Investigation into the potential of using UV-treated sporulated oocysts of *Eimeria tenella* as a local solution to immunization of chickens against caecal coccidiosis

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Abstract

In this study, we aim to evaluate the immune response of chickens to UV-treated sporulated oocysts as a means of protection against caecal coccidiosis caused by field strains of *Eimeria tenella*. Two groups of chicks were immunized using prepared UV-treated oocysts of *E. tenella* and challenged at day 20 post hatching. The first group was immunized only once at day 1 post hatching, the second group was immunized twice (day 1 and day 8 post hatching). Two non-immunized control groups were used: the first group was challenged with *E. tenella*, while the second group remained uninfected. The effectiveness of vaccination on production and animal health was evaluated by the following criteria: body weight, feed conversion ratio, blood in faeces, mortality, lesion scores and oocyst output. The two immunized groups showed a significantly better performance in body weight, weight gain and lesion scores than the non-immunized group. However, all three groups performed significantly worse than the unchallenged group. The mortality of the non-immunized infected group was high (70%) while mortality in both immunized and unchallenged groups of chickens was significantly lower (range 2.2 to 4.4%) than the infected group ($p<0.05$). The production of oocysts in faeces, post-infection, was significantly higher in the non-immunized group compared to the immunized group ($p<0.05$) and both were significantly higher than the uninfected group ($p<0.05$). In conclusion, immunization by prepared UV-treated sporulated oocysts of *E. tenella* is effective in stimulating at least a partial protective immunity in immunized chickens against caecal coccidiosis.

Keywords: UV-treated; oocysts; *Eimeria tenella*; coccidiosis; Immunity

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Introduction

Coccidiosis caused by *Eimeria tenella* is the best known of the avian protozoan diseases. This is partly because of the serious disease it causes and partly because of its widespread economic importance in poultry farming. This parasite inhabits the caeca and causes high morbidity and mortality, hemorrhagic diarrhea, major weight loss and emaciation (McDougald and Reid, 1997). Anti-coccidial drug resistance in broiler flocks is a growing global problem (Witcomb and Smith, 2014) and is significant in tropical areas such as the study location for this work in Algeria (Djemai et al. 2016). To this is added the low availability and the high cost of the anticoccidial vaccines imported to tropical locations.

It is well known that UV radiation can be used for attenuating or inactivating micro-organisms. In particular, this is widely used in the drinking water industries. Ware et al. (2010), concluded that UV treatment of water (10-mJ/cm^2 UV fluence) appears to be effective at inactivating *Toxoplasma gondii* oocysts under ideal conditions. *Cryptosporidium* oocysts are also highly resistant to chemical inactivation but several studies show that *Cryptosporidium parvum* oocysts are inactivated by UV radiation (Clancy et al., 1998; Bukhari et al., 1999; King et al., 2017). Kniel et al. (2007) were able to induce protective immunity in chickens immunized with sporulated oocysts of *E. acervulina* inactivated by UV radiation (261 mW / cm^2). The idea of decreasing the pathogenicity of a strain by irradiation has been around for some time (Yvoré et al., 1993). It is an applicable approach in practice. For example, it is used in helminths in the production of a commercial vaccine against lungworm infection in cattle (e.g., Bovilis Huskvac®, MSD Animal Health) and is based on the use of viable gamma irradiated *Dictyocaulus viviparus* L_3 larvae.

Different types of irradiation have been tried on *Eimeria* spp oocysts, in an attempt to reduce viability, with some success. For example: in various studies *E. tenella* oocysts were exposed to ultraviolet rays (El-Ashram et al. 2019), X-rays (Jenkins et al., 1991) and to gamma rays

(Abu Ali et al., 1972); the advantage of irradiation is that it does not appear to affect immunogenicity at a given dose of UV or gamma radiation.

The aims of this study were to investigate the potential for UV treated sporulated oocysts of *E. tenella* to be used as an immunization strategy to improve animal health and production performance in chickens.

Materials and methods

Experimental Design

Our Experimental design conforms to the guidelines set out for evaluating the efficacy and safety of anticoccidial vaccines (Chapman et al., 2005). Based on this we used four groups of chickens for our study: (1) unvaccinated unchallenged control birds, (2) unvaccinated challenged control birds, and (3) vaccinated challenged birds (two different regimes). A total of 240 one-day-old Cobb 500 broilers (non-sexed) were purchased from a commercial hatchery. Ethical approval and authorisation to conduct the animal experimentation was obtained from the University of Constantine (Veterinary Institute) and the State Veterinary Departments of both Constantine and Jijel. Experimental and rearing conditions conform to the standards of the COBB Broiler Management Guide and standards set out by other studies (Chapman and Rayavarapu, 2007; Bedrnik et al., 1995; Long and Millard, 1977). Broilers were reared in clean conditions, groups were maintained separately and animals exposed to the same environmental conditions.

