tenella* 3-1E protein delivered by *E. faecalis* MDXEF-1 enhance the immune protection against homologous challenge? To answer the previous question, we first constructed recombinant *E. faecalis* expressing cell wall-anchored *E. tenella* 3-1E protein based on our previous research. The results from oral immunization and challenge showed that the levels of sera IgG, secretory IgA (sIgA) in cecal lavage, proportion of CD4+ cells in peripheral blood, and mRNA expression levels of IL-2 and IFN-γ in spleen from chickens in the MDXEF-1/pTX8048-SP-NAΔ3-1E-CWA group were significantly higher than those in the MDXEF-1/pTX8048 and PBS group ($P < 0.05$), suggesting that target 3-1E protein delivered by symbiotic bacteria *E. faecalis* MDXEF-1 induced effective humoral and cellular immunity.

Previous studies have demonstrated that DC-targeting 12-mer peptide (DCpep) selected from a phage display library enhanced the uptake of the target antigen by intestinal DC, then produced a stronger specific immune responses, and therefore provided more efficient protection against pathogens such as *Bacillus anthracis* and NDV (Mohamadzadeh et al., 2009; Jiang et al., 2015). Will DCpep fused with *E. tenella* 3-1E protein enhances the immunogenicity of the target protein? Therefore, in the present study, the surface expression system of *E. faecalis* MDXEF-1 was further modified by fusing DCpep to *E. tenella* 3-1E protein encoding gene, and the recombinant *E. faecalis* MDXEF-1/pTX8048-SP-DCpep-3-1E-CWA displaying cell wall-anchored DCpep-NAΔ3-1E fusion protein was constructed with expection of enhancing the immunogenicity of target 3-1E protein. The expected results in this study were observed, showing the humoral and cellular immunity induced by recombinant *E. faecalis* MDXEF-1 expressing Dcpep-NAΔ3-1E fusion protein was higher than that expressing NAΔ3-1E ($P < 0.05$), demonstrating DCpep effectively enhanced the immunity elicited by NAΔ3-1E protein through elevating the levels of IgG in sera and IgA in in cecal lavage, increasing $CD4^+$ and $CD8^+$ T lymphocyte subtypes, and improving relative expression of mRNA levels of IL-2 and IFN-γ in spleen. It was generally accepted that both cellular and humoral immunity plays an important role against *Eimeria* challenge (Lillehoj, 1998; Wallach et al., 2010). The previous expected results could be explained by the fact that DCpep displayed on the surface of recombinant-positive *E. faecalis* specifically binds to the receptor existed on DC surface, and then, fusion protein DCpep-NAΔ3-1E was taken up by DCs and presented on the surface of DC, which further recognize and activate T cells to initiate effective immune responses, and meanwhile, the secretion of key cytokines from activated T cells indirectly modulate the function of immune cells (Curiel et al., 2004; Subramaniam et al., 2017).

Accordingly, the enhanced immune responses from chickens in the group orally immunized with *E. faecalis* expressing DCpep-NAΔ3-1E generated more immune efficacy against homologous challenge, which were reflected by significant improved BWG, increased oocyst decrease ratio, and reduced average lesion scores in cecum compared with live *E. faecalis* expressing NAΔ3-1E protein. The pathologic lesions in cecum of chickens immunized with the two 3-1E-expressing *E. faecalis*, especially DCpep-NAΔ3-1E-expressing bacteria were relatively slight compared with the control group, which suggesting the enhanced immunity based on DCpep reduced the cecal general and histopathologic changes caused by *Eimeria*.

CONCLUSION

Overall, oral immunization to chickens with recombinant live *E. faecalis* expressing *E. tenella* 3-1E protein elicited systemic immune responses and offered protective efficacy against homologous challenge. The induced immunity and protection could be enhanced by fusing DCpep with target 3-1E protein. The recombinant bacteria expressing DCpep and interested protein using intestinal symbiotic bacteria as delivery tool could be a potential approach for prevention of *Eimeria* infection.

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SUPPLEMENTAL DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.03.014.

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