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RESEARCH ARTICLE

Recombinant production of SAG1 fused with xylanase in *Pichia pastoris* induced higher protective immunity against *Eimeria tenella* infection in chicken

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Abstract

Chicken coccidiosis is an intestinal disease caused by the parasite *Eimeria*, which severely damages the growth of chickens and causes significant economic losses in the poultry industry. Improvement of the immune protective effect of antigens to develop high efficiency subunit vaccines is one of the hotspots in coccidiosis research. Sporozoite-specific surface antigen 1 (SAG1) of *Eimeria tenella* (*E. tenella*) is a well-known protective antigen and is one of the main target antigens for the development of subunit, DNA and vector vaccines. However, the production and immunoprotective effects of SAG1 need to be further improved. Here, we report that both SAG1 from *E. tenella* and its fusion protein with the xylanase XynCDBFV-SAG1 are recombinant expressed and produced in *Pichia pastoris (P. pastoris)*. The substantial expression quantity of fusion protein XynCDBFV-SAG1 is achieved through fermentation in a 15-L bioreactor, reaching up to about 2 g/L. Moreover, chickens immunized with the fusion protein induced higher protective immunity as evidenced by a significant reduction in the shedding of oocysts after *E. tenella* challenge infection compared with immunized with recombinant SAG1. Our results indicate that the xylanase enhances the immunogenicity of subunit antigens and has the potential for developing novel molecular adjuvants. The high expression level of fusion protein XynCDBFV-SAG1 in *P. pastoris* holds promise for the development of effective recombinant anti-coccidial subunit vaccine.

Chen Liu and HanBing Wei contributed equally to this work.

INTRODUCTION

Chicken coccidiosis, an intestinal disease caused by *Eimeria* parasites, seriously compromises the feed utilization and growth of chickens and is responsible for major economic losses of the poultry industry (Blake et al., 2020; Blake & Tomley, 2014; Shirley et al., 2005). Up to now, anti-coccidial drugs and live or attenuated parasite vaccines are available to control the occurrence and outbreaks of coccidiosis (Taylor et al., 2022). However, alternative strategies for disease control have attracted widespread attention due to the emergence of drug resistance, tighter regulations on antibiotic use in poultry and concerns about the pathogenicity of live or attenuated vaccines (Soutter et al., 2020). Recently, recombinant anti-coccidial subunit vaccines have emerged as a promising approach in inducing protective immunity against parasite infection (Blake & Tomley, 2014). To date, numerous protective antigens in *Eimeria* have been characterized, including surface proteins, internal proteins associated with subcellular organelles, such as the rhoptries, microneme and refractile bodies (Madlala et al., 2021; Song et al., 2020; Subramanian et al., 2008; Sun et al., 2014; Tang et al., 2019).

SAG1 (surface antigen 1), also known as TA4, is a major sporozoite-specific glycosylphosphatidylinositol (GPI) anchored surface antigen of *E. tenella* and involved in host cell attachment, invasion and signal transduction (Mineo & Kasper, 1994; Wang & Yin, 2014). It has been used as a potential target for immune response and shown to provide protection against *Eimeria* infection as DNA or subunit vaccines (Pagheh et al., 2020). Enhancements to its immune protective capacity have been achieved through fusion or co-administration with certain proteins including IL-2, IL-18, HSP90, et al. (Liu et al., 2010; Sanchez-Lopez et al., 2021; Song et al., 2009; Xu et al., 2008). In addition, multiple antigen delivery systems, including PLGA nanoparticle, *Salmonella typhimurium*, *Lactobacillus plantarum*, improve its protective effect (Allahyari et al., 2022; Liu et al., 2020; Pogonka et al., 2003).

Xylanases, key enzymes in lignocellulose hydrolysis, which are responsible for the hydrolysis of the 1,4-β-xylosidic linkages of xylan and have wide applications in the food, feed, paper and cellulose industries (Angural et al., 2023; Kaur et al., 2023; Qeshmi et al., 2020; Suchova et al., 2022). The supplementation of xylanase catalyses the depolymerization of the xylan into shorter chains in food or feed, changing its chemical structure and impacting the digestive physicochemical properties and which may lead to alterations on intestinal health parameters, especially in the composition and diversity of the intestinal microbiota (Dahiya et al., 2020; Jiang et al., 2005; Moita & Kim, 2022). Some fungal-derived GH11 (Glycosyl hydrolase family 11) xylanases including from *Trichoderma*, *Fusarium* and *Botrytis* species have been described as being able to activate plant immune responses independently of its enzymatic activity (Frias et al., 2019; Sella et al., 2013). At present, many recombinant xylanases have been stably and efficiently expressed (Fang et al., 2014). Moreover, xylanase has become an effective biotechnological tool as a fusion partner to enhance the expression level of proteins in *P. pastoris* recently (Lu et al., 2023).