At one-day-old, birds were randomly divided into four groups (V_1I, V_2I, NVI, NVNI), each containing 60 birds, divided into 3 replicates each containing 20 chicks. Group V_1I (single immunization challenge group) received only one dose of the UV- treated *E. tenella* oocysts before challenge (summarized in Table 1). Birds from group V_2I (double immunization challenge group) received two doses of the UV- irradiated oocysts and were then challenged. The control groups were, NVI and NVNI; chicks of both groups have not been immunized, however the NVI group (non-immunized challenge group) was infected and the NVNI group (non-immunized non-challenge group) was not infected. Standard, non-medicated feed and water (i.e. without coccidiostats) were provided *ad libitum* for days 1-28 to all groups (Chapman and Rayavarapu, 2007; Bedrnik et al., 1995; Long and Millard, 1977).

Eimeria tenella oocyst collection and sporulation

E. tenella oocysts used in the inoculum and those irradiated with UV used for immunization were collected from clinical episodes of hemorrhagic caecal coccidiosis in several broiler farms located in the Wilaya of Jijel (Algeria). *E. tenella* oocysts used for the immunization of chickens and those used for inoculation originated from different broiler farms. Oocysts were isolated from the caeca of infected chickens and, after separation of the faecal material, were recovered using standard procedures (Ryley et al., 1976). Isolated *Eimeria* oocysts were suspended in 2.5% potassium dichromate and sporulated for 72 h at 29°C by aeration and continuous agitation in a shaking water bath. When sporulation had reached > 80%, the *Eimeria* oocysts were stored at 4°C until use (Djemai et al. 2016).

Identification of *Eimeria* species

Prepared inoculums and immunization isolates could potentially contain a number of species of chicken *Eimeria* which could potentially confound our study. To test that all samples contained only *E. tenella*, morphological and molecular analyses were conducted. Parasites were examined by microscopy, at 1000X magnification, and differentiated using known average lengths and widths of the 7 *Eimeria* species infecting chickens as described (Long and Reid, 1982). Species designation was verified by PCR amplification using *Eimeria* species-specific ITS1 sequences following standard procedures (Jenkins et al., 2006a, 2006b). Genomic DNA of purified oocysts was extracted using phenol chloroform extraction (Duncanson et al., 2001; Bajnok et al., 2015) with modifications for small amounts as described (Dodd et al., 2014). ITS1-PCR revealed that prepared oocyst suspensions contained only *E. tenella*.

Preparation of the inoculum

Sporulated *E. tenella* oocysts, stored in potassium dichromate solution, were washed 3-4 times in water and resuspended in phosphate-buffered saline solution (PBS) (pH 7.2) and counted using the modified McMaster method (Taylor et al., 1995). A concentration of $2.5x10^4$ oocysts/ml was prepared.

Preparation of UV-treated sporulated oocysts

Sporulated *E. tenella* oocysts were collected, washed and counted as described above and a concentration of $5x10^3$ oocysts/ml was prepared. A UV Lamp, TUV T8 (TUV 30W G30T8, Philips, Holland), with an output at 253.7 nm was used to treat the oocysts. A 180ml sample of PBS containing $5x10^3$ oocysts/ml, was divided into 18 clear glass dishes with a 5-10 ml suspension of oocysts in each dish to a depth of ≤1 mm (Zhao et al., 2013; Abdel-Baki et al., 2009). Then each dish was placed at the centre of a closed wooden box (100 cm in length, 50 cm in width, and 50 cm in height) adjacent to a UV light meter (UV Light Meter, YK-35UV) for measuring the UV intensity and wavelength. The UV lamp was positioned above the sample and UV meter at the center of the box. The ceiling and the interior walls of the box were covered with a layer of aluminum foil (Thickness: 0.2mm) as an efficient reflector of ultra-violet light (Abdel-Baki et al., 2009). The oocyst suspensions were exposed to UV light (160 mW/cm^2) for 60 min (Abdel-Baki et al., 2009) and agitated using a magnetic agitator (Drehzahl electronic- IKA-COMBIMAG REO) to ensure homogeneous exposure of the oocysts to UV.

