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Original Article

Evaluation the Expression of IFN-γ, IL-4, IL-17, and IL-22 Cytokines in Birds Immunized with a Recombinant Chimeric Vaccine Containing Alpha Toxin, NetB, and ZMP against Necrotic Enteritis

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Abstract

Introduction: Necrotic enteritis (NE), an infection of the gastrointestinal tract of birds, is a major concern of the poultry industry due to its huge economic losses. The disease is caused by the Gram-positive bacterium *Clostridium perfringens (C. perfringens)*. Due to the ban on antibiotic usage in the poultry industry, the incidence of NE has increased significantly in recent years. We have previously shown that immunization of chickens with a subunit chimeric antigen composed of the most effective *C. perfringens* toxins in NE pathogenesis (alpha toxin, B-like toxin (NetB), and zinc metallopeptidase (ZMP)) can protect birds against this disease.

Materials and Methods: In the present study, the chickens were subcutaneously immunized by the recombinant protein. Then, the expression profile of cytokines in immunized birds was evaluated. For this purpose, following the immunization regimen, samples were taken from the intestines of the birds, mRNAs were extracted and the expression of four different cytokines (IFN-γ, IL-4, IL-17, and IL-22) was investigated using quantitative real-time PCR. The mentioned cytokines are representatives of helper T lymphocytes and have roles in several immune system activities, such as cellular, humoral, and mucosal immunity responses, as well as inflammation.

Results: According to the results of the cytokine assay, subcutaneously-administered recombinant protein elicited humoral and cellular immune systems but it could not stimulate the mucosal immune system. The candidate vaccine elicited the immune system so that the differences between the adjuvant recombinant protein (Adj-rNAM group) and the control group were significant (p <0.001).

Conclusions: The results, in addition to our previous study outputs, indicate that our strategy, after completing adequate investigations, can provide an alternative solution to using antibiotics in NE treatment.

Keywords: Vaccine Candidate, Necrotic Enteritis, *Clostridium perfringens,* Cytokine Assay, Cellular Immunity

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Introduction

Necrotic enteritis (NE), a worldwide gastrointestinal disease of birds, has been one of the major concerns of the poultry industry in the last decade due to reasons including production losses, reduced profits, increased mortality, reduced welfare of birds, and growing risk of contamination of poultry product to humans.[1] NE was first described by Parish in 1961 and has been well-controlled for many years using antibiotic growth promoters.[2] The damage caused by the outbreak of NE in chicken farms is estimated to be about six billion dollars annually.[3]

The causative bacterium of NE disease is *C. perfringens*, a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium that lives everywhere, from animal intestines to soil, especially manure soil.[4] Most of the time, *C. perfringens* can be found

in chicken intestines without causing NE because the existence of some predisposing conditions is necessary for the exacerbation of *C. perfringens* to produce toxins and cause the clinical disease.

