Preliminary evaluation of the protective effects of recombinant AMA1 and IMP1 against *Eimeria stiedae* infection in rabbits

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**Abstract**

**Background:** *Eimeria stiedae* parasitizes the bile duct, causing hepatic coccidiosis in rabbits. Coccidiosis control using anticoccidials led to drug resistance and residues; therefore, vaccines are required as an alternative control strategy. Apical membrane antigen 1 (AMA1) and immune mapped protein 1 (IMP1) are surface-located proteins that might contribute to host cell invasion, having potential as candidate vaccine antigens.

**Methods:** Herein, we cloned and expressed the *E. stiedae* EsAMA1 and EsIMP1 genes. The reactogenicity of recombinant AMA1 (rEsAMA1) and IMP1 (rEsIMP1) proteins were investigated using immunoblotting. For the vaccination-infection trial, rabbits were vaccinated with rEsAMA1 and rEsIMP1 (both 100 μg/rabbit) twice at 2-week intervals. After vaccination, various serum cytokines were measured. The protective effects of rEsAMA1 and rEsIMP1 against *E. stiedae* infection were assessed using several indicators. Sera were collected weekly to detect the specific antibody levels.

**Results:** Both rEsAMA1 and rEsIMP1 showed strong reactogenicity. Rabbits vaccinated with rEsAMA1 and rEsIMP1 displayed significantly increased serum IL-2 ($F_{(4, 25)} = 9.53, P = 0.000$), IL-4 ($F_{(4, 25)} = 7.81, P = 0.000$), IL-17 ($F_{(4, 25)} = 8.55, P = 0.000$), and IFN-γ ($F_{(4, 25)} = 6.89, P = 0.001$) levels; in the rEsIMP1 group, serum TGF-β1 level was also elevated ($F_{(4, 25)} = 3.01, P = 0.037$). After vaccination, the specific antibody levels increased and were maintained at a high level. The vaccination-infection trial showed that compared with the positive control groups, rabbits vaccinated with the recombinant proteins showed significantly reduced oocyst output ($F_{(5, 54)} = 187.87, P = 0.000$), liver index ($F_{(5, 54)} = 37.52, P = 0.000$), and feed conversion ratio; body weight gain was significantly improved ($F_{(5, 54)} = 28.82, P = 0.000$).

**Conclusions:** rEsAMA1 and rEsIMP1 could induce cellular and humoral immunity, protecting against *E. stiedae* infection. Thus, rEsAMA1 and rEsIMP1 are potential vaccine candidates against *E. stiedae*.

**Keywords:** *Eimeria stiedae*, AMA1, IMP1, Recombinant protein-based subunit vaccine, Protective efficacy

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Background
Rabbit coccidiosis is a highly contagious protozoan disease, which can affect rabbits of all ages, especially those between 1 and 4 months old [1]. Coccidiosis in adult rabbits is usually subclinical and asymptomatic. However, adults can become carriers and suffer from poor feed conversion and growth performance [2, 3]. Among the *Eimeria* species that infect rabbits, only *Eimeria stiedae* parasitizes the liver bile duct [4, 5]. *Eimeria stiedae* completes its endogenous stages in rabbit bile duct epithelial cells and causes liver dysfunction during its reproduction, resulting in severe hepatic coccidiosis [6].

Currently, rabbit coccidiosis control relies mainly on anticoccidials, which have led to problems such as drug resistance and residues [7]. Vaccines are promising alternative control strategies for chemotherapy. However, live vaccines are relatively expensive and carry the risk of pathogen transmission or reversal of virulence [8]. Recombinant subunit vaccines may circumvent these drawbacks [8]. Coccidia undergo four life-cycle stages and need to migrate in the host; therefore, their antigenic composition is very complex, making screening of protective antigens for next-generation vaccines particularly important [9]. Studies have screened for candidate antigens [10]; however, only CoxAbic® (Netanya, Israel) is currently commercialized [11].