In this study, the codon optimized SAG1 and the gene fused with *XynCDBFV* were recombinantly expressed in *P. pastoris*. Chicken were immunized using the purified SAG1 and XynCDBFV-SAG1 proteins, humoral immune response and oocyst output of the immunized chickens were evaluated. We observed that both SAG1 and XynCDBFV-SAG1 proteins induced immune protection against *E. tenella*, but XynCDBFV-SAG1 exhibited higher IgY titres and obviously reduced oocyst output in immunized chickens. In addition, the fusion protein XynCDBFV-SAG1 can be produced in substantial quantity by fed-batch fermentation. Our results demonstrate that fusion with xylanase enhanced immune protection of SAG1 against *E. tenella* in chicken, and the fusion protein XynCDBFV-SAG1 is a promising candidate vaccine for *E. tenella* infection.

EXPERIMENTAL PROCEDURES

Microbial strains, parasites and animals

E. coli TOP10 was used for vector construction and amplification. *P. pastoris* GS115 strain was used for recombinant protein expression of SAG1 and XynCDBFV-SAG1. *E. tenella* (XJ strain) was propagated in coccidian-free Arbor Acres (AA) broilers, aged between 2 and 5 weeks. Specific-pathogen-free (SPF) chickens, 1 week old, were purchased from Merial Animal Health Co., Ltd. (Beijing, China) and were maintained on a pathogen-free diet with unrestricted access to water.

Plasmid construction and transformation

The expression vectors pPIC9K and pPIC9K-*XynCDBFV* carrying xylanase gene *XynCDBFV* (GenBank accession number: KP691331.1) expression cassettes were maintained in our laboratory (Huang et al., 2021; Lu et al., 2023). The *SAG1* gene from *E. tenella* (GenBank accession number: AJ586531.2) was optimized to enhance expression, utilizing the codon usage bias of *P. pastoris* as referenced from the codon usage database (http://www.kazusa.or.jp/codon/). To facilitate detection and purification, a 6×His tag sequence was appended to the 5′-end of downstream primers for both *SAG1* and *XynCDBFV-SAG1*. The optimized *SAG1* gene sequences, synthesized by

Sangon Biotech (China), were amplified using specific primers and subsequently inserted into either the *Eco*RI and *Not*I sites of pPIC9K or the *Eco*RI and *Spe*I sites of pPIC9K-*XynCDBFV* (Table S1). The full-length *XynCDBFV* and *SAG1* genes measure 675 bp and 639 bp, encoding 225 and 213 amino acids, respectively. Positive transformants were validated by PCR amplification and further confirmed by Sanger sequencing.

Competent *P. pastoris* GS115 cells were prepared following to the protocol outlined by Invitrogen. The plasmids were individually linearized by *Bgl*II, subsequently transformed into *P. pastoris* GS115 via electroporation. The transformants were then cultured on MD plates for subsequent screening.

Induced expression in plastic test tubes and shake flasks

Induced expression was conducted in plastic test tubes and shake flasks following the method described previously (Lu et al., 2023). Approximately twelve individual transformant colonies were selected at random from MD plates and inoculated into plastic tubes containing 3 mL of BMGY medium, followed by incubation with shaking at 30°C and 220 rpm. After 48 h, the cultures were centrifuged at 4500 rpm, the supernatants removed, and the pellets were resuspended in 1.5 mL of BMMY medium containing 1% (v/v) methanol for inducing protein expression. Post 48-h induction, the culture supernatants were assessed for protein expression through SDS-PAGE and Western blot using an anti-His monoclonal antibody (Lablead His tag mouse mAb). The strain exhibiting the highest level of protein expression was subsequently chosen for both flask and fed-batch fermentations.

In order to confirm the positive transformants, the shake flask fermentation was performed. In summary, selected positive transformants were grown in 300 mL of BMGY media at 30°C, 220 rpm for 36 h. The cells were then collected by centrifugation at 4500 rpm for 5 min and resuspended in 200 mL of BMMY media containing 1% (v/v) methanol. Induction continued for an additional 48 h with periodic addition of 0.5% methanol every 12 h. Finally, the cultures were concentrated by centrifugation at 10,000 × *g* for 10 min, and the supernatants were subjected to analysis using SDS-PAGE and Western blot with anti-His monoclonal antibodies.

SDS-PAGE (sodium dodecyl Sulfate-Polyacrylamide gel electrophoresis)

Protein samples were prepared in SDS sample buffer and boiled for 5 min to denature. Electrophoresis was performed using a 12% resolving gel with a 5% stacking gel. The gel was run at 120 V for approximately 90 min until the tracking dye reached the bottom of the gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue for protein visualization.