For more than 60 years, antibiotics such as Virginiamycin and Bacitracin Methylene Disalicylate have been widely used as growth promoters (AGPs) to improve the conversion rates and growth performance of birds.[5] However, the use of antibiotics in the chicken diet has led to the development of antibiotic resistance. Furthermore, through the food chain, antibiotic resistance may be transmitted to human bodies due to the chemical similarities between human and veterinary antibiotics. For this reason, European countries have banned the use of antibiotics in the treatment of poultry disease and as a growth promoter. As a result, production levels in the poultry industry have decreased and the incidence of NE has increased.[6] Therefore, our strategies for bird immunization must be redefined or adjusted. Modulation of the birds' gut microflora, potentiation of the immune responses, pathogen reduction by management of the feed supplements, and vaccination are some alternative strategies to deal with NE in the poultry industry. Among them, vaccination is very efficient in protecting birds against NE. Various types of vaccines have been developed to reduce NE infection in chickens. Live-attenuated vaccines, inactivated *C. perfringens* toxins, recombinant or native proteins, etc. have been used for this purpose and have had promising results.[7] A live-attenuated *Salmonella* strain was used to deliver up to three *C. perfringens* antigens. This vaccine candidate led to higher antibody levels, lower lesion scores, and higher body weight. NetB and *C. perfringens* α- toxin toxoids and fructose-1,6-bisphosphate aldolase (Fba), a metabolic enzyme with an unidentified pathogenic function, were the exploited antigens.[8] In a study conducted by Valipouri et al., using a recombinant protein vaccine containing only α-toxin, immunized chickens developed partial protection against NE.[9] Different live-attenuated vaccine candidates have varying degrees of effectiveness in preventing NE, according to Thompson et al. 2006. A substantial decrease in intestinal lesions was seen after hens were immunized with a live, virulent strain of *C. perfringens*. However, immunization with an avirulent strain of *C. perfringens* did not provide protection against challenge with a virulent strain.[10] The immunogenic protein components of *C. perfringens* have been evaluated for the creation of immunity against NE. For instance, several researchers have looked at the anti-NE effects of *C. perfringens* -secreted toxins in culture supernatants. Despite how easily they may be made, care should be taken to make sure toxoid vaccines are made appropriately because formalin inactivation may reduce their immunogenicity.[11] Subcutaneous vaccination of broiler chickens with a formalin-inactivated NetB toxoid or NetB genetic toxoid offers some protection against experimental NE, according to an *in vivo* investigation.[12] Even, some vaccine candidates have been delivered using nanomaterials and have had promising results.[13]

In our previous work[14] we showed that a chimeric recombinant vaccine containing immunogenic epitopes derived from three virulence factors from the non-toxic carboxyl-terminal domain of Net-B, α-toxin, and the peptidase domain of a ZMP can efficiently raise the IgY level in the sera of the immunized chickens. In the present study, the potential of this protein in the expression of four cytokines, related to different types of immune cells, including interleukin-4 (IL-4), interleukin-17 (IL-17), interleukin-22 (IL-22), and interferon-γ (IFN-γ) in the intestinal tissue of birds following the completion of the immunization regimen was investigated using real-time PCR.

Materials and Methods

Preparation of the Vaccine Candidate

The details of the vaccine candidate have been presented in a previous work.[15] Briefly, the chimeric vaccine candidate is comprised of immunogenic epitopes of α-toxin (amino acids 284 to 398), NetB (amino acids 146 to 322), and ZMP (amino acids 698 to 1022) fused by an alpha-helix forming linker A(EAAAK)4A. The protein was expressed in the expression system *Escherichia coli* (*E. coli)*. Due to the presence of a histidine tag, the protein was purified by a nickel column and confirmed by Western blotting.

Immunization of Chickens

Preparation of Chickens

Thirty 1-day-old Ross 308 broiler chickens whose parent flocks had not been vaccinated with any *C. perfringens* vaccine or candidate vaccine, were taken part in this study. The chickens were fed on an antibiotic-free starter diet containing 20% (w/w) proteins for 20 days. From day 20 onwards, the starter diet was replaced by the high protein-based feed containing 40% fishmeal protein as a predisposing factor for experimental NE infections.

The experiments performed on chickens were approved by the guidelines of the poultry disease section, Faculty of Veterinary Medicine, University of Tehran, and according to the recommendation of the NIGEB animal care and use committee (ethical code NO. IR.NIGEB.EC.1397.11.30F).[16]

Immunization Schedule

The chickens were randomly divided into 2 experimental groups (Table 1): a group that received the rNAM in combination with Montanide ISA 71 VG adjuvant (Seppic, France) (abbreviated as Adj-rNAM), and a group that received only the Montanide ISA 71 VG adjuvant (15 chickens in each group). Each group was housed in separate rooms. On days 7, 13, and 21, chickens of the test group were immunized subcutaneously in the neck with 30 µg of purified recombinant NAM protein mixed with 70 mg of Montanide.[17]

Table 1. Experimental Design of Immunization Study*

Group No.	Group Name	Bird Number	Vaccination D7,D13.D21	Challenge D28-D32	Sacrifice D40
1	Adjvant Control(Adj)	15	Adj+PBS	*C.p*	S
2	Adj-rNAM	15	Adj-rNAM	*C.p*	S

*Vaccination birds were challenged orally with (10^8 to 10^9 CFU) of *C. perfringens* strain that containing netB, phospholipase, (Alpha toxin), and ZMP on days 28,29,30,31, and 32. Adj = Adjvant; T.n = *N. Tabacum;* C.p = *C. perfringens;* S = Sacrificed; PBS = phosphate-buffered salen.

