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RESEARCH ARTICLE

Immunogenicity and Protective Efficacy of Probiotics with EtIMP1C against *Eimeria tenella* Challenge

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ABSTRACT

Coccidiosis is an endemic problem in broilers inflicting disastrous losses to worldwide poultry industry. Vaccines are generally effective in controlling the infectious diseases; the subunit vaccines effectiveness can hopefully be improved with concurrent use of probiotics as assessed in this experiment using an *Eimeria tenella* challenge. Immune mapped protein-1 (IMP1) is a novel immunogenic protein of Apicomplexans, including in *Eimeria tenella*. Anticoccidial performance of the cumulative effect of probiotics and EtIMP1C (Part of *Eimeria tenella* immune mapped protein1) was evaluated based on various parameters such as an intestinal lesion, oocyst scores, feed conversion ratio, and organ weight. Data were also analyzed on both immunological and hematological parameters. The EtIMP1C and probiotics administered group showed less intestinal lesion, decreased oocyst shedding, satisfactory feed conversion ratio (FCR) and improved hematological parameters as compared to EtIMP1C emulsified with FCA group. However, there was no statistically significant difference between the two groups (P>0.05), in aspect of lesion and oocyst scores, as well as immunological and hematological parameters. The experimental work showed that probiotics could be a good hope due to its antioxidant, immunomodulatory and growth promoting effect against poultry coccidiosis alone or in combination with vaccines, including IMP1C based vaccine, but further studies are required to formulate its dose with the vaccine as well as a different strain of probiotics effect against coccidiosis.

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INTRODUCTION

Coccidiosis is one of the major protozoal diseases of poultry, causing a significant reduction in feed conversion ratio and growth performance (Abbas *et al.,* 2017a, 2020). The *Eimeria* parasite invades intestinal epithelium causing extensive destruction and necrosis of epithelial cells, leading to decreased weight gain (Zhang *et al.,* 2020). According to an estimate, coccidiosis causes about $127 million losses to US poultry industry annually and similar losses may occur worldwide including Pakistan (Ramadan *et al.*, 2015; Abbas *et al.,* 2017b, 2017c). The economic losses may fall into various categories, including - prophylactic feed additives cost, medication cost, mortality, and poor feed conversion ratio in infected birds (Idris *et al*., 2017; Khater *et al.,* 2020).

Various control strategies are routinely used against coccidiosis. However, chemoprophylaxis and anticoccidial drugs have mostly been practiced in feed or drinking water against *Eimeriasis* (Abbas *et al*., 2019a, 2019b). Due to drug resistance, novel alternatives such as adjuvant based vaccines (Lin *et al*., 2020) and probiotics (Awais *et al.,* 2019) can serve as excellent source against poultry coccidiosis (Abbas *et al*., 2019). One of the cost effective methods to manage infectious diseases in the veterinary practice to induce a protective adaptive and innate immune response by vaccinating the animals with one (or more) antigens combined with an adjuvant (Lin *et al*., 2020).

A newly discovered Immune mapped protein-1 (IMP1) in *Eimeria maxima* has shown to possess the immunogenic potential and protect the chickens against *E. maxima* challenge. The IMP1 protein has also been proven as an immunoprotective protein against the other apicomplexans species, including *Neorospora* and *Toxoplasma* (Yin *et al.,* 2013). Usage of IMP1 with and without adjuvant is considered good approach against parasitic problems in modern era (Chen *et al*., 2020; Lin *et al*., 2020). The C-terminal part of EtIMP1 includes the immune dominant portion of the protein, just like in EmIMP1 (Chen *et al*., 2020).

Many advancements have been achieved to find new adjuvants that improve cell-mediated and humoral immunity by improving vaccine efficacy (Nanishi *et al*., 2019), especially relevant to avian coccidiosis. FCA can act as an adjuvant due to the immunomodulatory effect in different animal experiments (Lin *et al*., 2020).

There is also a growing interest in the use of probiotics before and during the vaccination to regulate the immune response (Awais *et al*., 2019) and improve the efficacy of vaccinations against viral (Santos *et al*., 2020), bacterial (Ritzi *et al*., 2016) and parasitic infections (Awais *et al*., 2019). Probiotics can be defined as living microbes having the ability to improve host immune responses, with toll-like receptor expression (Sato *et al*., 2009; Praharaj *et al*., 2015), cytokine stimulation (Brisbin *et al*., 2010), enhanced antibody development (Awais *et al*., 2019), as well as balanced the beneficial host microbes (Ritzi *et al*., 2014). Based on these findings, Probiotics have been suggested as potential adjuvants to vaccines, but there is limited confirmation (Praharaj *et al*., 2015). In this experiment, we evaluated the comparative effect of *Eimeria tenella* immune mapped protein 1C (EtIMP1C) alone and in combination with probiotics and FCA (Adjuvant) against the caecal coccidiosis.