Western blot (WB)

Following SDS-PAGE, proteins were transferred to a nitrocellulose (NC) membrane at 300 mA for 90 min in a cold environment. The membrane was blocked with 5% non-fat dry milk in TBST (Tris-buffered saline, 0.1% Tween 20), then incubated overnight at 4°C with a His tag mAb. Following this, the membrane was washed with TBST and incubated with a Goat anti-Mouse IgG-HRP conjugated secondary antibody. After a final series of washes to clear away excess secondary antibody, protein detection was performed using an enhanced chemiluminescence (ECL) detection reagent with signals captured on a digital imaging system.

Purification of SAG1 and XynCDBFV-SAG1

The recombinant proteins SAG1 and XynCDBFV-SAG1 were purified through Ni^{2+} affinity chromatography. Initially, the post-cultivation clarified supernatant was loaded onto a Ni^{2+} affinity column pre-equilibrated with binding buffer (20 mM citric acid-phosphate, pH 7.4, containing 0.5 M NaCl and 10 mM imidazole). Subsequently, the column was washed using a buffer containing 20 mM citric acid-phosphate (pH 7.4), 0.5 M NaCl, 20 mM imidazole. The proteins were then eluted with an elution buffer of the same composition but with 300 mM imidazole. The purified proteins underwent dialysis and were stored in phosphate-buffered saline (PBS) at −80°C. The purified protein was detected by SDS PAGE to access its purity, and then the signal intensity of the specific and non-specific band was calculated by ImageJ, thereby estimating the percentage of purity.

Vaccination and challenge infection

One-week-old SPF chickens were randomly allocated into four groups, each comprising seven individuals. Chickens in the SAG1 and XynCDBFV-SAG1 groups were immunized intramuscularly (IM) injection in the breast with 100 μg of the respective recombinant antigen proteins emulsified in Freund's complete adjuvant (FCA). The unchallenged and challenged group was administered PBS. Fourteen days post-primary immunization, each group received a booster of the same antigen dose. Two weeks following the secondary immunization, all groups were exposed to 2000 *E.*

tenella sporulated oocysts, except unchallenged group. One week post-exposure, cecal dissection was performed, and oocyst production was quantified using a McMaster Egg Slide Counting Chamber. The oocyst decrease ratio for each group was calculated. Serum samples were collected on days 0, 14 and 28 after the first immunization and preserved at −20°C for subsequent analysis.

McMaster counting method

The collected caeca were crushed and then adjusted to a volume of 50 mL using a 2.5% potassium dichromate solution. Oocysts were diluted with saturated saline to achieve a concentration that allowed for the observation of 50 to 300 oocysts under a microscope within the counting chamber. Each sample was subjected to counting three times for accuracy.

Serum antigen-specific antibody detection by ELISA

Antigen-specific IgY in serum was detected via indirect ELISA (enzyme-linked immunosorbent assay). ELISA plates were coated with recombinant protein SAG1 or XynCDBFV-SAG1 (1 μg/mL) and incubated overnight at 4°C. Plates were washed three times with PBST containing 0.05% Tween 20, following by blocking with 5% skim milk in PBST for 2 h. Serum samples, diluted 1:2000, were then added and incubated at 37°C. After further washing with PBST, plates were incubated for 1 h at 37°C with HRP-conjugated goat anti-chicken IgY (Abcam, UK), diluted at 1:10,000. Colour development was achieved using TMB solution, and optical density at 450 nm (OD450) was measured using an automated microplate reader. Samples were processed in triplicate.

Fed-batch fermentation

Fed-batch fermentation of *P. pastoris* was conducted in a 15 L fermenter (EastBio Co. Ltd., Jiangsu, China), following the method previously described (Lu et al., 2023). Initially, a positive transformant was cultured in 50 mL YPD at 30°C and 220 rpm for 48 h. Subsequently, this culture was expanded to 200 mL YPD with a 1% inoculation until an OD of 6.0 was achieved. The entire cell culture was then transferred to a 15-L fermenter filled with 7.5 L of basal salt medium and PTM1 solution. For large-scale fermentation, the conditions were maintained at a stirring speed of 600 rpm and a temperature of 30°C. The pH was stabilized at 5.4 using 28% ammonium hydroxide. Upon a sharp increase in dissolved oxygen (DO) due to glycerol depletion, a feed containing 50% glycerol and PTM1 solution was introduced at 18 mL/(L·h) over 6 h until biomass reached approximately 200 g/L. Following this, methanol was used to induce protein expression, meanwhile, the temperature was adjusted to 25°C and DO was controlled at 20%–40%. Cell samples were collected every 24 h for wet weight analysis, and the fermentation supernatant was evaluated for protein expression using Bradford Protein Assay Kit and SDS PAGE analysis.