Table 2. Primers Sequences

Primer		Primer Sequence	Tm. °C
IFN-γ	Forward primer	ATGTAGCTGACGGTGGACCTAT	60.69
	Reverse primer	TTCACCTTCTTCACGCCATCAG	60.87
IL-4	Forward primer	ACATCCAGGGAGAGGTTTCCT	60.20
	Reverse primer	TGACGCATGTTGAGGAAGAGAC	60.61
IL-17A	Forward primer	TCCCCCAAACTGTGAGAGTCA	60.69
	Reverse primer	ACTGGGCATCAGCAACCAAG	60.90
IL-22	Forward primer	GGATGGGTTGTCTTCTGCTGT	60.27
	Reverse primer	ATGTAGGGCTGCTGGAAGTTG	60.34

Montanide.[17] The control group received only adjuvant (Adj group) on the same dates and via the same route.

Bacterial Challenge

One week after the completion of the immunization regimen, chickens were challenged by a strain of *C. perfringens* containing NetB, phospholipase c (α-toxin), and ZMP. For this aim, the strain was grown for 18 h cooked meat medium (Difco, Lebanon) at 37 °C under anaerobic conditions. Before the challenge, the chickens were starved for a night. Then, for five consecutive days, chickens received (10^8 to 10^9 CFU) of the bacteria through the oral route, twice a day.

Sample Collection

Following the challenge, chickens were sacrificed and their intestines were sampled by a veterinary pathologist. Samples were stored in liquid nitrogen till the cytokine assay.

RNA Isolation

Total RNA was isolated from intestinal tissue by RiboEx kit following the manufacturer's protocol (Geneall-Seoul) with certain modifications. 1 ml of RiboEx solution was added to 200 mg of the tissue and homogenized by vortex. After homogenization, samples were incubated at room temperature for 5 min, and then 200 µl of chloroform was added to the samples and incubated for 5 min in similar conditions. Then, the samples were centrifuged at 12000 x g for 15 min at 4 °C. The aqueous phase was collected and the RNA pellet was air-dried and dissolved in 25 µl DEPC nuclease-free water and stored at -80 °C for further analysis.

DNase Treatment

Genomic DNA could be amplified in the next steps and lead to false positive results. To remove residual genomic DNA in samples, RNA samples were treated with DNase I, an enzyme that selectively degrades DNA.

RNA Quantity and Quality Assessment

RNA quality was assessed with a 1.5% agarose gel electrophoresis. RNA with good quality shows two bands (28s and 18s) on the gel. RNA quantity was measured by spectrophotometer. Absorbance readings were performed at wavelengths of 260 nm and 280 nm. The optical density (OD) ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid. An OD ratio of less than 1.8 indicates the contamination with protein and an OD ratio above 2.0 indicates contamination with organic compounds, such as phenol or chloroform.

1st-Strand cDNA Synthesis

The RNA was used as a template to synthesize 1st-strand cDNA by using FIREScript RT cDNA synthesis KIT (Solis BioDyne, Estonia). The cDNA synthesis reaction was primed using Oligo dT primer at 50 °C for 60 min.

Quantitative RT-PCR

Four immune system genes representative of cytokines IFN-γ, IL-4, IL-17, and IL-22 were chosen to detect the immune responses of chickens challenged with pathogenic strains of *C. perfringens* after vaccination with the vaccine candidate. Real-time PCR Was conducted using primers are listed in Table 2 as well as GAPDH as an internal control. The reaction was carried out in 10 µl volume containing 5 µl SYBR green master mix (SolisaBioDyne-Estonia), 1.08 µl PCR buffer (10x), 0.24 µl $MgCl_2$, 0.36 µl dNTPs mix (10mM each), 1.08 each forward and reverse primer (2.5 mM), 0.044 µl Taq polymerase and 1 µl of cDNA. The PCR reaction (10 µl) was initiated with one cycle of 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 20 s, primer annealing at 60 °C for 20 s, and extension at 72 °C for 5 s.