Immunization and challenge

Immunization was carried out in two batches in groups V_1I and V_2I. One-day old chicks (18 h post-hatching) from the group V_1I received only one dose of $5x10^3$ UV-treated *E. tenella*

158 sporulated oocysts suspended in 1 ml of PBS solution (per bird). Chicks from group V_2I
159 received two doses (also $5x10^3$ oocysts per ml per bird): the first was given at one-day old
160 (18 h post-hatching) and the second at 8 days post-hatching. UV-treated oocysts were
161 administered by gavage into the crop using a syringe (5 ml) connected to a cat urethral
162 catheter (Buster Cat Catheter; sterile, 1.0 x 130mm, China). The immunization strategy is
163 summarized in Table 1.
164 The *E. tenella* sporulated oocysts, used to challenge the immunized chickens, were non-
165 treated wild strains of *E. tenella* (collected from broiler flocks during clinical events of
166 hemorrhagic caecal coccidiosis) (Li et al., 2005). At 20 days post-hatching, each chick from
167 groups V_1I, V_2I and NVI received 50 000 sporulated *E. tenella* oocysts (in 2 ml of PBS) by
168 gavage as described (Table 1) (Long and Millard, 1977; Jenkins et al., 1991; Li et al., 2005).
169
170 **Evaluation of the efficacy and safety of immunization using UV-treated *E. tenella***
171 In order to evaluate the efficacy and safety of the immunization by UV-treated *E. tenella*, we
172 collected the following parameters (Bedrnik et al., 1995):
173 (a) Body weight and average weight gain. Birds were individually weighed at day 1; the day
174 of inoculation (day 20 post-hatching); and days 2, 4, 6 and 8 post-challenge (at day 22, 24,
175 26, 28). Average weights gains were calculated for all groups of chicks (including dead
176 birds).
177 (b) Feed conversion ratio (FCR). The feed conversion ratio (FCR) was calculated by
178 dividing the average amount of feed consumed by the average weight gain in each replicate
179 of each group.
180 (c) Oocyst output in faeces. Oocysts were counted as described (Taylor et al.,1995) for
181 determination of oocyst output per gram of faeces (OPG). Fecal samples for OPG counting
182 were taken from four random spots from the floor of each pen. They were samples at the

frequency of 4 morning samples/repetition/day between the following time intervals: 5th and 9th day of post-hatching (after the first immunization); 12th and 16th day post-hatching (after the second immunization); 5th and 8th day post-challenge (25th and 28th day post-hatching). The average oocyst output was calculated per gram of faeces (OPG) at each interval and for each group.

(d) Faecal score. A scoring system was used to record the daily occurrence of bloody faeces seen on the litter for each repetition of all groups. The evaluation was applied according to the method suggested by Williams (1997): (-) = no bloody droppings seen; ($\pm$) = $\leq$ 1 bloody dropping per chick; (+) = > 1 $\leq$2 bloody droppings per chick; (++) = > 2 bloody droppings per chick. Measurements were taken at the following time intervals: 5th and 16th day post-hatching (after immunization); 5th and 8th days post-challenge.

(e) Caecal lesion score. On 5th, 12th and 25th (after-challenge) days post-hatching, 15 chicks/group/day (5 chicks/repetition/day) were killed. The caeca were removed and opened. The infected caeca of each bird were examined and scored on a scale of 0–4 according to the method described by Johnson and Reid (1970). To confirm the attribution of the lesions observed during the autopsy to the coccidiosis, scrapings of the caecal mucosa were inspected by microscopy (100x and 400x magnification), for unsporulated oocysts, schizonts and gametocytes. Unsporulated oocysts present in the pool of the products of the scrapings were purified and suspended in 2.5% potassium dichromate solution. The oocysts in the suspension were sporulated by incubation in a stirring water bath at 29°C for 3 days (Coudert et al., 1995). DNA extraction and ITS1 PCR (Jenkins et al., 2006a, 2006b) were carried out, as described above, to confirm the presence of *E. tenella*. The only species identified in the products of these scrapings, from the inoculated and immunized chicks, was *E. tenella*.

(d). Mortality. Between 20^{th} and 28^{th} day (day 1 to day 8 post-challenge), mortality (%) was recorded for each of the 4 batches. Additionally, any dead subjects were also weighed and autopsied to determine possible coccidiosis lesions.