Apical membrane antigen 1 (AMA1) is a key molecule for apicomplexans to invade host cells [12]. Recently, research on AMA1 as an antigen has shown a certain level of protection against apicomplexan infections such as *Plasmodium* spp. [13], *Toxoplasma gondii* [14], *Babesia* spp. [15], and *Eimeria* spp. [16]. Immune mapped protein 1 (IMP1), identified in 2011, is a surface-located protein that might contribute to host cell invasion [17]. In subsequent research, recombinant *Emax*IMP1 induced protection against *E. maxima* infection [18]. Furthermore, the homologous gene of IMP1 was found in *Toxoplasma gondii* and *Neospora caninum* [19, 20]. So far, there has been no report of a recombinant subunit vaccine for rabbit coccidiosis. In this study, the *EsAMA1* and *EsIMP1* genes were selected based on *E. stiedae* transcriptome data for prokaryotic expression [21]. Then, the recombinant proteins, r*EsAMA1* and r*EsIMP1*, were used as subunit vaccines. The results showed that r*EsAMA1* and r*EsIMP1* conferred immune protection against *E. stiedae* by stimulating both humoral and cellular immune responses. This research provides a reference for developing a recombinant protein-based subunit vaccine for *E. stiedae*.

Methods
Parasites, Animals, and Sera
The *E. stiedae* Sichuan strain was propagated in our laboratory. Sixty coccidia-free New Zealand White rabbits (45 days old, 1.086 ± 0.068 kg, 30 females and 30 males), with five female and five male rabbits in each group, were randomly grouped. Experimental groups included the r*Es*-IMP1 and r*EsAMA1* groups (r*EsIMP1* or r*EsAMA1* proteins vaccinated and *E. stiedae* infected). Positive control groups included PBS-infected (sterile phosphate-buffered saline mock-vaccinated and *E. stiedae* infected), Quil-A-infected (saponin derivative Quil-A mock vaccinated and *E. stiedae* infected), and Trx-His-S-infected (pET-32a tag protein mock vaccinated and *E. stiedae* infected) groups. The PBS-uninfected group comprised sterile phosphate-buffered saline mock vaccination without *E. stiedae* infection (Table 1). The rabbits were housed in pairs in flame-sterilized steel cages, with a plastic partition placed at the bottom to prevent contact with feces. The rabbits were raised based on the method described by Wei’s research [22]. Anticoccidial drugs were discontinued 1 week before the challenge infection was performed, and pathogenic examination was performed every other day to ensure that no coccidia oocysts were detected. The rabbits were vaccinated with a bivalent vaccine against rabbit hemorrhagic disease virus and *Pasteurella multocida* at 35 days old.

Rabbits were inoculated orally with 5 × 10⁴ *E. stiedae* sporulated oocysts for positive sera collection. Negative sera were obtained from the 1-month-old coccidia-free rabbits without past exposure to any *Eimeria* species. All sera were stored at −20 °C.

Bioinformatic analysis
The open reading frames (ORF) and amino acid sequences of *EsAMA1* and *EsIMP1* were obtained using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The ExpASy Proteomics Server (http://www.expasy.org/protparam/) was used to predict the molecular weight (MW) of the proteins. The TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/#opennewwindow) and the SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/) server were used to analyze the transmembrane regions and signal peptides of the proteins, respectively. B-cell epitopes were predicted using the IEBD Analysis Resource (http://tools.immuneepitope.org/bcell/). The multiple sequence alignment was performed using Jalview 2.11.2.0 [23].

Cloning, expression, and protein purification
Total RNA of *E. stiedae* sporulated oocysts was extracted using a commercial kit (Tiangen, China). First-strand cDNA was synthesized from the total RNA and then used for second-strand cDNA synthesis (Thermo, Waltham, MA, USA). The *EsAMA1* and *EsIMP1* specific primers were designed based on transcriptome data [21]: *EsAMA1*-F 5′-CGGGATCCATGTGGAAGATGAGG
CTTGT-3', and EsAMA1-R 5'-CCCTCGAGTTAAGAGTCCTGGTCACGG-3', with BamHI and XhoI restriction enzyme sites (underlined) (Takara, Dalian, China); Es1IMP1-F 5'-CGGGATCCATGGGGGCCCCTCTGTTCG-3', Es1IMP1-R 5'-GGCTGCACTCAATCATCCTTGCTTCTCCTGTG-3', with BamHI and SalI restriction enzyme sites (underlined).