Statistical Analysis

Statistical differences between each target group and its control were analyzed with REST Software which uses the formula below to calculate expression ratio between control group and sample group:

$$R = E_{(target)}^{\Delta Ctarget\ (\ MEAN\ control\ -\ MEAN\ sample)} / E_{(ref)}^{\Delta CPref\ (\ MEAN}$$

control – MEAN sample)

p <0.05 was considered to be statistically significant. Gene expression level was calculated using ΔCt method according to the flowing formula $\Delta Ct = Ct_{target} - Ct_{ref}$, where Ct_{target} refers to cytokine gene and Ct_{ref} refers to the control gene GAPDH.[18] The significance of means differences was analyzed with unpaired student's t-test.

Results

Expression and Confirmation of the Recombinant Protein

The recombinant protein (the immunogen was expressed in *E. coli* 21 (DE3) and, owing to its His-tag, purified using nickel column chromatography. Figure 1 shows the result of the expression. As can be seen in the figure a 78 kDa protein can be seen in the IPTG-induced samples (Thermo fisher-Canada protein ladder SM0331 has been used for bond measurement). The expression was confirmed using Western blotting. Figure 2 confirms the over-expressed protein is the desired protein (Thermo fisher-Canada protein ladder SM0331 has been used for bond measurement).

RNA Isolation

Figure 3 shows the result of the isolation of total RNAs from the intestine tissues. As can be seen in the figure, two different bands are visible on agarose gel, which indicates the proper extraction of total RNAs. Cinaclon- Iran 1kb ladder has been used.

Investigation of the Cytokines Gene Expression in the Subcutaneously-Immunized Group (Adj-rNAM)

As demonstrated in Figure 4, the expression of *IFN-γ*, *IL-4*, and *IL-22* genes were upregulated in the Adj-rNAM group

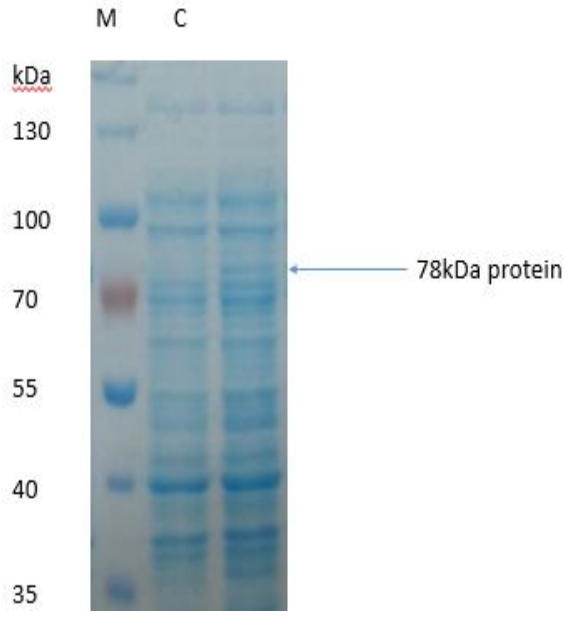

Figure 1. Detection of r-NAM 78 kDa Protein on SDS-PAGE. M: marker, C: control, 78 kDa protein: r-NAM protein.