Statistical Analysis

Data were analyzed by one-way ANOVA using SPSS 10.0 software (Statistical Package for the Social Sciences). Treatment groups were compared by ANOVA using the following parameters, weight gain (the first day post-hatching; 20^{th} day post-hatching; 2^{nd}, 4^{th}, 6^{th}, 8th days post-challenge), feed conversion ratio (2^{nd}, 4^{th}, 6^{th} and 8^{th} days post-challenge), and lesion scores (5^{th}, 12^{th} and 25^{th} days post-hatching), oocyst output in the faeces (between 5^{th} and 9^{th} day post-hatching; between 12^{th} and 16^{th} day post-hatching; between 5^{th} and 8^{th} days post-challenge), and cumulative mortality (20^{th} and 28^{th} day post-hatching). Immunized groups were compared to the non-immunized challenge control group (NVI) and non-immunized non-challenge control group (NVNI) and a statistically significant difference of $p<0.05$ was used. Tukey's post-test was used to identify significantly different groups.

Results

The average body weight and average weight gains recorded in the immunized challenge groups (V_1I, V_2I) were significantly lower ($p< 0.05$) than those of the non-immunized non-challenge group (NVNI), but significantly higher ($p< 0.05$) than those of the non-immunized challenge group (NVI) (Table 2). Significantly poor growth performance was observed in inoculated non-immunized chickens (NVI) compared to the non- immunized non-challenge group (NVNI) ($p<0.05$). The administration of the UV-treated oocysts of *E. tenella* using the two immunization regimes (V_1I, V_2I) caused a significant reduction in the average weight gain after the challenge compared to the uninfected group (V_1I= 24.24%; V_2I= 20 %).

However, this compared positively to the non-immunized challenge group which showed a very low weight gain (NVI= 50%) compared to the uninfected controls. Interestingly the repeated immunization of chickens with the UV-treated *E. tenella* produced no significant increase in growth performance when compared to the group immunized only once.

After immunization, the two immunized groups (V_1I, V_2I) developed light caecal lesions, scoring from 0 to 0.6 (5^{th}, 12^{th} days post-hatching), low oocyst output in faeces (from 0 to 600 OPG) with light output scores between 5 - 9 days of age and between 12-16 days of age (Table 3). These were not significantly different from the uninfected groups ($p>0.05$).

After challenge, the caecal lesion scores (day 5 post-challenge), the oocyst excretion (day 5 - 8 post-challenge) and mortality (day 1 - 8 post-challenge) in the 2 immunized groups (V_1I, V_2I) were significantly lower ($p< 0.05$) than those observed in the non-immunized challenge group (NVI). There was no significant difference between the singly and doubly immunized groups. Mortality of the non-immunized group (NVI) is significantly higher at 70% than either the immunized or uninfected control group ($p<0.05$) (Table 3). Post-mortem testing, as described (Materials and Methods), indicated that this mortality was due to infection with *Eimeria*. There is no significant effect of immunization on mortality. Faecal caecal scores (day 5 to 8 post inoculation) observed in the 2 immunized groups show some damage caused by the immunization but this later attenuates (Table 4). The faecal caecal score observed in the non-immunized challenge group (NVI) showed that the birds were all very seriously affected compared to the immunized or uninfected groups (Table 5).

Discussion

In this study, the efficacy of the UV-treated *E. tenella* immunization was determined primarily on comparative growth rates of immunized and non-immunized chickens following virulent challenge as described previously (Williams and Catchpole, 2000), with feed

conversion ratios and lesion scores used as secondary parameters (Crouch et al., 2003). The pathogenic potential of an *E. tenella* challenge was confirmed by the ability to individually cause significant reductions in weight gain. We demonstrate that immunization with UV-treated oocysts significantly improves weight, weight gain, compared with non-immunized birds. However, the effect is not complete since the uninfected birds gained significantly higher weights which was achieved more rapidly than those immunized. Furthermore, feed conversion was unaffected by either infection of immunization. Broadly, this shows that this immunization has the potential to improve productivity in part. Where UV-treated oocyst immunization showed a clear effect was in reducing mortality from 70% to between 2.2 to 4.4%. This would have a major influence on productivity. Taken together these data suggest that this approach shows promise as an approach to improving productivity compared to the non-immunized state.