The EsAMA1 and Es1MP1 genes were amplified by polymerase chain reaction (PCR), and the amplicons were sequenced (Sangon, Shanghai, China). The target EsAMA1 and Es1MP1 fragments were digested with BamHI/XhoI and BamHI/SalI, respectively, and then ligated into the expression vector pET32a(+) (Takara). Then, *Escherichia coli* BL21 (DE3) was used to express the proteins (induced by 1 mM isopropyl β-d-thiogalactopyranoside). rEsAMA1 and rEs1MP1 were purified using a Nuvia Ni-Charged IMAC Cartridge (Bio-Rad, Hercules, CA, USA). After purification, rEs1MP1 and rEsAMA1 were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified fusion Trx-His-S tag protein (with no insert fragment) was cryopreserved in our laboratory. The recombinant protein that was expressed in the inclusion bodies of *E. coli* was dialyzed at 4 °C according to the method detailed in Shi’s study [24].

**Western blotting analysis**

The separated rEsAMA1 and rEs1MP1 were transferred onto nitrocellulose (NC) membranes (Boster, Wuhan, China), separately. After blocking for 2 h using Tris-buffered saline (TBS) containing 5% (w/v) skimmed milk at room temperature, the NC membranes were incubated with positive or negative sera (1:200 v/v dilution) overnight at 4 °C. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (EarthOx Life Sciences, Millbrae, CA, USA, 1:2000 v/v dilution) for 2 h at room temperature. Then, detection of the specific bands was performed using a Metal Enhanced DAB Substrate Kit (20 ×) (Solarbio, Beijing, China) after further washing.

**Design of the vaccination-infection trial**

The details of trial design and vaccine procedures are summarized in Table 1. The timings of sample collection are shown in Fig. 1.

**Evaluation of protective effect**

Safety evaluation: The health status of all experimental rabbits was observed after vaccination. The bodyweight of each rabbit was recorded before the first vaccination, booster vaccination, and infection. The weight gain after vaccination was determined as the weight before infection minus the weight before the first vaccination to verify whether the vaccination affects the weight gain of the experimental rabbits.

The protective effects of rEsAMA1 and rEs1MP1 against *E. stiedae* infection were assessed according to several indicators, calculated as follows: (1) The survival rate in each group was obtained by dividing the number of surviving rabbits by the initial number of rabbits. (2) The body weight gain after infection was assessed according to the method detailed in Shi’s study [24].

| Groups          | Number of rabbits | Immunogen and dosage | Vaccination weeks | Vaccination route | infection dose/week/route |
|-----------------|-------------------|-----------------------|-------------------|-------------------|--------------------------|
| PBS-uninfected  | 10                | 1 ml Sterile PBS      | 0, 2              | Subcutaneous injection in the neck | –                        |
| PBS-infected    | 10                | 1 ml Sterile PBS      | 0, 2              | Subcutaneous injection in the neck | 1 × 10⁴ sporulated oocysts/week 4/oral |
| Quil-A-infected | 10                | 1 mg Quil-A dilution in 1 ml PBS | 0, 2 | Subcutaneous injection in the neck | 1 × 10⁴ sporulated oocysts/week 4/oral |
| Trx-His-S-infected | 10          | 100 μg Trx-His-S tag + 1 mg Quil-A dilution in 1 ml PBS | 0, 2 | Subcutaneous injection in the neck | 1 × 10⁴ sporulated oocysts/week 4/oral |
| rEs1MP1         | 10                | 100 μg rEs1MP1 + 1 mg Quil-A dilution in 1 ml PBS | 0, 2 | Subcutaneous injection in the neck | 1 × 10⁴ sporulated oocysts/week 4/oral |
| rEsAMA1         | 10                | 100 μg rEsAMA1 + 1 mg Quil-A dilution in 1 ml PBS | 0, 2 | Subcutaneous injection in the neck | 1 × 10⁴ sporulated oocysts/week 4/oral |
Estimation of serum AST/ALT levels
Blood samples were collected into vacuum blood collection tubes without any anticoagulants before sacrifice. The serum alanine aminotransferase (AST) and aspartate aminotransferase (ALT) levels were then measured using enzyme-linked immunosorbent assay (ELISA) kits (RUIXIN Biotech, Quanzhou, China).

Determination of serum anti-rEsAMA1 and rEsIMP1 IgG levels
Pre-vaccine sera (week 0) were collected, and then sera were collected weekly after vaccination. All sera were stored at −20 °C. Specific antibody levels in the sera (OD450 values of serum samples) were evaluated using indirect ELISAs based on the recombinant proteins rEsAMA1 and rEsIMP1. The concentrations of the recombinant proteins and sera were determined using standard checkerboard titration procedures [26]. The optimal concentration of rEsAMA1 was 0.78 μg/well, while it was 0.96 μg/well for rEsIMP1. The optimal serum dilution was 1:160.