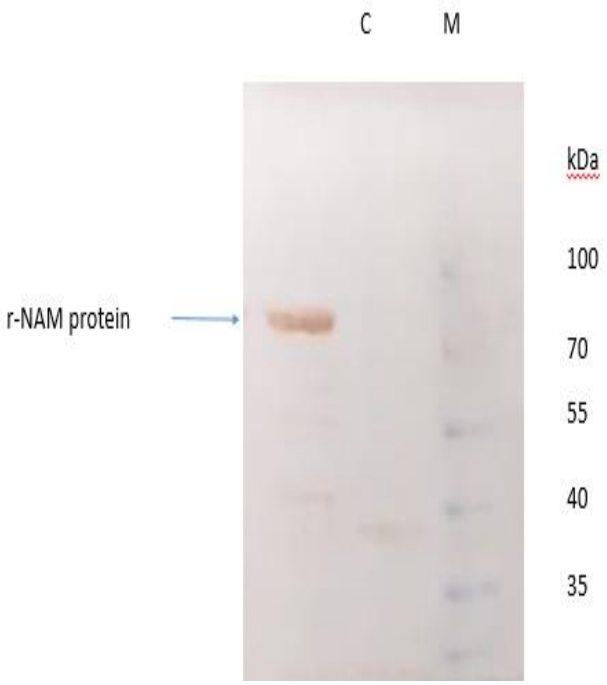

Figure 2. Detection of r-NAM 78 kDa Protein by Immunoblotting.

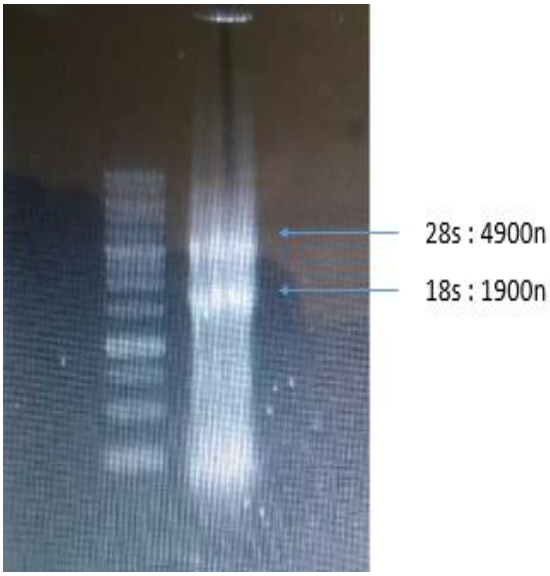

Figure 3. Detection of Extraction RNA bond on Agarose Gel.

compared to the control group, while the *IL-17* gene was downregulated in the Adj-rNAM group. The expression ratios of genes *IFN-γ*, *IL-4*, *IL-17*, and *IL-22* in the Adj-rNAM group compared to the control group were 32.985, 1.209, 0.323, and 1.340, respectively. ΔΔCt (the difference in ΔCt) between Adj-rNAM group and the control group with regard to genes *IFN-γ*, *IL-4*, *IL-17*, and *IL-22* were significant (p <0.001) as shown in Table 3.