In addition to investigating productivity, this study considered parameters that influenced the health of the birds. Immunization was shown to have a beneficial effect on the development of caecal lesions as judged by lesion scores and blood in the droppings – both were significantly reduced. Furthermore, oocyst production was significantly reduced in immunized birds compared to their non-immunized counterparts thus potentially reducing the degree of transmission from bird to bird. Overall, while immunization using UV-treated oocysts was not absolute, there may be benefits to tropical countries or communities where other anti-coccidial approaches are restricted. We were unable to demonstrate any value in giving a second immunization – there was no significant difference between the two immunized groups of chickens.

In other studies, Crouch et al.(2003) indicated that protection against the reduced weight gain induced by virulent challenge could be demonstrated in chicks vaccinated at 1-day old for *E. acervulina, E. maxima, E. mitis, E. tenella* using the commercial PARACOX^{TM}-5 vaccine.

Maes et al. (1991), observed very good growth performance in a group of chickens challenged by 200000 oocysts of *E. tenella*/bird and immunized by repeated doses of 2000 virulent oocysts of *E. tenella*/bird/day (at day 4, 6, 8, 11 and 13). Nakai et al. (1992) obtained an improvement in the growth performance in chickens immunized daily throughout 13 successive days (50 virulent oocysts of *E. tenella*/bird/day). These studies all demonstrate the value of immunization and the measurement of growth and growth rate as an indicator of success.

On the other hand, it has been shown that the use of the severity of coccidial lesions, as a major criterion in the evaluation of the protective immunity induced by a anticoccidial vaccines, can lead to incorrect conclusions due to a lack of correlation between body weight and the coccidial lesions (Williams, 1997). According to Norton et al. (1989), Weber (1989), Shirley and Long (1990) and Williams (1997), the absence of coccidial lesions following a virulent challenge of an immunized bird, is a sign of immune protection, while the presence of the lesions does not necessarily indicate a weak protection and a significant depression of growth rate. According to the above-mentioned studies, we can conclude that immunization by the prepared UV-treated sporulated oocysts of *E. tenella*, carried out in this study, generates a good protection against caecal coccidiosis. However, we observe that the above-mentioned parameters are slight in spite of the large immunization dose administered ($5x10^3$ oocysts/bird).

There are a number of hypotheses that could explain what we are observing with the UV-treated oocyst immunization. Firstly, we may be observing attenuation which preserves the capacity for sexual and asexual reproduction but has resulted in the attenuation of pathogenicity. Secondly, there could be a proportion of the UV-treated *E. tenella* sporulated oocysts which remain viable and provide low level infection but which stimulates an immune response. Thirdly, inactivation of a part of the population of UV-treated *E. tenella* oocysts

(dead oocysts) which results in attenuation of viable oocysts by stimulation of the immune response against them. Unfortunately, we are unable to confirm the proportion of oocysts that the UV-treatment attenuates or kills and, at present, we are unable to distinguish between these possibilities or determine the full extent of the immunity to *E. tenella*. We also think that vaccination with a large number of UV-treated sporulated oocysts of E. tenella can cause clinical signs, hence the importance of studying immunizations with lower doses of oocysts. According to Rose (1970), the term immunity, as used in most studies, refers to the acquisition of resistance to an infection with an *Eimeria* species. Resistance may be measured by a reduction in the pathogenic effects of infection, a reduction in the extent of macroscopically visible lesions, or a decrease in the numbers of parasites, as measured by the production of oocysts in the feces.

In this study, immunization of chickens with sporulated oocysts of *E. tenella* treated with UV radiation does not prove effective at completely preventing the development of caecal lesions, oocyst excretion and bloody droppings irrespective of the single or double immunization regime. However, it clearly influences survivability and growth rate and shows potential to be a useful tool in countries or regions where other approaches are restricted or too costly.

The cost of commercial anticoccidial vaccines is high and this may be prohibitive in some parts of the tropical world. Currently, the cost of the PARACOX^{TM}-8 vaccine in Algeria, as an example location, is 0.35-0.45 € per laying pullet – which scales up to large sums of money in an animal production system that relies on large numbers of animals. Furthermore, in many parts of the tropical world, little is known of the identity of *Eimeria* strains and species present in local chicken farming systems. Development of methods that enable the use of local strains or species may offer opportunities to vaccinate in areas where traditional vaccines may not have been tried or might not be effective. While, we recognise the value of

using established vaccines there may be a role for the use of UV-treated oocyst vaccines in local settings to make improvements in animal health and production.

