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The effects of a novel adjuvant complex/*Eimeria* profilin vaccine on the intestinal host immune response against live *E. acervulina* challenge infection☆

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ABSTRACT

The effects of a novel adjuvant composed of Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol (QCDC) on protective immunity against avian coccidiosis following immunization with an *Eimeria* recombinant protein were determined. Broiler chickens were subcutaneously immunized with isotonic saline (control), *Eimeria* recombinant profilin alone, or profilin emulsified with QCDC at 1 and 7 days post-hatch, and orally challenged with live *Eimeria acervulina* at 7 days following the last immunization. Body weight gains, gut lesion scores, fecal oocyst outputs, profilin serum antibody titers, lymphocyte proliferation, and intestinal cytokine transcript levels were assessed as measures of protective immunity. Chickens immunized with profilin plus QCDC showed increased body weight gains and decreased intestinal lesion scores compared with the profilin only or control groups. However, no differences were found in fecal oocyst shedding among the three groups. Profilin serum antibody titers and antigen-induced peripheral blood lymphocyte proliferation in the profilin/QCDC group were higher compared with the profilin only and control groups. Finally, while immunization with profilin alone or profilin plus QCDC uniformly increased the levels of intestinal transcripts encoding all cytokines examined (IL-1β, IL-10, IL-12, IL-15, IL-17F, and IFN-γ) compared with the control group, transcripts for IL-10 and IL-17F were further increased in the profilin/QCDC group compared with the profilin only group. In summary, this study provides the first evidence of the immunoenhancing activities of QCDC adjuvant in poultry.

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1. Introduction

Avian coccidiosis, an intestinal disease caused by multiple species of *Eimeria*, has had significant economic effects on the poultry industry [1]. Prophylactic medication has been successfully used to control avian coccidiosis, but alternative strategies are sought due to the increasing emergence of drug-resistant coccidia in commercial production settings [2,3]. Although there is a growing reliance on the use of *Eimeria* parasite vaccines for coccidiosis control [4,5], live vaccines pose the risk of unintended infection under the immunosuppressive conditions associated with high-density commercial rearing conditions. On the other hand, while subunit protein and DNA vaccines do not present the possibility of field infections, both are of limited immunogenicity and there is a lack of suitable immunoenhancing adjuvants for the poultry industry [6–9].

Profilin is a major immunogenic protein of *Eimeria* and vaccination of chickens with this recombinant polypeptide induces partial protection against avian coccidiosis [10,11]. Profilins are essential components of all Apicomplexan parasites, and are involved in actin-dependent gliding motility, parasite migration across biological barriers, and host cell invasion [10]. The profilin gene encodes a ~20 kDa protein that is highly conserved among the various genera of Apicomplexans and expressed during most stages of the parasite's life cycle [6,11,12]. Toxofilin, the profilin of *Toxoplasma gondii,* binds to TLR11, inducing a potent IL-12 response in murine dendritic cells, and plays a critical role in $CD4^+$ T cell-driven immunity to the pathogen [10,13]. In our previous study, profilin was identified from the merozoites of *Eimeria acervulina* as an immunogenic protein that induced high levels of IFN-γ production by splenic T cells [14]. Subcutaneous immunization of young broiler chickens with recombinant profilin protein in the absence of adjuvant, or *in ovo* vaccination of 18-day-old embryos with a DNA plasmid encod-

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ing the profilin gene, provided limited, but statistically significant protection against challenge infection with live *E. acervulina* [6,8].