Detection of serum cytokine levels
The levels of circulating interleukin (IL)-2, IL-4, IL-10, IL-17, interferon gamma (IFN-γ), and transforming growth factor beta 1 (TGF-β1) were estimated after two vaccinations using ELISAs. The rabbit IFN-γ ELISA kit was purchased from MABTECH (Nacka Strand, Sweden), and the other ELISA kits were purchased from CUSABIO (Wuhan, China).

Statistical analysis
The differences among the groups were assessed using one-way analysis of variance (ANOVA) employing IBM SPSS statistics 22.0 (IBM Corp., Armonk, NY, USA). P < 0.05 was considered significant, and P < 0.01 was considered extremely significant.

Results
Cloning and bioinformatic analysis
The sequences of EsAMA1 (GenBank accession number: MZ934414) and EsIMP1 (GenBank accession number: MZ934415) were successfully amplified. The ORF of EsAMA1 was 1644 bp (encoding a protein with a predicted MW of 60 kDa). EsAMA1 has a predicted transmembrane region (amino acids 458–480), but no predicted signal peptide. The ORF of EsIMP1 was 1191 bp (encoding a protein with a predicted MW of 43 kDa). EsIMP1 has no predicted signal peptide or transmembrane region.

Multiple sequence alignments revealed that EsIMP1 and EsAMA1 proteins are highly variable (Fig. 2). The amino acid sequences of EsIMP1 and EsAMA1 shared 37.02–44.15% and 37.67–53.66% identity with IMP1 and AMA1 proteins from different Apicomplexan species, respectively.
Expression, purification, and western blotting analysis of rEsAMA1 and rEsIMP1

rEsIMP1 (~63 kDa) was expressed in the supernatant, and rEsAMA1 (~80 kDa) was expressed in inclusion bodies of E. coli. rEsAMA1 was mainly dissolved in 6 M and 8 M urea (Fig. 3, lanes 1–4). The MW of the recombinant proteins included the approximately 20-kDa fusion tag protein encoded by vector pET32a(+). After purification using an Ni²⁺-affinity column, rEsIMP1 and rEsAMA1 were analyzed using SDS-PAGE (Fig. 3, lane 5). Purified rEsAMA1 was dialyzed at 4 °C according to the method detailed in Shi's study [2624].

Fig. 2 Multiple sequence alignments of IMP1 and AMA1 from different species. (a) Multiple alignment of E. stiedae IMP1 with IMP1 proteins from other parasites: Eimeria maxima (UniProt: F4MK46), Eimeria acervulina (UniProt: U6GH29), Eimeria tenella (UniProt: F4MK47), Eimeria mitis (UniProt: U6G27), Eimeria praecox (UniProt: U6GP2), (b) multiple alignment of E. stiedae AMA1 with AMA1 proteins from other parasites: Eimeria tenella (UniProt: U6KTA0), Eimeria brunetti (UniProt: U6LLB9), Toxoplasma gondii (UniProt: B6KAM0), Besnoitia besnoiti (UniProt: A0A2A9MBX4), Neospora caninum (UniProt: F0VH85). Blue shading indicates conserved residues. Dashed red boxes represent B-cell epitopes. The transmembrane region is marked with a solid red box.
rEsIMP1 and rEsAMA1 reacted with anti-E. stiedae positive sera and specific bands were observed on the NC membranes (Fig. 3, lane 6), while incubation with the sera from coccidia-free rabbits showed no specific bands (Fig. 3, lane 7). These results indicated that both rEsIMP1 and rEsAMA1 have strong reactogenicity.

**Evaluation of the protective effect of rEsAMA1 and rEsIMP1**

No statistically significant differences were observed for weight gain after vaccination among the six groups \((F(5, 54)=0.16, P=0.977)\) (Table 2). No obvious adverse reactions were observed in the vaccinated rabbits. This result suggested that the recombinant proteins rEsAMA1 and rEsIMP1 have good safety at the doses used in our experiments.

Gross postmortem examination showed that the livers of the infected groups were enlarged, and their surfaces were full of different-sized and -shaped yellowish-white nodules. The gallbladders were distended with yellowish fluid (Fig. 4).