MATERIALS AND METHODS

Parasite propagation: Chicks (160-one day old) were obtained from the hatchery located in Fuzhou. They were housed in a coccidian free cage system and given feed and water *ad libitum*. For *Eimeria tenella* unsporulated oocyst collection, 15-day old chickens were infected through oral route (3×10^4 oocysts per bird). Post-infection period of 6 to 9 days, the oocysts were identified, separated, cleaned, and sporulated as mentioned by the procedure (Yin *et al*., 2013; Chen *et al*., 2020; Lin *et al*., 2020).

Cloning and expression of EtIMP1C gene: The primers were designed according to the fragment of the EtIMP1C gene. They introduced the restriction enzyme sites EcoR I and Xho I, synthesized by Shengong Bioengineering (Shanghai) Co., LTD. The underlined part was the restriction enzyme sites in the primer sequence. The EtIMP1C EcoRI-f (5'-GAATTCGCCACCATGGGAAG CAACGCGAACCTGCCG-3') and EtIMP1C XhoI-r (5'-CTCGAGTCAGTGGTGGTGGTGGTGGTGAGTTGCT GCCGCCACATTTC-3'). Gene extraction, purification and cloning were done by pEASY-Blunt Simple Cloning Vector [Trans Gen Biotech, Beijing, China (Yin *et al*., 2015; Chen *et al*., 2020; Lin *et al*., 2020)]. Protein expression was checked by the Western blotting method.

Experimental design: Two-week-old male layer chickens were taken from the local poultry company located in Fuzhou, and randomly divided into 7 (n=22) groups. Group A was immunized intramuscularly with 200µg EtIMP1C protein while probiotics (*Lactobacillus plantarum*) were given @ 1×10^8 CFU (Colony forming unit) per liter of drinking water in different experimental days such as 1-3, 10,17, 24, 31, 38, 41-44, and 52 days. However, further studies are needed to check the effects of probiotics alone or in combination with subunit vaccines with extended time in layer experiment. Groups B was treated with 200µg of the EtIMP1C protein in Freund's complete adjuvant (FCA). Group C was supplemented with probiotics @ 1×10^8 CFU per liter of drinking water, while group D was administered with 200µl of FCA. Group E was kept as a control group with 200µg of EtIMP1C. Groups F and G were treated as infected and non-infected control groups, respectively. The same protocol was repeated after 14 days. *Eimeria tenella* virulent oocyst infection was administered to all groups with a concentration of 3×10^4 oocysts except group G at 42 days of the experiment.

Anticoccidial efficacy: At 8 days of post-infection, the anticoccidial effect of all groups mentioned above was assessed in terms of oocyst score (Sun *et al*., 2014), lesion score (Johnson and Reid, 1970).

Feed conversion ratio: The following formula determined the feed conversion ratio for all the treated groups at 54 days of the experiment. Feed conversation ratio (FCR) = Mean feed consumption/Mean weight

Organ weight and Hematological parameters: At the end of the experiment (56 days), the remaining chickens were slaughtered and weighed the different organs using a digital weighing balance. Blood samples were checked for Packed Cell Volume (microhematocrit method), hemoglobin level (Sahli's apparatus) and the serum was also isolated from the blood and stored it at -80ºC and processed for further experiment. The method mentioned by Natt and Herrick (1952) is used to calculate RBCs and WBCs value.

Anti-EtIMP1C antibodies detection assessment: ELISA assay was used to detect the chicken immunoglobulin G (IgG) by the method (Yin *et al*., 2015; Chen *et al*., 2020; Lin *et al*., 2020).

Statistical analysis: Statistical analysis was performed by ANOVA and Tukey's HSD test (Chen *et al*., 2020; Lin *et al*., 2020).

RESULTS

Protein expression: Protein expression was observed by using western blotting (Mouse anti- his 6 antibody) technique. This method showed that the 29 KDa protein expression band of EtIMP1C is shown in Fig. 1.

Lesion score and oocyst shedding in feces: Mean lesions score are shown in Table 1. The lesion score augmented by EtIMP1C- *Lactobacillus Plantarum* was comparable with EtIMP1C-FCA. However, less caecal lesion score was observed in EtIMP1C- *Lactobacillus Plantarum* treated group as compared to all other groups. (P>0.05). Although fewer oocysts in feces were seen in the probiotic, EtIMP1C-FCA and EtIMP1C-*Lactobacillus Plantarum* groups than that of all other treated groups, as shown in Table 2 (P>0.05).