Recently, substantial progress has been made in the formulation of novel adjuvants that enhance the immunogenicity of peptides and proteins without unwanted side effects [15–17]. For example, saponins such as Quil A are derived from the South American tree *Quillaja saponaria molina* and are currently used in several veterinary applications [18]. Quil A is combined with cholesterol and phospholipids to form immunostimulatory complexes (ISCOMs) that avoid the hemolytic and viricidal properties of Quil A alone [19]. However, ISCOMs may stimulate an undesirable autoimmune reaction, and some hydrophilic proteins cannot be efficiently incorporated into their structure. Alternatively, dimethyl dioctadecyl ammonium bromide (DDA) and avidine are lipophilic quaternary amine adjuvants that stimulate robust cell-mediated and humoral immune responses [20]. The efficacy of DDA as an adjuvant for proteins, haptens, viruses, protozoa, and bacteria has been reported [21–23]. By itself, DDA is sparingly soluble in water and is more effective when used with polymers such as dextran, polyethelyne glycol, or polyacrylic acids like Carbopol. QCDC is a new adjuvant complex composed of Quil A, cholesterol, DDA, and Carbopol that enhances immune responses against multiple pathogen vaccines in a variety of veterinary settings [24]. However, the adjuvanticity of QCDC in poultry has not been reported. Therefore, the current study was undertaken to evaluate the adjuvant effect of QCDC during vaccination against avian coccidiosis.

2. Materials and methods

2.1. Experimental animals

Experiments were approved by the Agricultural Research Service Institutional Animal Care and Use Committee (ARS IACUC). One-day-old broiler chickens (Ross/Ross, Longenecker Hatchery, Elizabethtown, PA) were housed in Petersime starter brooder units and randomly assigned to 4 groups (20 birds/group). Chickens were kept in brooder pens in an *Eimeria*-free facility for 2 weeks and then transferred into large hanging cages (2 birds/cage) at a separate location where they were infected and kept until the end of the experimental period.

2.2. Expression and purification of recombinant profilin

The profilin gene was originally cloned by immunoscreening an *E. acervulina* cDNA library using a rabbit antiserum against *E. acervulina* merozoites [6]. The 1086-base pair profilin cDNA was subcloned into the pMAL plasmid (New England Biolabs, Ipswich, MA) with an NH_2-terminal maltose-binding protein epitope tag and a Factor Xa protease cleavage site between maltose-binding protein and profilin. Transformed *Escherichia coli* DH5a bacteria were grown to mid-log phase, induced with 1.0 mM of isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, collected by centrifugation, and disrupted by sonication on ice (Misonix, Farmingdale, NY). The recombinant profilin protein was isolated on an amylose affinity column (New England Biolabs) according to the manufacturer's instructions, digested with Factor Xa to release profilin from the solid phase, and repassed through the amylose column to remove any contaminating maltose-binding protein. Final purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with profilin-specific rabbit antibody.

2.3. Profilin immunization

One-day-old chickens were immunized subcutaneously with sterile isotonic saline or with 100 μg of profilin alone or profilin plus QCDC in a 50 μl emulsion. The formulation of QCDC consisted of 12.0 μg/ml of Quil A (E.M. Sergeant Pulp & Chemical Co., Clifton, NJ), 12.0 μg/ml of cholesterol (FabriChem, Trumbull, CT), 0.6 μg/ml of DDA (Fluka, Buchs, Switzerland), and 0.75 mg/ml of Carbopol® 974P (Lubrizol, Wickliffe, OH). Booster immunization was given at the same injection site as the primary immunizations at 7 days following primary immunization.

2.4. Eimeria infection and measurements of body weight gains and fecal oocyst shedding

At 7 days following secondary immunization, chickens (20/group) were orally infected with 1.0×10^4 sporulated oocysts of *E. acervulina* as described [25,26]. Body weights were measured between 0 and 9 days post-infection. For the determination of fecal oocyst shedding, birds (12/group) were placed in oocyst collection cages and fecal samples were collected between 6 and 9 days post-infection. Oocyst numbers were determined using a McMaster chamber according to the following formula: total oocysts/bird = [oocyst count × dilution factor × (fecal sample volume/counting chamber volume)]/2 [27,28].