According to the survival rate, weight gain, oocyst output, liver index, and the feed conversion ratio, rEsAMA1 and rEsIMP1 showed good protection against E. stiedae infection (Table 2). The average body weight gain after infection in the two protein vaccinated groups (78.1% and 94.4%, respectively) was significantly higher than that in the three positive control groups: PBS-infected, Quil-A-infected, and Trx-His-S-infected groups \((F(5, 54)=28.82, P=0.000)\).

The oocyst output decreased significantly in the rEsAMA1 and rEsIMP1 groups \((F(5, 54)=187.87, P=0.000)\), displaying 74.6% and 80.0% oocyst output reductions, respectively.

The rEsAMA1 and rEsIMP1 groups had better feed conversion ratios compared with the positive control groups from week 4 to week 7. The feed conversion

| Table 2 | Protective effects of rEsAMA1 and rEsIMP1 against E. stiedae infection under different evaluation indicators |
|---------|---------------------------------------------------------------------------------------------------------|
| Groups  | Average body weight gain after vaccination (g) | Average body weight gain after infection (g) | Relative body weight gain rate (%) | Oocyst shedding per rabbit \((\times 10^5/g)\) | Oocyst decrease ratio (%) | Average liver index | Feed conversion ratio | Survival rate (%) |
|---------|-------------------------------------------------|---------------------------------|----------------------------|---------------------------------|-----------------|------------------|------------------|-----------------|
| PBS-uninfected | 902.00±90.16a | 656.60±99.5a | 100 | 0a | – | 3.06±0.26a | 3.20±1.0 | 100 |
| PBS-infected | 870.00±42.69a | 347.00±104.25b | 52.9 | 15.84±2.42b | 0 | 6.92±0.94b | 6.05±1 | 100 |
| Quil-A-infected | 907.00±160.77a | 361.80±89.51b | 55.2 | 16.57±2.80b | – 4.6 | 6.84±0.85b | 5.80±1 | 100 |
| Trx-His-S-infected | 886.00±105.75a | 341.80±78.95b | 52.1 | 16.15±2.12b | – 1.9 | 7.02±1.21b | 6.14±1 | 100 |
| rEsIMP1 | 881.00±105.67a | 619.00±70.00a | 94.4 | 3.16±0.66c | 800 | 4.27±0.68c | 3.39±1 | 100 |
| rEsAMA1 | 897.78±116.06a | 512.00±48.26c | 78.1 | 4.02±0.39c | 74.6 | 5.21±0.83a | 4.10±1 | 100 |

The data are presented as the mean ± standard deviation (SD). In each column, there is a significant difference between the data marked with different letters (a, b, c; ANOVA, \(P<0.05\)), and there is no significant difference between the data marked with the same letter \((P>0.05)\).
ratios of the rEsAMA1 and rEsIMP1 groups were 4.10:1 and 3.39:1, respectively, while the PBS-infected, Quil-A-infected, and Trx-His-S-infected groups reached ratios of 6.05:1, 5.80:1, and 6.14:1, respectively. The liver indices of the rEsAMA1 and rEsIMP1 groups were lower than those of the three positive control groups ($F_{(5, 54)} = 37.52$, $P = 0.000$).

**Estimation of serum AST/ALT levels in different groups of the experiment**

Compared with the PBS-uninfected group, the serum ALT levels of the five infected groups increased significantly ($F_{(5, 30)} = 3.43$, $P = 0.014$). There was no statistical difference in serum AST levels ($F_{(5, 30)} = 1.05$, $P = 0.408$) (Table 3).

**Serum anti-rEsAMA1 and rEsIMP1 antibody levels**

In the rEsIMP1 group (Fig. 5a), the anti-rEsIMP1 antibody level increased rapidly after the first vaccination and reached its highest level in the second week. In the rEsAMA1 group (Fig. 5b), the anti-rEsAMA1 antibody level increased more slowly than that in the rEsIMP1 group and reached its highest level in the third week. After two vaccinations with rEsIMP1 and rEsAMA1, the specific antibody levels were maintained at a high level.
of *Eimeria piriformis* were selected in rabbits. The PL PBS-uninfected 21.82 serum levels of IL-2 (*F*<sup>28</sup>, *r*<sup>Es</sup>) groups (PL) of *Eimeria flavescens* [29], *Eimeria media* [30], and *Eimeria piriiformis* [31] were selected in rabbits. The PL of *E. intestinalis* had strong immunogenicity: the vaccinia of six oocysts was sufficient to reduce the oocyst output by about 60%, while vaccination with 600 or more oocysts provided complete protection in rabbits [32]. Mohamed et al. [33] reported a 97% oocyst output reduction in rabbits following vaccination with 3500 PL of *E. magna*. However, the PL of *E. flavescens* showed weak immunogenicity [34]. Additionally, it is possible to use the gamma-ray radiation-attenuated *Eimeria* spp. as a vaccine to prevent coccidiosis [35].