Feed conversation ratio: EtIMP1C- *Lactobacillus Plantarum* administered group showed an improved feed conversion ratio than the infected untreated control group, as shown in Table 3.

Organ weight and hematology: The favorable effect of EtIMP1C- *Lactobacillus Plantarum* was observed on organ weight as compared to the infected non-medicated control group, as shown in Table 4 (P>0.05). Mean hematological parameters (HB, PCV, RBCs & WBCs) values were observed in the EtIMP1C- *Lactobacillus Plantarum* administered group was close to the EtIMP1C-FCA and probiotics treated group but significantly different as compared to infected non-medicated group as shown in Table 5 (P>0.05).

Immunoglobulin (IgG) value: The EtIMP1C-FCA and EtIMP1C-*Lactobacillus Plantarum* groups showed a higher concentration of EtIMP1C- specific IgG comparison to control groups (P<0.05). Consequently, there is a higher proportion of IgG in the EtIMP1C-FCA vaccinated chicken group than in the EtIMP1C- *Lactobacillus Plantarum* treated group; no significant difference is observed. There was no significant difference between the treatment groups such as FCA and EtIMP1C, as shown in Fig. 2.

DISCUSSION

In vaccination research, FCA is a widely used adjuvant, particularly for animal's experiment (Grzywa *et al*., 2015), leading to the increased immune response in the form of higher production of specific antibodies due to immune stimulant function (Stills, 2005). However, it is poisonous and difficult to manage (Fodey *et al*., 2008). The current study revealed that probiotics established best results against coccidiosis and fewer toxic effects than FCA. The FCA induced granulomas and discomfort in chickens and was not examined in the case of probiotics treated group as well as exposed non statistically significant difference in immune-protective results as compared to EtIMP1C-FCA treated group. In improving immunity against parasitic diseases, probiotics may therefore be an appealing and effective approach.

Probiotics are live microorganisms having a beneficial effect on the health of humans as well as animals by improving the host immune system and playing their role as an antioxidant (Awais *et al*., 2019). Different types of probiotics such as *lactobacillus bifidobacterium, bacillus* based probiotics are available in the market, having a beneficial effect against infectious diseases. Lactobacillus based probiotics have shown a positive impact against the lesion score.

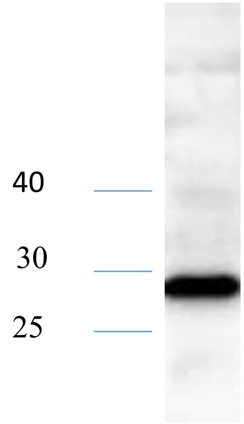

Fig. 1: EtIMP1C expression band by using Western blotting method (Mouse anti His6).

Table 1: Lesion Score in chickens (n=6) with experimentally induced coccidiosis in various treatment groups

Groups	0	+1	+2	+3	+4	Mean ± SD
A	2	1	2	1	-	1.33±0.2[c]
B	2	-	3	1	-	1.5±0.3[c]
C	0	4	1	1	-	1.5±0.3[c]
D	-	1	1	2	2	2.83±0.1[b]
E	-	-	2	2	2	3.0±0.1[b]
F	-	-	-	3	3	3.5±0.1[a]
G	6	-	-	-	-	0

Means with various superscripts within a column are significantly different (P<0.05). A= EtIMP1C and probiotics treated group, B= EtIMP1C-FCA treated group, C= Probiotics treated group, D= FCA treated group, E= EtIMP1C treated group, F= Infected, non-medicated treatment group, G= Non-infected, non-medicated treated group.

Table 2: Oocyst Score in chickens (n=6) with experimentally induced coccidiosis in various treatment groups

Groups	0	+1	+2	+3	+4	+5	Mean ± SD
A	1	2	2	1	-	-	1.5±0.2[c]
B	1	1	3	1	-	-	1.67±0.2[c]
C	-	2	4	-	-	-	1.67±0.3[c]
D	-	1	1	2	2	-	2.83±0.4[b]
E	-	-	2	2	2	-	2.66±0.4[b]
F	-	1	-	2	2	1	3.5±0.6[a]
G	6	-	-	-	-	-	0

Means with various superscripts within a column are significantly different (P<0.05). A= EtIMP1C and probiotics treated group, B= EtIMP1C-FCA treated group, C= Probiotics treated group, D= FCA treated group, E= EtIMP1C treated group, F= Infected, non-medicated treatment group, G= Non-infected, non- medicated treated group.