Statistical analysis

GraphPad Prism v.9.0.0 (GraphPad Software) was used for statistical analysis. Differences among experimental treatments were assessed using ANOVA followed by Duncan's multiple range test, with significance set at $p \leq 0.05$.

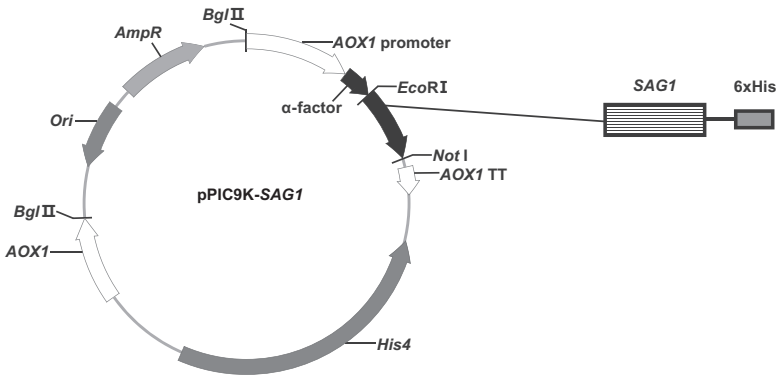
RESULTS

Construction of *SAG1* and *XynCDBFV-SAG1* vectors

As fusion with xylanase XynCDBFV improves the protein expression of hen egg white lysozyme (HEWL), somatostatin and IGF-1/LR3 IGF-1, and the XynCDBFV-somatostatin displays excellent antigenicity (Cui et al., 2022; Huang et al., 2021), we investigated whether xylanase could similarly enhance the expression level and antigenicity of SAG1. To test the hypothesis, the code optimized and synthesized *SAG1* and *XynCDBFV-SAG1* gene sequences were inserted into the *P. pastoris* expression vector pPIC9K with the α-factor leader sequence for recombinant proteins secretion. A flexible linker GGGGSGGGGS between XynCDBFV and SAG1 was designed to facilitate proper folding (Chen et al., 2013). Additionally, a 6 × His tag was fused to the SAG1 carboxyl terminus to simplify detection and purification. The schematic details and identification of the plasmids are shown in Figures 1 and S1.

Heterologous expression of *SAG1* and *XynCDBFV-SAG1* in *P. pastoris*

The plasmids for *SAG1* and *XynCDBFV-SAG1* were linearized and individually transformed into *P. pastoris* GS115 via electroporation. The positive transformants were screened through protein detection of the culture supernatants by SDS PAGE after induction expression with methanol in test tubes. The results demonstrated that distinct protein bands were observed in expected size of SAG1 and XynCDBFV-SAG1 in pPIC9K-*SAG1/*



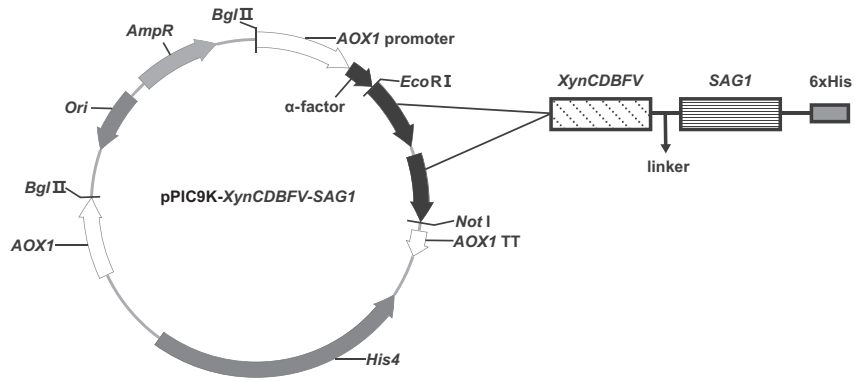


FIGURE 1 SAG1 expression vector construction in *P. pastoris*. Schematic representation of the SAG1 and XynCDBFV-SAG1expression vector genetic map.