However, live anticoccidial vaccines have drawbacks such as high production cost and the risk of virulence reversal [8]. Therefore, it is necessary to explore a new generation of anticoccidial vaccines to overcome these shortcomings. Recombinant subunit vaccines are easier to mass produce than live vaccines and have a longer shelf life, making them an ideal alternative strategy [36]. In recent years, some antigens have shown good protective effects in chicken coccidiosis and have been identified as vaccine candidate antigens, including adhesion and invasion-related antigens [16, 18], sexual reproduction-stage-related antigens [37], and common antigens [38]. Omata [39] and Hanada et al. [40] found that the soluble antigens in the bile from *E. stiedae*-infected rabbits could induce protection against *E. stiedae* infection. Rabbits vaccinated with *E. stiedae* coproantigen in Freund’s adjuvant showed a high level of IgG response, and vaccination resulted in a decline in the oocyst count [41]. These studies showed that it is feasible to develop vaccines using the immunodominant antigens of rabbit coccidia.

AMA1 is involved in apicomplexan host cell invasion and has been considered a candidate vaccine antigen [12, 16]. Lei et al. [42] found that BALB/c mice vaccinated with *rPoAMA1* (recombinant AMA1 protein from *Plasmodium ovale*) showed high antibody titers. *rPoAMA1* induced IFN-γ-secreting cells and increased the lymphocyte proliferation response. In chickens, AMA1 has also been studied as a candidate vaccine antigen against coccidial infection [43, 44]. A previous study reported a 25.37–33.33% oocyst output reduction and a 75.45–81.50% weight gain in chickens following vaccination with live bacteria expressing *EtAMA1* and infection with 4×10<sup>4</sup> *E. tenella* sporulated oocysts [16]. Another study reported a 77.4% oocyst output reduction in chickens following vaccination with recombinant *E. tenella* AMA1 protein and infection with 250 *E. tenella* sporulated oocysts [44]. In our study, rabbits vaccinated with *rEsAMA1* showed improved weight gain, and the reduction rate of oocyst output in feces reached 74.6%. We consider the reasons for the difference between studies to be as follows: (1) The two previous studies [16, 44] were performed with different infectious doses and *E. tenella* isolates. (2) The *E. stiedae* used in this study parasitizes the rabbit liver, and there are marked differences from chicken coccidia in terms of biological characteristics such as host, parasitic site, and pathogenicity, which will also lead to differences in the results.

Recombinant IMP1 could provide immune protection against chicken coccidiosis [18, 45–47]. In this study, rabbits vaccinated with *rEsIMP1* displayed a 94.4% body

### Table 3 Biochemical estimation of ALT and AST levels in the six groups

| Groups            | ALT (U/l)     | AST (U/l)    |
|-------------------|---------------|--------------|
| PBS-uninfected    | 21.82 ± 1.86<sup>a</sup> | 15.93 ± 1.55<sup>a</sup> |
| PBS-infected      | 30.33 ± 3.82<sup>b</sup>  | 19.34 ± 1.61<sup>a</sup>  |
| Quil-A-infected   | 29.55 ± 3.15<sup>b</sup>  | 18.43 ± 3.58<sup>b</sup>  |
| Trx-His-S-infected| 29.05 ± 5.44<sup>c</sup>  | 18.74 ± 3.77<sup>a</sup>  |
| *rEsAMA1*         | 27.96 ± 3.90<sup>c</sup>  | 18.23 ± 3.33<sup>b</sup>  |
| *rEsIMP1*         | 28.75 ± 5.24<sup>b</sup>  | 17.45 ± 2.44<sup>a</sup>  |

The data are presented as mean ± standard deviation (SD). In each column, there is a significant difference between the data marked with different letters (a, b, c; ANOVA, *P* < 0.05), and there is no significant difference between the data marked with the same letter (*P* > 0.05).