Table 3: Feed conversion ratio in chickens with experimentally induced coccidiosis in various treatment groups

Groups	Feed consumption (g)	Final weight (g)	FCR
A	1380	735	1.87
B	1370	693	1.97
C	1367	708	1.93
D	965	383	2.51
E	1034	412	2.50
F	965	351	2.74
G	1500	838	1.78

A= EtIMP1C and probiotics treated group, B=EtIMP1C-FCA treated group, C= Probiotics treated group, D=FCA treated group, E=EtIMP1C treated group, F=Infected, non-medicated treatment group, G=Non-infected, non-medicated treated group.

In this study, we investigated the effect of EtIMP1C-probiotic products on Chicken and their resistance to an *Eimeria tenella* infection. In the lactobacillus treatment group, birds had less severe intestinal lesion scores that indicated a healthier intestine. In the water source, birds receiving a high probiotic dose on irregular days shed fewer oocysts in the feces than the positive control group.

Table 4: Effect on Organ weight in chickens (n=6) with experimentally induced coccidiosis in various treatment groups

Groups	Bursa of fabricius	Heart	Liver	Spleen	Intestine	Kidney
A	0.21 ± 0.03^{ab}	1.16 ± 0.3^{ab}	4.06 ± 0.2^{ab}	0.18 ± 0.01^{b}	20.8 ± 1.8^{ab}	1.20 ± 0.17^{bc}
B	0.18 ± 0.01^{abc}	1.13 ± 0.3^{ab}	3.86 ± 0.1^{b}	0.19 ± 0.01^{b}	18.8 ± 2.2^{ab}	1.40 ± 0.14^{b}
C	0.19 ± 0.01^{abc}	1.10 ± 0.4^{ab}	3.81 ± 0.3^{bc}	0.17 ± 0.02^{b}	19.01 ± 1.7^{ab}	1.0 ± 0.05^{bcd}
D	0.11 ± 0.03^{bc}	0.59 ± 0.1^{b}	2.68 ± 0.1^{d}	0.09 ± 0.02^{b}	14.5 ± 1.1^{bc}	0.62 ± 0.02^{de}
E	0.13 ± 0.01^{bc}	0.57 ± 0.2^{b}	2.86 ± 0.2^{cd}	0.12 ± 0.02^{b}	15.8 ± 1.5^{bc}	0.70 ± 0.08^{cde}
F	0.083 ± 0.01^{c}	0.46 ± 0.2^{b}	2.10 ± 0.2^{d}	0.09 ± 0.02^{b}	10.9 ± 1.7^{c}	0.32 ± 0.11^{e}
G	0.32 ± 0.04^{a}	2.42 ± 0.1^{a}	4.95 ± 0.05^{a}	0.37 ± 0.03^{a}	25.8 ± 2.8^{a}	2.67 ± 0.20^{a}

Means with various superscripts within a column are significantly different (P<0.05). A= EtIMP1C and probiotics treated group, B= EtIMP1C-FCA treated group, C= Probiotics treated group, D= FCA treated group, E= EtIMP1C treated group, F= Infected, non-medicated treatment group, G= Non-infected, non-medicated treated group.

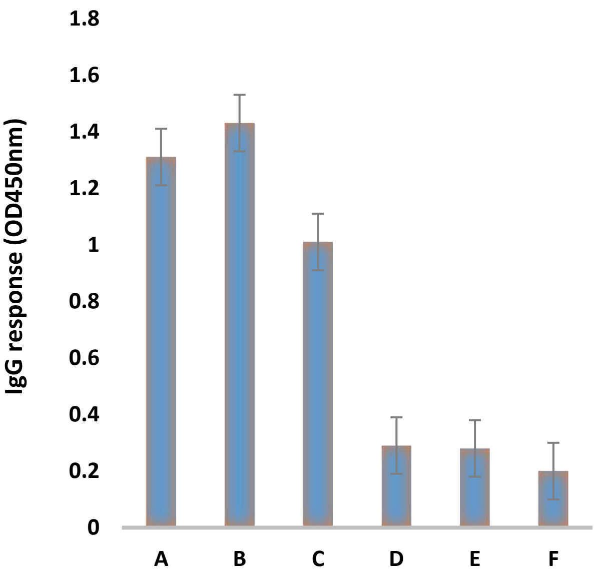

Fig. 2: Anti-IMP1C IgG in chickens sera after the second vaccination on 14 days using different treated groups. Results are showed as OD 450nm readings (P<0.05). A=EtIMP1C and probiotics treated group, B=EtIMP1C-FCA treated group, C=Probiotics treated group, D=FCA treated group, E= EtIMP1C treated group, F= PBS treated group.