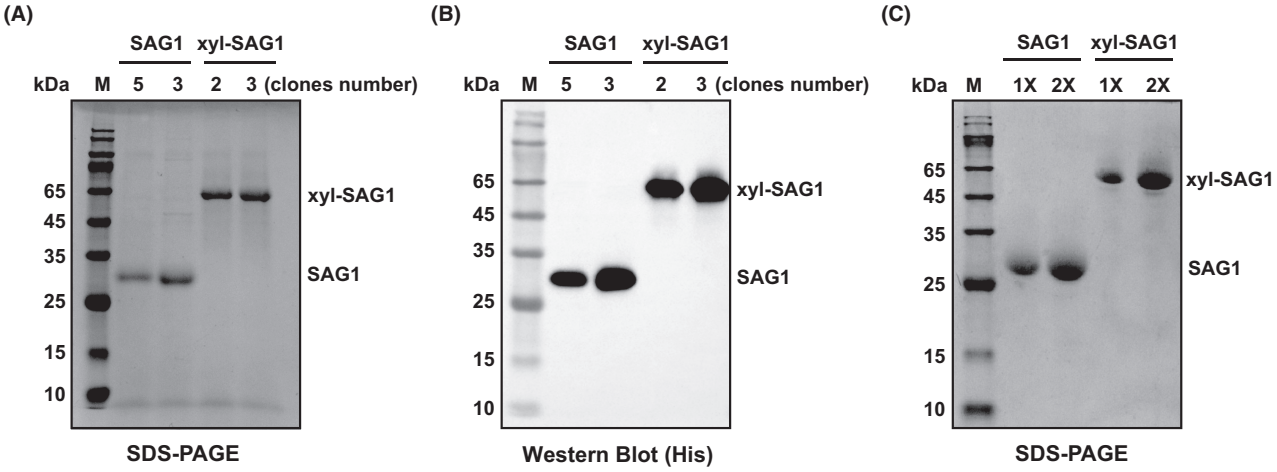


FIGURE 2 Heterologous expression and purification of SAG1 and XynCDBFV-SAG1 in *P. pastoris*. (A) SDS-PAGE analysis of the recombinant SAG1 and XynCDBFV-SAG1 in the flask culture supernatant. (B) Western blot analysis of the recombinant SAG1 and XynCDBFV-SAG1 in the flask culture supernatant. (C) Analysis of purified SAG1 and XynCDBFV-SAG1 by SDS-PAGE. M: protein marker; Xyl-SAG1: XynCDBFV-SAG1.

XynCDBFV-SAG1 clones (Figure S2). In order to verify the heterologous expression of SAG1 and XynCDBFV-SAG1, two positive transformants for each plasmid were chosen and expressed in shaking flasks. After the induction of methanol for 2 days, we observed that both protein band and His signal were detected at expected size for SAG1 and XynCDBFV-SAG1 in the supernatant of the chosen positive clones (Figure 2A,B). These results indicated that both SAG1 and XynCDBFV-SAG1 could be heterologous expressed in *P. pastoris*.

Purification of SAG1 and XynCDBFV-SAG1

In order to gain further insights into the antigenicity of recombinant SAG1 and XynCDBFV-SAG1, we next

purified them through affinity chromatography. As a 6×His tag was fused to the C terminus of SAG1 and XynCDBFV-SAG1, a Ni-NTA affinity column was used for purification from the culture supernatants. Finally, both SAG1 and XynCDBFV-SAG1 achieved up 95% purity after affinity purification (Figure 2C).

Higher immune protection against *E. tenella* in chicken induced by XynCDBFV-SAG1 than SAG1

To assess the protective efficacy of SAG1 and its fusion protein XynCDBFV-SAG1 as a subunit vaccine against *Eimeria*, we evaluated antigen-specific immune responses based on IgY antibody titre and Oocyst output of *E. tenella*. First, we evaluated the adaptive humoral immune responses elicited by immunized with either SAG1 or the fusion protein XynCDBFV-SAG1. IgY antibody specific to SAG1 or XynCDBFV-SAG1 was assessed in the immunized chickens by ELISA. Initially, IgY antibody titres specific against SAG1 or XynCDBFV were found to be minimal at day 14 post-primary immunization. However, these titres exhibited a significant increase on day 14 following the booster immunization in both SAG1 and XynCDBFV-SAG1 groups, while it remained unchanged in the unimmunized groups (Figure 3A). To better compare the humoral immunity response elicited by SAG1 and XynCDBFV-SAG1, we detected the titre of IgY antibodies specific to SAG1 in the sera of SAG1 and XynCDBFV-SAG1 groups in the same ELISA microplate. We observed that there was no significant difference in IgY titres in both groups at day 0 and day 14 post-first immunization but XynCDBFV-SAG1 group showed higher IgY titres against SAG1 than the SAG1 group at day 28 post-immunization (Figure 3B). The findings imply that XynCDBFV augments the humoral immune response elicited by SAG1.

Additionally, we observed that the oocyst outputs were reduced in two immunized groups compared to the unimmunized challenged group, with a particularly significant reduction in the XynCDBFV-SAG1 group (unchallenged: 0; challenged: 5.66 [±1.67] × 10^6; SAG1: 2.58 [±0.38] × 10^6; xyl-SAG1: 9.86 [±4.73] × 10^5) (Figure 3C). And the oocyst decrease ratios were 54.33% (±6.71%) in SAG1 group and 82.67% (±8.30%)