In addition, the Trx-His-S-infected group also showed an increase in antibody levels, indicating that the inclusion of the Trx-His-S tag in the recombinant *rEsIMP1* and *rEsAMA1* proteins increased the antibody levels; however, the Trx-His-S-infected group’s antibody levels were lower than those of the *rEsIMP1* and *rEsAMA1* groups. The PBS-infected and Quil-A-infected groups showed almost no changes in antibody levels.

### Cytokine levels induced by *rEsAMA1* and *rEsIMP1*

The serum cytokine levels were estimated 2 weeks after the booster vaccination. Rabbits vaccinated with *rEsAMA1* and *rEsIMP1* displayed significantly increased serum levels of IL-2 (*F*<sup>4, 25</sup> = 9.53, *P* = 0.000), IL-4 (*F*<sup>4, 25</sup> = 7.81, *P* = 0.000), IL-17 (*F*<sup>4, 25</sup> = 8.55, *P* = 0.000), and IFN-γ (*F*<sup>4, 25</sup> = 6.89, *P* = 0.001); the TGF-β1 level was also elevated in the *rEsIMP1* group (*F*<sup>4, 25</sup> = 3.01, *P* = 0.037). The IL-10 level increased significantly in all groups except the PBS-infected group (*F*<sup>4, 25</sup> = 10.53, *P* = 0.000) (Fig. 6).

### Discussion

At present, control of coccidiosis mainly relies on the addition of anticoccidials. However, the emergence of drug resistance and drug residues forced researchers to focus on vaccine development [7]. The precocious lines (PL) of *Eimeria intestinalis* [27], *Eimeria magna* [28], *Eimeria flavescens* [29], *Eimeria media* [30], and *Eimeria piriiformis* [31] were selected in rabbits. The PL of *E. intestinalis* had strong immunogenicity: the vaccination of six oocysts was sufficient to reduce the oocyst output by about 60%, while vaccination with 600 or more oocysts provided complete protection in rabbits [32]. Mohamed et al. [33] reported a 97% oocyst output reduction in rabbits following vaccination with 3500 PL of *E. magna*. However, the PL of *E. flavescens* showed weak immunogenicity [34]. Additionally, it is possible to use...
weight gain and an 80.0% oocyst output reduction. Our results are similar to Yin’s reports [45, 47], in which chickens vaccinated with rEtIMP1 or its fusion expression product, rEtIMP1-CD40L, showed significantly reduced lesion scores, reduced oocyst output (by 62.58–77.6%), and a relative weight gain rate of 70.86–86.03%. Kundu et al. [46] found that in chickens vaccinated with rEtIMP1, the cecal parasite genome numbers were reduced by 67–79%. Based on the above test results, the recombinant proteins rEsAMA1 and rEsIMP1 showed good protective effects against E. stiedae infection.

Cellular immunity performs an essential function in defending the host from coccidiosis, whereas the effect of humoral immunity is limited [48, 49]. Th1-type cytokines, such as IFN-γ and IL-2, partially regulate T-cell responses against Eimeria [50, 51]. IFN-γ enhances macrophage, natural killer (NK) cell, and cytotoxic T lymphocyte (CTL) functions to defend against Eimeria infection [52]. IL-2 might contribute to the proliferation of T cells involved in cytotoxic effector mechanisms [53]. Early studies found that chicken IFN-γ has an anticoccidial effect and can inhibit the development of E. stiedae.
*Eimeria* resistance of chicks might be weakened because of reduced IFN-γ and IL-2 levels [55]. Vaccination with expression products containing IFN-γ or IL-2 further improved the anticoccidial index of chickens [56, 57]. Quil-A can stimulate both humoral and cellular responses and enhance Th1 and CTL responses [58]. Quil-A, which is ideal for vaccines against intracellular pathogens, has been studied in *Toxoplasma gondii* and *Neurospora caninum* with good results [58, 59]. Therefore, we chose Quil-A as the adjuvant for the recombinant proteins in this study. Compared with the control groups, serum IL-2 and IFN-γ levels were significantly elevated after vaccination with rEsIMP1 and rEsAMA1 (P<0.05), indicating that rEsIMP1 and rEsAMA1 can stimulate Th1-type immune responses. Additionally, the ability to elicit Th1-type cytokine response might be further enhanced by Quil-A. Similarly, the serum IL-4, IL-17, and TGF-β1 levels were also significantly increased in the rEsIMP1 and rEsAMA1 groups. The Th2-type immune response is characterized by elevated levels of IL-4 and other cytokines. IL-4 plays a role in regulating B cells and inducing humoral immune reactions [60, 61]. Thus, rEsIMP1 and rEsAMA1 can also stimulate a Th2-type immune response.