2.5. Measurement of profilin antibody responses

Blood samples (4 birds/group) were collected by cardiac puncture immediately following euthanasia at 7 days post-secondary immunization. Sera was obtained by low speed centrifugation, and used in an ELISA to measure the profilin-specific antibody response as described [27,29]. In breif, 96-well microtiter plates were coated overnight with 1.0 μg/well of purified recombinant profilin. As a negative control, wells were coated with a recombinant microneme protein from *E. tenella*, EtMIC2 [8]. EtMIC2 was cloned from *E. tenella* and was shown during host cell invasion to be localized at the point of parasite entry and secreted from the host–parasite interface. The plates were washed with phosphate-buffered saline containing 0.05% Tween (PBS-T) and blocked with PBS containing 1% BSA. Diluted sera (1:100) were added (100 μl/well), incubated with agitation for 2 h at room temperature, and washed with PBS-T. Bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgG and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma, St. Louis, MO). Optical density at 450 nm (OD_{450}) was measured with an automated microplate reader (Bio-Rad, Richmond, CA). All samples were analyzed in quadruplicate to ensure accuracy.

2.6. Determination of intestinal lesion scores

At 9 days post-infection, lesion scores (5 birds/group) were determined using a numerical scale from 0 (normal) to 4 (severe) [28,30] and evaluated by 3 independent observers.

2.7. Lymphocyte proliferation

Peripheral blood lymphocytes (PBL) were collected from uninfected chickens (4 birds/group) by cardiac puncture immediately after euthanasia at 7 days following secondary immunization as described [31]. The cells were adjusted to 5.0×10^6 cells/ml in RPMI medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified incubator at 41 °C and 5% CO_2 for 48 h with 10 μg/ml of profilin in 96-well plates. Cell proliferation was measured using WST-8 (Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) as described [32] and was expressed as OD_{450} (profilin) − OD_{450} (medium alone). Each sample was analyzed in quadruplicate.

Table 1
Oligonucleotide primers used in quantitative RT-PCR.

Type	mRNA target	Primer sequences	PCR product size (bp)	GenBank accession no.
Reference	GAPDH	F: 5′-GGTGGTGCTAAGCGTGTTAT-3′ R: 5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
Pro-inflammatory	IL-1β	F: 5′-TGGGCATCAAGGGCTACA-3′ R: 5′-TCGGGTTGGTTGGTGATG-3′	244	Y15006
	IL-17F	F: 5′-CTCCGATCCCTTATTCTCCTC-3′ R: 5′-AAGCGGTTGTGGTCCTCAT-3′	292	AJ493595
Th1	IFN-γ	F: 5′-AGCTGACGGTGGACCTATTATT-3′ R: 5′-GGCTTTGCGCTGGATTC-3′	259	Y07922
	IL-12	F: 5′-AGACTCCAATGGGCAAATGA-3′ R: 5′-CTCTTCGGCAAATGGACAGT-3′	274	NM_213571
	IL-15	F: 5′-TCTGTTCTTCTGTTCTGAGTGATG-3′ R: 5′-AGTGATTTGCTTCTGTCTTTGGTA-3′	243	AF139097
Th2	IL-10	F: 5′-CGGGAGCTGAGGGTGAA-3′ R: 5′-GTGAAGAAGCGGTGACAGC-3′	272	AJ621614

2.8. Quantification of cytokine mRNA levels

Intestinal duodenum tissues were obtained from uninfected chickens (4 birds/group) at 7 days post-secondary immunization. The tissues were cut longitudinally and washed 3 times with ice-cold Hanks' balanced salt solution containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. The mucosal layer was carefully scraped away using a surgical scalpel and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μl of 10× reaction buffer (Sigma) and incubated for 15 min at room temperature. Then, 1.0 μl of stop solution was added to inactivate DNase I, and the resulting mixture was heated to 70 °C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide primers for chicken cytokines (IL-1β, IL-17 F, IFN-γ, IL-12, IL-15 and IL-10) and the GAPDH internal control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA using the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA and the levels of individual transcripts were normalized to those of GAPDH by the Q-gene program [33]. Each sample was analyzed in triplicate. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression prior to calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression (MNE) using the Q-gene program [32,34].