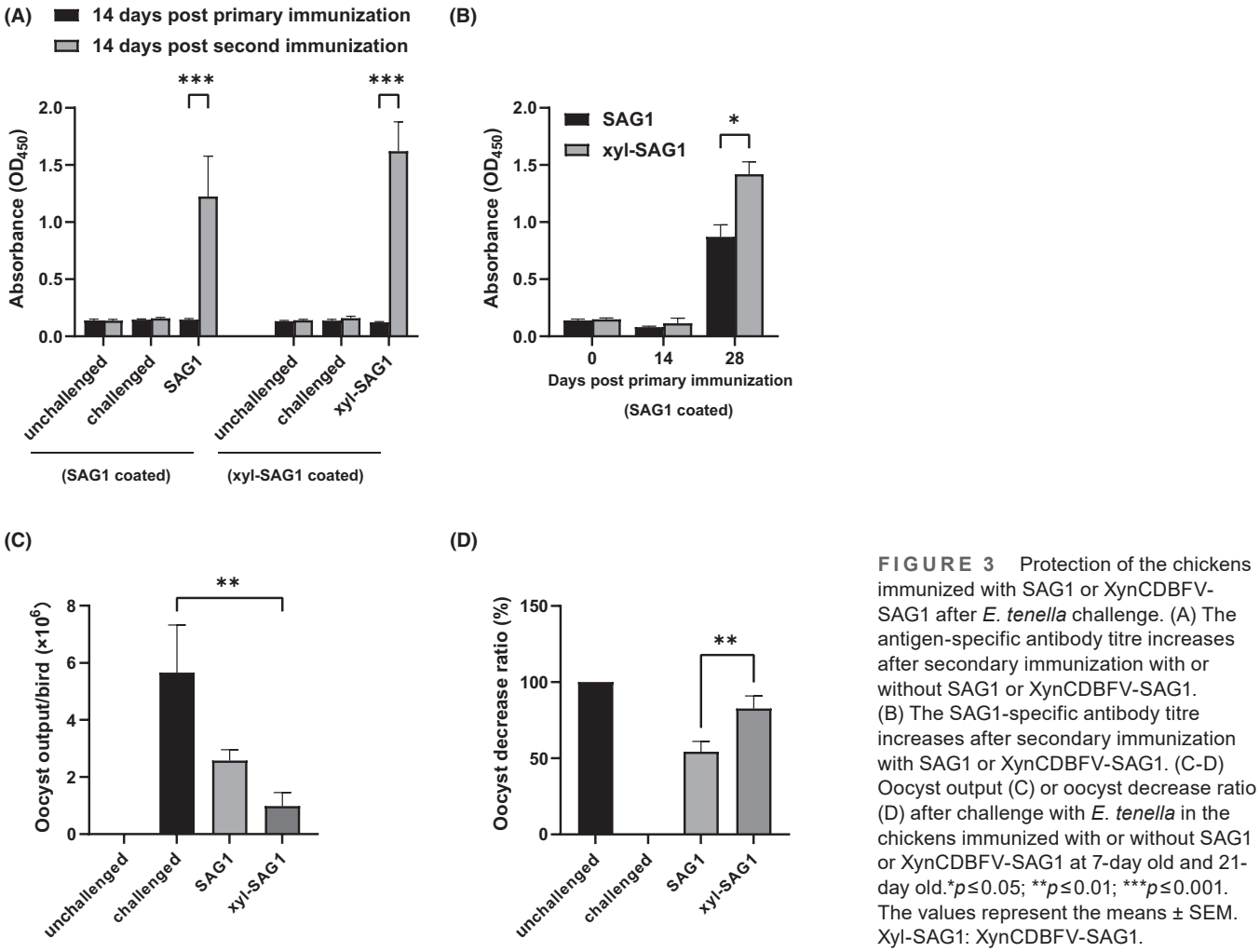


FIGURE 3 Protection of the chickens immunized with SAG1 or XynCDBFV-SAG1 after *E. tenella* challenge. (A) The antigen-specific antibody titre increases after secondary immunization with or without SAG1 or XynCDBFV-SAG1. (B) The SAG1-specific antibody titre increases after secondary immunization with SAG1 or XynCDBFV-SAG1. (C-D) Oocyst output (C) or oocyst decrease ratio (D) after challenge with *E. tenella* in the chickens immunized with or without SAG1 or XynCDBFV-SAG1 at 7-day old and 21-day old.*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. The values represent the means ± SEM. Xyl-SAG1: XynCDBFV-SAG1.

in the XynCDBFV-SAG1 group compared to the challenged group, indicating that XynCDBFV-SAG1 provided enhanced immune protection against *E. tenella* than SAG1 alone (Figure 3D).