Discussion

In the present study, the expression of four cytokines, IFN-γ,

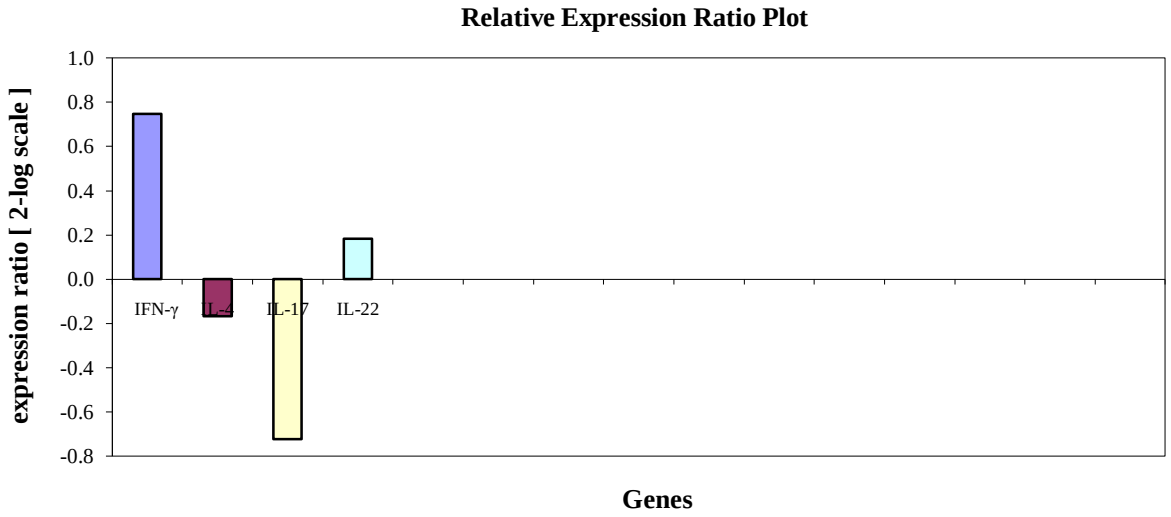

Figure 4. The Relative Expression Ratio Plot of Genes IFN-γ, IL-4, IL-17, and IL-22 for Adj-rNAM Group Compared to the Control Group. Expression ratio has been calculated according to the formula $R = E_{(target)}^{\Delta CTarget\ (MEAN\ control - MEAN\ sample)} / E_{(ref)}^{\Delta CPref\ (MEAN\ control - MEAN\ sample)}$.

Table 3. Differences in Expressions of the IFN-γ, IL-4, IL-17, and IL-22 Genes between the Adj-rNAM Group and the Adj Group

Gene	IFN-γ	IL-4	IL-17	IL-22
Expression Ratio	32.985	1.209	0.323	1.340
p-Value	≤0.001	≤0.001	≤0.001	≤0.001

IL-4, IL-17, and IL-22, in the gut of chickens following the immunization with a recombinant vaccine candidate against *C. perfringens* was evaluated. The results showed that the expression of IFN-γ, IL-4, and IL-22 was upregulated while the expression of IL-17 was downregulated in immunized birds compared to control.

The chimeric protein whose immunogenicity was evaluated in this research is based on the most critical toxins of NE-causative bacteria: α-toxin, NetB toxin, and ZMP. α-toxin is the main pathogenic factor in NE disease that induces membrane disruption.[20] The NetB toxin, in *C. perfringens* type G, also has a crucial role in the pathogenesis of the bacterium by forming hydrophilic heptameric pores in the membrane. ZMP toxin has also been shown to induce apoptosis by disrupting the Ras signaling pathway.[19]

Immune system cells located inside the gut of birds play an important role in the tissue defense against bacterial pathogenesis.[3,20] They play their role through the expression of various genes, especially the genes of interleukins and cytokines. The expression of these genes indicates the activation of that particular birds' immune response. There are three different types of T helper cells: Th1, Th2, and Th17, with distinct roles in the birds' immunity. Th1 induces INF-γ, TNF-β, IL-2, and IL-10.[2] IFN-γ plays an important role in promoting innate and adaptive immunity systems toward bacterial, viral, and protozoan attacks. Interferons are secreted from infected cells and activate the innate immune response, which in turn leads to the production of cytokines and the activation of natural killer cells and antigen presentation. IFN-γ is a key link that switches the innate immune response and then potentiates the adaptive immune response, especially the cellular arm of adaptive immunity.[21] Th2 is mainly responsible for the secretion of IL-4, IL-5, and IL-13. Th17, which differs from other Th cells, and secretes many cytokines, like IL-6, IL-17A, IL-17F, IL-22, IL-26, IL-20, and TNF-α.[22] A recent study has shown that birds with greater resistance to NE showed higher levels of IFN- γ gene expression,[20] which was also observed in the group of immunized birds compared to the control group in our experiment. Chickens infected with highly virulent strains of *C. perfringens* increased IFN-γ transcription in caecal tonsils compared to chickens infected with avirulent strains.[23]

IL-4, produced by lymphocytes, plays an important role in the adaptive immune system and mast cells, inducing the differentiation of CD4+T progenitors into the Th2 subset[24] and B cells into plasma cells[25] and preventing their differentiation into the Th1 cells when bacterial infections activate the immune system.

In a study by Alimolaei et al., immunization of BALB/c mice with recombinant *Lactobacillus casei* expressing mutant ε toxin strongly induced protective mucosal, humoral, and cellular immune responses against activated ε toxin. They found that IL-4 and IFN-γ levels were increased in mice immunized against *C. perfringens*.[26] In the present study, it was observed that these two cytokines have been upregulated in immunized mice.