2.9. Statistical analyses

All data was expressed as mean ± SEM values of 4–20 chickens/group with 3–4 replicates/sample. Comparisons of the mean values were performed by one-way analysis of variance followed by the Duncan's multiple range test using SPSS software (SPSS 15.0 for Windows, Chicago, IL). Differences between groups were considered statistically significant at $P < 0.05$.

3. Results

3.1. The effects of vaccination with profilin plus QCDC on body weight gain and fecal oocyst shedding

In the absence of profilin immunization, *E. acervulina*-infected chickens showed significantly reduced body weight gains (596.4 ± 11.3 g) compared with the uninfected controls (691.0 ± 15.0 g) (Fig. 1A). Chickens immunized with profilin alone and infected with parasites exhibited weight gains (665.3 ± 8.4 g) equal to uninfected controls and chickens immunized with profilin plus QCDC exhibited additional weight gains (719.1 ± 17.0 g) compared with the profilin alone group. In contrast, no differences were found in fecal oocyst shedding among the 3 immunized and infected groups (Fig. 1B).

3.2. The effect of vaccination with profilin plus QCDC on profilin serum antibody responses

Serum antibody levels in the profilin plus QCDC group increased compared with those of the control or profilin only groups (Fig. 2A).

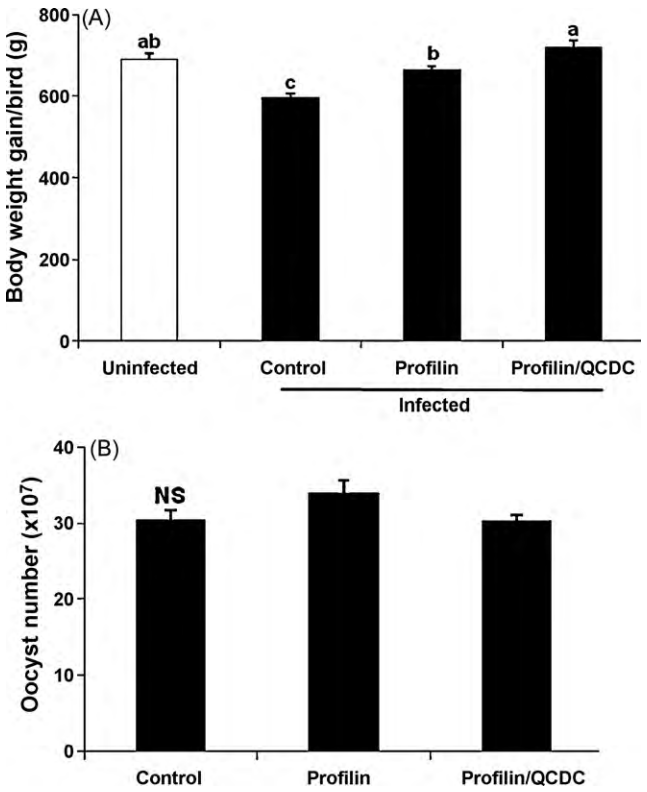

Fig. 1. The effects of vaccination with profilin plus QCDC on body weight gain and fecal oocyst shedding. Chickens were immunized 1 and 7 days post-hatch with PBS (control), profilin alone, or profilin plus QCDC and orally infected with 1.0×10^4 sporulated oocysts of *E. acervulina* at 7 days post-secondary immunization. (A) Body weight gains were calculated between 0 and 9 days post-infection. Each bar represents the mean ± SEM values ($N = 20$). (B) Fecal oocyst numbers were measured between 6 and 9 days post-infection. Each bar represents the mean ± SEM values ($N = 12$). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$). NS indicates that the bar is not significant among the experimental groups.