Fed-batch production of XynCDBFV-SAG1

Given its robust antigenicity and protective efficacy against *E. tenella*, the fusion protein XynCDBFV-SAG1 may have the potential to serve as subunit candidate vaccine. Therefore, we continued to assess whether XynCDBFV-SAG1 can be produced in large quantity. In a 15-L bioreactor, we observed that the wet cell weight for XynCDBFV-SAG1 reached 361 g/L after 96 h, and the secreted crude proteins in the fermentation supernatant increased from 0.9 g/L at 24 h to 3.6 g/L at 120 h following methanol induction (Figure 4A,B). Furthermore, we also observed that XynCDBFV-SAG1 accumulated continuously by SDS PAGE gel examination (Figure 4C). Based on the SDS PAGE analysis and the concentration of crude proteins, we evaluated that about up to 2 g/L XynCDBFV-SAG1 were produced after methanol induction in a 15-L bioreactor. These results indicated the feasibility of large-scale production of the fusion protein XynCDBFV-SAG1.

DISCUSSION

SAG1, a crucial surface antigen of parasites, is known to elicit robust humoral and cellular immune responses against parasitic infections (Khan et al., 1991; Wang & Yin, 2014). Owing to the high conservation of its sequence across different parasite strains, SAG1 has become one of the most promising vaccine candidates (Lekutis et al., 2001). Specifically, SAG1 of *E. tenella* has been identified as the target of two neutralising monoclonal antibodies, indicating that it plays a significant role in immune protection against *E. tenella* (Wang & Yin, 2014). However, the immune protective efficiency of SAG1 as an efficient vaccine requires enhancement, despite its notable strong antigenicity and immunogenicity. Xylanases are a group of glycoside hydrolases that catalyse the degradation of the polysaccharide xylan into oligoxylose or xylose. These enzymes are predominantly produced by microorganisms including bacteria, fungi, actinomycetes, protozoa and marine algae, with fungi being a notable source (Li et al., 2022; Sadeer et al., 2023).

In this study, a subunit vaccine fused SAG1 of *E. tenella* with xylanase XynCDBFV showed stronger vaccine immunity compared to SAG1 alone. The fusion protein XynCDBFV-SAG1 group showed an increase of nearly 30% in the reduction of oocyst count compared

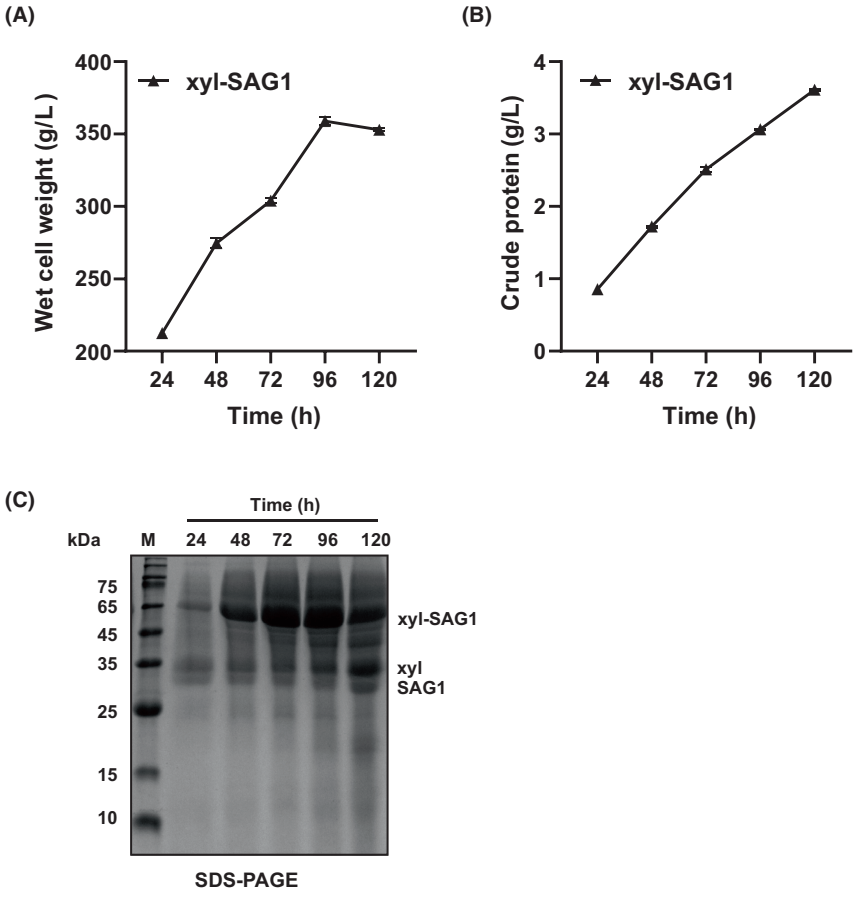


FIGURE 4 Fed-batch production of XynCDBFV-SAG1. (A) Wet weight of XynCDBFV-SAG1 strains during the induction time. (B) Crude protein in fermentation supernatant of XynCDBFV-SAG1 strains. (C) SDS-PAGE analysis for expression during fermentation. M: protein marker; Xyl: XynCDBFV; Xyl-SAG1: XynCDBFV-SAG1.