Table 5: Effect on Hematological parameters in chickens (n=6) with experimentally induced coccidiosis in various treatment groups

Groups	WBC 10^3/µL	RBC 10^6/µL	PCV %	HB g/dL
A	28.66 ± 1.67^{a}	4.36 ± 0.50^{b}	32.23 ± 1.3^{ab}	17.2 ± 0.8^{ab}
B	29.96 ± 1.50^{a}	4.03 ± 0.64^{bc}	31.45 ± 1.1^{ab}	16.8 ± 0.9^{ab}
C	26.7 ± 1.30^{a}	3.65 ± 0.52^{bc}	30.43 ± 0.9^{b}	15.7 ± 1.1^{b}
D	15.79 ± 1.23^{b}	2.3 ± 0.45^{bc}	19.08 ± 1.2^{c}	9.55 ± 0.5^{c}
E	16.23 ± 1.40^{b}	2.8 ± 0.85^{bc}	20.8 ± 1.1^{c}	8.9 ± 0.7^{cd}
F	13.7 ± 1.70^{b}	1.64 ± 0.82^{c}	16.35 ± 1.2^{c}	5.53 ± 0.7^{d}
G	31.42 ± 1.50^{a}	7.64 ± 0.30^{a}	36.55 ± 1.1^{a}	20.69 ± 1.2^{a}

Means with various superscripts within a column are significantly different (P<0.05). A=EtIMP1C and probiotics treated group, B=EtIMP1C-FCA treated group, C=Probiotics treated group, D=FCA treated group, E=EtIMP1C treated group, F=Infected, non-medicated treatment group, G=Non-infected, non-medicated treated group.

Lesion scoring is an important parameter used to determine the severity of coccidiosis. Less intestinal lesion scores suggest less damage and increased chances of recovery of infected birds (Ritzi *et al*., 2014). Corroborating our findings, Dalloul *et al.* (2003, 2005) found that broilers provided significantly fewer *E. acervulina* for a *Lactobacillus*-based probiotic in the feed shed Oocysts of compared with the challenged control group. In a study, Eimeriasis by ingestion of *Bacillus*-based probiotics was observed with fewer lesion scores than non-supplemented food served to infected birds (Lee *et al*., 2010). In order to diagnose the extent of avian coccidiosis, there is a great need to count oocysts that come in a bird dropping. It shows the importance of coccidiosis and also reflects a decreased level of host immunity (Küçükyilmaz *et al*., 2012). The birds receiving a high probiotic dose in the feed and the drinking water shed less oocysts in the feces than the positive control group.

Corroborating our findings, the supplementation of probiotics is an excellent antibody producer and an antioxidant agent, resulting in fewer numbers of oocysts falling against *E. acervulina* & *E. tenella* infection. Chicken supplemented probiotics increased the lymphocytes of CD+3, CD+4, and CD+8 and Immunoglobulin levels against Eimeria parasites (Dalloul *et al*., 2003; Noujaim *et al*., 2008). In *E. acervulina* and *E. tenella* infection, oocyst in feces can be minimized by using probiotics in Chicken (Awais *et al*., 2019; Ritzi *et al*., 2016). *E. maxima* infection was reduced by using *Bacillus* species-containing probiotics (Lee *et al*., 2010).

The significant symptoms of parasite infection with Eimeria are growth retardation, including reducing weight gain, weakness ultimately great economic losses to the poultry industry (Ritzi *et al*., 2016). The findings of improved weight gain and different organ weights in broiler chicken are presented in Table 4 and were influenced by various treated groups. The previous study has investigated the positive effect of probiotics and vaccines alone and in combination with the weight gain of the Chicken, which is supporting our research (Ritzi *et al*., 2016). The oral administration of the probiotics had a positive effect on improved blood cell count, corroborating our findings (Alkhalf *et al*., 2014). EmIMP1 has a C-terminal component of immune dominant protein, which showed an improved immunity level in terms of IgG (Chen *et al*., 2020), which is supporting our study.

Conclusions: The role of probiotics alone and in combination with vaccines, including IMP1 against poultry coccidiosis, are economical and modern approaches. In this research, probiotics have shown favorable results with EtIMP1C against *Eimeria tenella* infection and improved the hematological and FCR values and the positive effect on the immunity level of infected Chicken. However, further study is needed on a different strain of probiotics and dosage rate alone and in combination with immune mapped protein-based vaccine.

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Author contributions: GWY and RZA supervised and designed the experiment. MM experimented and wrote the manuscript. MTA, MZA and AA polished the manuscript. LNL, YJH, AIS and XHH assisted in our experiment.

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