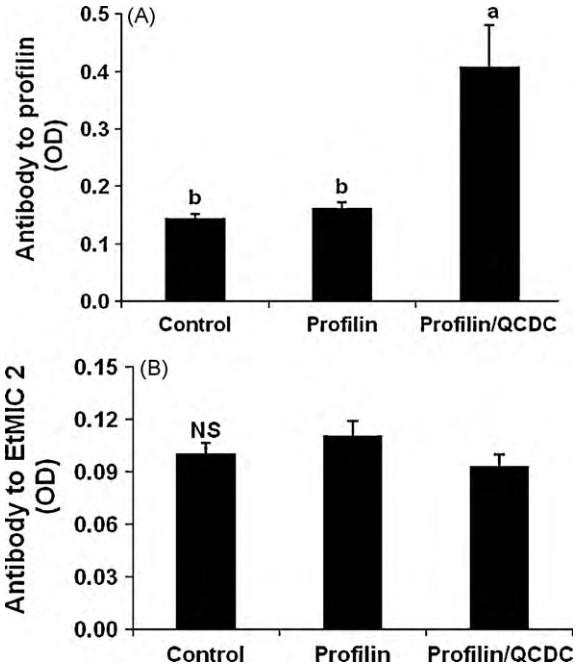

Fig. 2. The effect of vaccination with profilin plus QCDC on profilin serum antibody responses. Chickens were immunized and infected as in Fig. 1 and serum antibodies against profilin (A) and EtMIC2 (B) recombinant protein were measured at 7 days post-secondary immunization. Each bar represents the mean ± SEM values from quadruplicate samples/bird ($N = 4$ birds/group). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$). NS indicates that the bar is not significant among the experimental groups.

As a negative control, the levels of antibodies reactive with an unrelated *Eimeria* protein antigen, EtMIC2, were equal in the 3 immunization groups (Fig. 2B).

3.3. The effect of vaccination with profilin plus QCDC on intestinal lesion scores

Intestinal lesion scores in the profilin plus QCDC group (1.62 ± 0.2) decreased compared with those of the control group (2.5 ± 0.2) (Fig. 3). Comparison of scores between the profilin only (2.0 ± 0.2) and profilin plus QCDC groups, or between the control and profilin only groups, revealed no significant differences.

3.4. The effect of vaccination with profilin plus QCDC on lymphocyte proliferation

Profilin-stimulated PBL proliferative responses in the profilin plus QCDC group increased compared with those of the control or profilin only groups (Fig. 4).

3.5. The effect of vaccination with profilin plus QCDC on intestinal cytokine transcript levels

The levels of transcripts encoding proinflammatory (IL-1β, IL-17F), Th1 (IFN-γ, IL-12, IL-15), or Th2 (IL-10) cytokines in the duodenum increased in the profilin only and the profilin/QCDC groups compared with the control group (Fig. 5). The levels of transcripts encoding IL-17F and IL-10 further increased in the profilin/QCDC group compared with the profilin only group.

4. Discussion

This study documents the immunopotentiating effects of a new adjuvant formulation, QCDC, and its ability to augment the immunogenicity of an *Eimeria* recombinant protein vaccine in

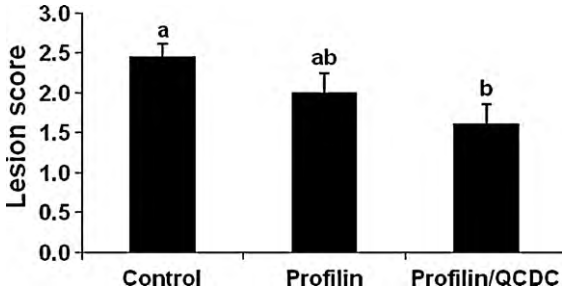

Fig. 3. The effect of vaccination with profilin plus QCDC on intestinal lesion scores. Chickens were immunized and infected as in Fig. 1, and lesions were scored from 0 (normal) to 4 (severe) at 9 days post-infection. Each bar represents the mean ± SEM values from 3 independent observers ($N = 5$ birds/group). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

chickens. The major findings are that immunization of chickens with profilin plus QCDC reduced body weight loss caused by *Eimeria* infection, induced higher serum antibody titers to profilin, decreased gut pathology, increased antigen-driven PBL proliferation, and increased IL-10 and IL-17F transcript levels in the intestine compared with chickens vaccinated with profilin alone.