to the SAG1 only group (Figure 3A). Furthermore, both SAG1 only group and XynCDBFV-SAG1 group elicited significant increase of specific IgY level at 14 days post the second immunization, and XynCDBFV-SAG1 group showed higher IgY titres against SAG1 than the SAG1 group only at day 28 post-immunization (Figure 3B). We also recorded the animal survival rate, body weight gains and intestinal lesson scores. However, the results showed that all animals were alive, their body weight did not decrease, and no significant intestinal lesions were observed 7 days after being exposed to 2000 *E. tenella* sporulated oocysts, which may be due to the low infection dose of sporulated oocysts. As it has been reported that oocyst output is the most important criterion for evaluating the vaccine's protective efficacy (Roberts et al., 1996), therefore, it is suggested that fusion with XynCDBFV enhanced immune protection against *E. tenella* of SAG1.

XynCDBFV is a thermostable alkaline xylanase derived from a rumen fungus *Neocallimastix patriciarum* and highly recombinant expressed in *P. pastoris* (Fang et al., 2014; Han et al., 2017). Due to the high expression of XynCDBFV in *P. pastoris*, we have successfully fused it to several difficult-to-express proteins, such as somatostatin, HEWL and IGF-1/LR3 IGF-1, and these fusions greatly increased their expression levels in *P. pastoris*, while preserving their bioactivity (Cui et al., 2022; Huang et al., 2021; Lu et al., 2023). As shown in this study, fusion XynCDBFV with SAG1 did not increase the expression level of SAG1, but we found surprisingly that the fusion with XynCDBFV improved the immune protection of SAG1 against *E. tenella*, which is the first report that the fusion of xylanase with antigen improves the immune protection of antigen. However, it remains an open question how XynCDBFV enhances immune protection of SAG1 against *E. tenella*, which is an interesting topic warranting future investigation. There may be several possible explanations for these results. First, the fusion of XynCDBFV may promote the stability and antigenicity of SAG1, which is partially verified by the result that XynCDBFV-SAG1 induces stronger humoral immune response than SAG1 alone (Figure 3D). Second, the fusion of XynCDBFV may induce stronger cellular immunity through some unknown mechanisms. Finally, XynCDBFV or its products may inhibit the growth of *E. tenella* by modulating the intestinal microbiota. All of these explanations require further confirmation.

This study shows that XynCDBFV-SAG1 as a candidate subunit vaccine exhibits excellent inhibitory effect on infection *E. tenella*, so it is particularly important to produce it in large quantities. SAG1 of *E. tenella* is usually recombinant expressed in *E. coli*. However, the production of SAG1 in *E. coli* results in the formation of inclusion bodies, which make it difficult to refold and purify (Appiah-Kwarteng et al., 2019; Chen et al., 2001). For instance, the expression of recombinant SAG1 was produced in *E. coli* to a final concentration of 200 μg/L (Jahn et al., 2009). In this study, we attempted to produce SAG1 and XynCDBFV-SAG1 in the *P. pastoris* expression system, as they have the advantages of proper folding, convenient purification and high product yield (Karbalaei et al., 2020; Tachioka et al., 2016). In this study, the yield of fusion protein XynCDBFV-SAG1 was up to about 2 g/L in a 15 litre fermenter, much higher than the production efficiency in previous reports. However, due to the high inorganic salt content in the medium used for high-density fermentation, the fusion proteins were partially fragmented (Figure 4C). Adjustments to the fermentation conditions will continue to be made to reduce the loss of fusion proteins.

AUTHOR CONTRIBUTIONS

Chen Liu: Conceptualization; data curation; investigation; methodology; writing – original draft; writing – review and editing. **HanBing Wei:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Ruiying Liang:** Investigation; methodology; writing – review and editing. **Yuan Wang:** Investigation; methodology; writing – review and editing. **Xiaoyun Su:** Investigation; methodology; writing – review and editing. **Tao Tu:** Investigation; methodology; writing – review and editing. **Huiying Luo:** Writing – original draft; writing – review and editing. **Bin Yao:** Conceptualization; methodology; supervision; writing – original draft; writing – review and editing. **Jiabo Ding:** Conceptualization; formal analysis; supervision. **Xinming Tang:** Data curation; investigation; writing – review and editing. **Huoqing Huang:** Data curation; methodology; writing – review and editing. **Honglian Zhang:** Data curation; methodology; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All materials are available by the corresponding author upon reasonable request.

ETHICS STATEMENT

The animal studies described in this work followed institutional guidelines for animal welfare and biosafety and were approved by Animal Welfare and Ethics Committee of Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS 2023-74).

CONSENT FOR PUBLICATION

All authors have read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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