![Fig. 6 Post-vaccination cytokine levels. The serum IL-2 (a), IL-4 (b), IL-10 (c), IL-17 (d), IFN-γ (e), and TGF-β1 (f) levels at 2 weeks after booster vaccination. Different superscripts (a, b) indicate a significant difference (P<0.05). The same superscript indicates no significant difference (P>0.05). The unit of TGF-β1 concentration was ng/ml, and the concentration unit of other cytokines was pg/ml](image-url)
Recent research has proven that antibodies play a role during *Eimeria* infection [62]. Lee et al. [63, 64] found that in chickens fed with hyperimmune IgY of egg yolk powder, passive immunity provided significant protection against *Eimeria* infection. CoxAbic® comprises *Eimeria maxima* gametocyte antigens, and breeder hens vaccinated with CoxAbic® produced large amounts of specific IgY maternal antibodies, which provided passive immunity for their offspring against *Eimeria* [11]. We found that rabbits vaccinated with rEsIMP1 and rEsAMA1 exhibited elevated serum levels of specific antibodies. The results indicated that both rEsIMP1 and rEsAMA1 induce significant humoral immunity; however, rEsIMP1 displayed a better performance.

*Eimeria stiedae* parasitizes the rabbit liver and completes its endogenous stages in bile duct epithelial cells [6]. However, it is not yet clear how *E. stiedae* migrate from the duodenum to the liver. Studies found sporozoites of *E. stiedae* in the mesenteric lymph nodes (MLN) and supposed that the sporozoites might be transported to the liver via the portal vein and lymphatic system [65, 66]. Owen et al. [67] observed *E. stiedae* sporozoites in the MLN, bone marrow, liver, and plasma and proposed that sporozoites steadily accumulate in the MLN. Suo et al. [68] proposed that the sporozoites are transported via the lymphatic vessels to the MLN and are then carried by lymph into the systemic circulation, after which the sporozoites continue their migration and finally enter the bile duct epithelial cells via the capillaries of intrahepatic bile ducts. AMA1 and IMP1 are expressed at high level in sporozoites and are located on the sporozoites surface [69, 70]. The anti-rEtAMA1 polyclonal antibodies had a potent inhibitory effect on *E. tenella* sporozoite invasion, decreasing it by approximately 70% [69]. Treatment of *Neospora caninum* tachyzoites with anti-rNcIMP1 polyclonal antibodies reduced cell invasion by approximately 44% [20]. Therefore, we speculated that when *E. stiedae* sporozoites migrate in the blood circulation, the anti-rEsAMA1 or anti-rEsIMP1 antibodies might interact with them and inhibit cell invasion by the sporozoites. Meanwhile, the high IFN-γ and IL-2 levels in the vaccinated rabbits further inhibited the intracellular infection of *E. stiedae*. Together, these effects might eventually lead to significant differences in oocyst output and body weight gain. There is currently no specific standard for the evaluation of recombinant subunit vaccines against rabbit coccidiosis; therefore, the protective efficacy was assessed through the survival rate, clinical symptoms, oocyst output reductions, and body weight gain. In the present study, rabbits vaccinated with rEsAMA1 or rEsIMP1 displayed a significantly reduced oocyst output ($F(5, 54) = 28.82, P = 0.000$). Thus, rEsAMA1 and rEsIMP1 are potential candidate vaccines against *E. stiedae*.

**Conclusions**

In this study, we obtained recombinant rEsIMP1 and rEsAMA1 proteins. The vaccination-infection trial showed that both rEsIMP1 and rEsAMA1 conferred protective immunity against *E. stiedae* infection. rEsIMP1 and rEsAMA1 could stimulate specific antibodies and the production of various cytokines. Thus, rEsIMP1 and rEsAMA1 could induce significant cellular and humoral immunity and alleviated the body weight loss and oocyst output, making them potential candidate vaccines against *E. stiedae*. The results showed that rEsIMP1 performed better than rEsAMA1.
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