Novel vaccination strategies using DNA or proteins derived from mucosal pathogens have successfully induced protective immunity against a variety of infectious diseases including avian coccidiosis [6,35]. For example, DNA immunization with plasmids encoding either the profilin or EtMIC2 *Eimeria* protein has been shown to induce both antigen-specific antibody and T cell responses, and to confer partial protection against live parasite challenge infection [6,8]. However, the levels of protective immunity against avian coccidiosis that are induced by subunit protein vaccines have been limited, due in large part to the complex parasite life cycle that includes intracellular and extracellular as well as sexual and asexual stages [1,14,23]. Each of these stages is associated with a unique pattern of gene expression by the parasite and not all *Eimeria* proteins are expressed in all stages. In addition, individual chickens can be simultaneously or sequentially colonized by multiple *Eimeria* species, and cross-protection of host immunity among the different parasites is severely limited. As a result of these practical limitations, recent interest has developed in using vaccine adjuvants as immunostimulators to enhance the immunogenicity of coccidia subunit vaccines.

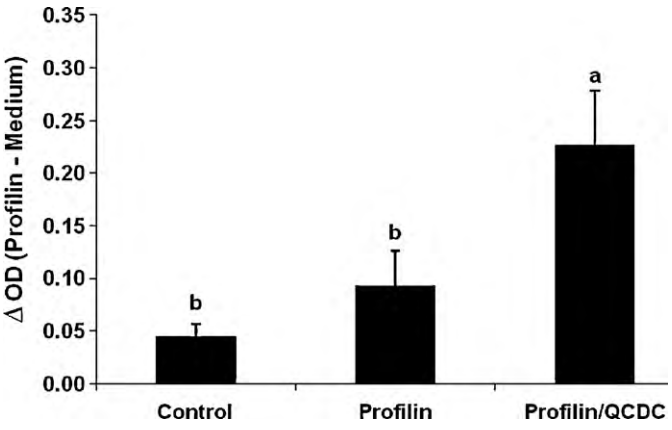

Fig. 4. The effect of vaccination with profilin plus QCDC on lymphocyte proliferation. Chickens were immunized as in Fig. 1, PBL were isolated at 7 days post-secondary immunization, and cell proliferation in response to stimulation with 10 μg/ml of profilin was measured and expressed as ΔOD (OD_{450} profilin − OD_{450} medium alone). Each bar represents the mean ± SEM values from quadruplicate samples/bird ($N = 4$ birds/group). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