In another study that investigated the effect of *C. perfringens* on the immunity and inflammation of birds, it was shown

that the virulent strain of *C. perfringens* were able to upregulate IL-4 gene expression, which is compatible with the results of the present study.

Indeed, it has been shown that in chicken immune responses, INF-γ and IL-4 affect intestinal epithelial cells, lamina propria lymphocytes, and intraepithelial lymphocytes, leading to their increased expression, which is necessary to combat antigens[23] IL-17 is an essential cytokine of the immune system which is important in defense against extracellular bacteria, especially in mucosal tissues, such as the intestines. This cytokine can also affect non-hematopoietic epithelial cells and fibroblasts in various tissues, such as intestine, skin, and liver.[27] Broom et al. showed that in NE-resistant birds, IL-17 was upregulated in intraepithelial lymphocytes of the jejunum.[20] In another study, subclinical NE activates Th17-mediated immune responses, which manifested itself by upregulation the expression of the IL-17 and INF-γ in cytotoxic T-cells in the jejunum.[28] However, in the present study, the level of IL-17 expression was not upregulated. This observation can be explained by the fact that subcutaneously-administration of antigens is not able to elicit mucosal immune systems. Indeed, this downregulation may be due to IFN- γ upregulation that occurs due to an inflammatory immune response in birds' intestines.[29]

Despite this, somehow, in agreement with our study, Yeh et al., have shown that co-infection with *Eimeria maxima* and *C. perfringens* negatively regulates the expression of the IL-17 and IFN- γ.[30]

In fact, host immune responses and parameters differ very widely between different studies on NE; the *C. perfringens* strain and adjustments of the NE disease model are among these factors.[28]

IL-22 can be secreted by a wide range of immune cells, such as Th1, Th22, and mast cells. However, some data have shown that IL-22 signaling limits Th1 responses and promotes regulatory T cells that inhibit the immune system and cytokines production.[29] This interleukin is upregulated in many chronic inflammatory diseases, however, understanding the biology of IL-22 can be complicated due to its dual role in inflammation.[31] It has been shown that *C. perfringens* infection increases IL-22 gene expression.[32] In the present study, IL-22 gene expression in Adj-rNAM was significantly higher than the control (p <0.001). It seems that the candidate vaccine used in this study was able to stimulate the innate and adaptive immune systems. Body response has been demonstrated by cytokines upregulating that results in differentiation and enhancing cellular immune systems, humoral immune systems, and activating immune cells like B cells to secret antibodies such as IgG and IgE. Our previous results also showed that this vaccine can make birds more resistant to NE and stimulate the humoral immune system by secreting IgY antibodies regulated by cytokine genes. However, before it can be widely used as a commercial vaccine, further research is needed such as investigating the efficacy of the vaccine after severe challenging of birds with more pathogenic strains of *C. perfringens*.

Conclusion

Birds immunized with a newly designed chimeric subunit protein based on α- toxin, netB, and ZMP can help birds develop resistance against *C. perfringens* by enhancing their immune systems and affecting their genes expression, such as cytokines and interleukins genes. Our strategy has demonstrated the ability of the candidate vaccines (α- toxin, NetB, and ZMP) to increase the expression of IFN-γ (the indicator of cellular immunity and the TH1 response), IL-4 (the indicator of humoral immunity and the TH2 response), and IL-22 (related to the inflammation), all of which play important protective roles in defense against pathogens. These results, in addition to our previous study outputs, indicate that our strategy, after completing adequate investigations, can provide an alternative solution to using antibiotics in NE treatment.

Authors' Contributions

BA did experimental investigation, methodology, investigation, technical analysis, formal analysis, writing the manuscript draft, data curation. AAM and GA supervised the research, provided resources, acquired funding, designed the experiments and contributed to the investigation. CK and AH contributed to editing manuscript, technical design and analysis. JR contributed to the investigation.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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