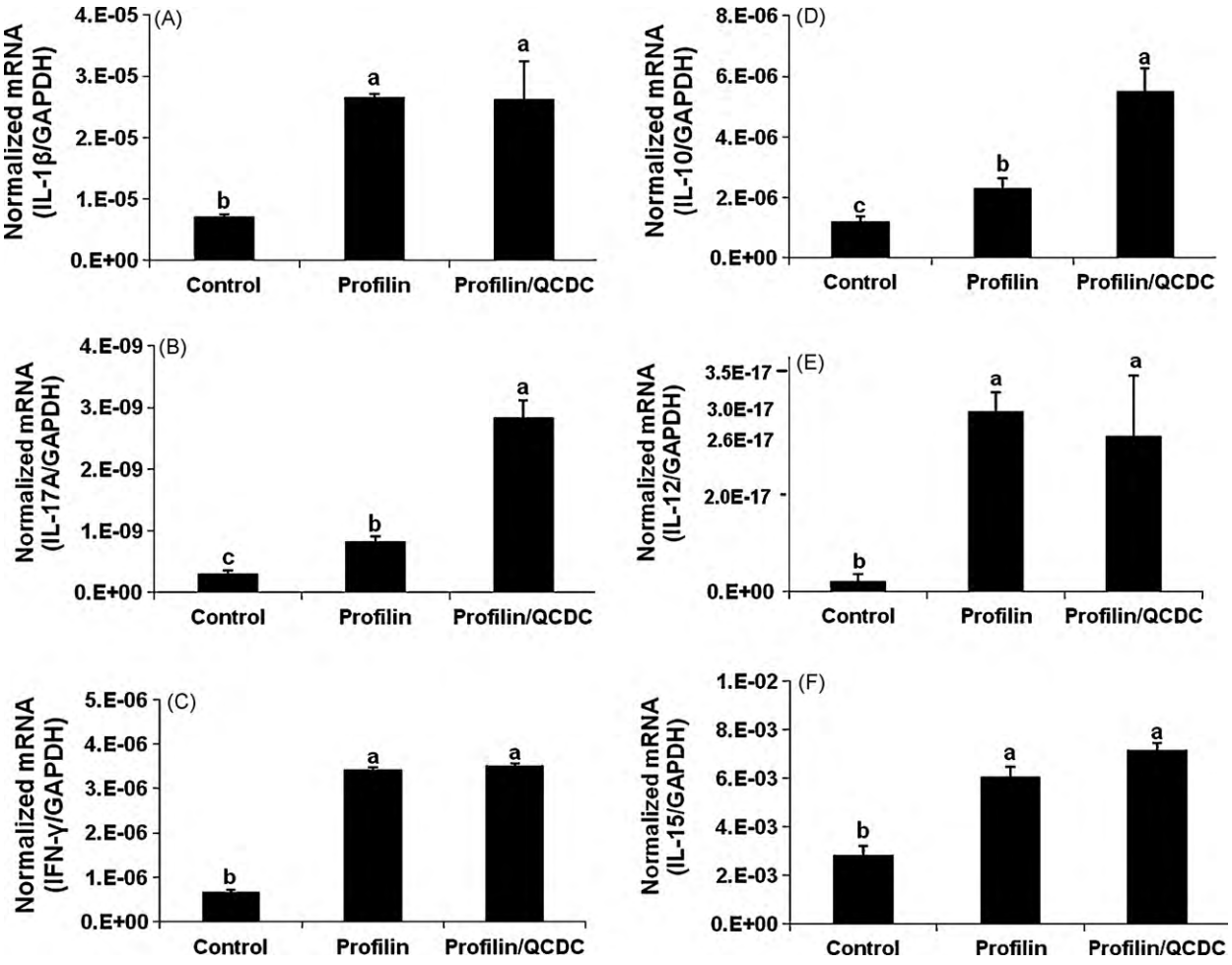

Fig. 5. The effect of vaccination with profilin plus QCDC on intestinal cytokine transcript levels. Chickens were immunized as in Fig. 1, intestinal duodenum tissues were isolated at 7 days post-secondary immunization, and transcripts encoding IL-1β (A), IL-17F (B), IFN-γ (C), IL-10 (D), IL-12 (E), and IL-15 (F) were quantified by real time RT-PCR, and normalized to GAPDH transcript levels. Each bar represents the mean ± SEM values from triplicate samples/bird ($N = 4$ birds/group). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

QCDC is an adjuvant complex composed of Quil A, cholesterol, DDA, and Carbopol, and its adjuvant effects have been demonstrated against *E. coli*, the feline leukemia virus, bovine viral diarrhea virus, mycoplasma hyopneumoniae, avian and canine influenza viruses, canine coronavirus, and bovine rotavirus [24]. Quil A by itself has been used as an adjuvant in many veterinary animal species, and defined saponins such as QS-21 are currently used in human clinical trials [36–39]. DDA provides a significant adjuvant effect when co-administered systemically or locally with antigen and stimulates predominantly Th1 cells with no reported toxic effects in humans [40–46]. In avians, DDA enhanced humoral and cell-mediated immune responses to a Newcastle disease virus vaccine and induced greater lymphocyte proliferation compared with complete Freund's adjuvant [22]. Chickens immunized with *Eimeria* merozoite antigens in combination with DDA displayed longer-lasting coccidial immunity compared with the *Corynebacterium parvum* adjuvant [23]. Carbopol is a mucoadhesive polymer that has been extensively investigated due to its high viscosity at low concentrations and low toxicity [47]. Carbopol enhanced the efficacy of recombinant canarypox viral vectors for equine herpes virus vaccination and significantly improved virus neutralizing antibody responses to this virus [48]. No prior studies have examined the effects of Carbopol on poultry vaccines.

The beneficial effects of profilin plus QCDC immunization on body weight gain and intestinal pathology indicate that the mode of action of this vaccine complex may involve effector mechanisms influencing these two clinical parameters of coccidiosis. In this regard, the importance of cell-mediated immunity in conferring protection against avian coccidiosis has been well documented [26,34,49–51], and humoral immunity also appears to be of some assistance [25,28]. Serum antibody levels showed higher titers against profilin in the group immunized with profilin plus QCDC, whereas no significant differences in anti-EtMIC2 antibody levels were seen among these groups (Fig. 2B). The reason why profilin plus QCDC had no effect on parasite fecundity/shedding compared with profilin alone is unclear, but this observation is in agreement with previously published reports indicating the absence of a direct correlation between *Eimeria*-induced body weight loss and fecal oocyst shedding [29,49].

In concordance with the evidence that supports the role of cell-mediated immunity in protection against avian coccidiosis, other studies have verified the significance of the gut cytokine response, including the role of proinflammatory, Th1, and Th2 cytokines following *Eimeria* infection [34,51–55]. In fact, increasing levels of gene transcripts encoding IL-10 and IL-17F were associated with enhanced protection against coccidiosis [55], although their role in local immune response needs to be investigated. IL-1β, a proinflammatory cytokine produced by macrophages, monocytes and dendritic cells, exerted an adjuvant effect when given with a profilin DNA vaccine and reduced fecal oocyst shedding following oral infection with viable parasites [7]. IL-17 is a cytokine that is secreted exclusively by activated memory T cells which induce fibrob-

lasts to secrete other proinflammatory or hematopoietic cytokines [56–61]. IL-17 was highly up-regulated following primary infection with *E. acervulina* or *E. maxima*, but not *E. tenella* [52,60]. Chicken IL-17 induced IL-6 production and both cytokines together may play an immunoregulatory role in the initiation, maintenance, and control of intestinal inflammatory responses [61]. IFN-γ has also been associated with protective immune responses to coccidiosis [26,62,63]. Administration of recombinant chicken IFN-γ significantly increased host protection against coccidiosis and reduced the intracellular development of *Eimeria* in chickens [64]. In contrast, IL-10 acts as a differentiation factor for a novel subset of T cells that function as suppressors [65]. IL-10 inhibits the synthesis of proinflammatory cytokines (including IL-1β, TNF-α, and IL-6), thus down-regulating inflammatory T cell responses [66,67]. Following *E. acervulina* infection, IL-10 expression was moderately increased [52]. Finally, IL-15, a cytokine that promotes the survival of T and NK cells [53,68], enhanced protective immunity to coccidiosis challenge when co-administered with the profilin vaccine [7].

In summary, the enhanced protection against experimental avian coccidiosis conferred by QCDC is a result of augmented cellular and humoral immune responses. Future studies to elucidate the molecular and cellular immune mechanisms mediated by QCDC in various avian clinical conditions will be beneficial to the commercial industry.

Acknowledgments

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