Compliance with ethical standards

Ethical approval. Our Experimental design conforms to the guidelines set out for evaluating the efficacy and safety of anticoccidial vaccines stated by Chapman et al. (2005). Experimental and rearing conditions conform to the standards of the COBB Broiler Management Guide and standards set out by other studies (Chapman and Rayavarapu, 2007; Bedrnik et al., 1995; Long and Millard, 1977).

Conflict of interest

The authors declare that they have no conflict of interests.

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Table 1. Immunization methodology.

Group	Immunization by UV-treated *E. Tenella* sporulated oocysts	Immunization dose	*E. tenella* sporulated oocysts challenge	Inoculum dose
V1I	Yes (once)	5000 UV- treated *E. tenella* oocysts/bird at18 h post-hatching.	Yes	50 000 sporulated oocysts of *E. tenella* at 20 days post-hatching.
V2I	Yes (twice)	5000 UV- treated *E. tenella* oocysts/bird at 18 h post-hatching. 5000 UV- treated *E. tenella* oocysts/bird at 8 days post-hatching.	Yes	50 000 sporulated oocysts of *E. tenella* at 20 days post-hatching.
NVI	No	None	Yes	50 000 sporulated oocysts of *E. tenella* at 20 days post-hatching.
NVNI	No	None	No	None

V_1I: Single immunization challenge group. V_2I: Double immunization challenge group. NVI: Non- immunized challenge group. NVNI: Non- immunized non-challenge group.

Table 2. Effects of UV-attenuated *E. tenella* immunisation on Body Weight, Weight Gain and Feed Conversion Ratio (FCR) in broiler chickens infected with *E. tenella.*

Group	Body Weight (g)*	Weight Gain (g)*	Feed Conversion*

V_1I	976.1± 8[b]	377.7± 7[b]	1.4± 0.03[a]
V_2I	998± 6[b]	399± 2[b]	1.37± 0.02[a]
NVI	849.5± 7[c]	250.9±9[c]	1.42± 0.03[a]
NVNI	1095.7± 8[a]	498.9± 8[a]	1.36± 0.02[a]

[*]Mean values sharing similar superscripts within a column are not significantly different ($p < 0.05$) from each other as determined using ANOVA and the Tukey post-test.

Table 3. Intensity of oocysts in faeces (OPG), lesion scores and mortality rate

Group	After immunization				After challenge		
	OPG (x 10^3)[*]	OPG (x 10^3) [**]	LS[***]	LS[****]	OPG (x10^3) [*****]	LS[******]	Mortality(%) [*******]
V_1I	0.6±0.5[a]	0.0 ± 0.0[a]	0.5± 0.1[a]	0.0 ± 0.0[a]	3.7±0.4[a]	1.7± 0.2[a]	4.4 [b]
V_2I	0.5±0.3[a]	0.2±0.3[a]	0.5± 0.4[a]	0.6 ± 0.0[a]	2.5±0.1[a]	1.5± 0.1[a]	2.2[b]
NVI	0.0±0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	13.5±0.5[b]	3.5 ± 0.2[b]	70.0[a]
NVNI	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[c]	2.2[b]

OPG: oocyst per gram of faeces, [*]Average oocyst output between 5[th] - 9[th] day post-hatching. [**] Average oocyst output between 12[th]-16[th] day post-hatching. LS : [***] Lesion

score at the 5th day post-hatching (15 Birds/group), **** Lesion score at the 12th day post-hatching (15 Birds/group), ***** Average oocyst output between 5th and the 8th day post-challenge (25th -28th day post-hatching), ****** Lesion score at the 25th day post-hatching (15 Birds/group). ******* Mortality recorded (as a percentage) after the challenge (1st - 8th day post-challenge). Mean values sharing similar super scripts within a column are not significantly different ($p < 0.05$) as determined by the ANOVA and Tukey post-test.

Table 4. Faecal caecal score following immunization.

Group	Faecal caecal score*									
	5	6	7	8	9	12	13	14	15	16
V_1I	+	+	+	-	-	-	-	-	-	-
V_2I	+	+	+	+	-	+	+	+	-	-
NVI	-	-	-	-	-	-	-	-	-	-
NVNI	-	-	-	-	-	-	-	-	-	-

* Day post-hatching ; The evaluation of faecal score was applied according to the method suggested by Williams (1997): (-) = no bloody droppings seen; ≤ 1 bloody dropping per chick; (+) = > 1; ≤2 bloody droppings per chick; (++) = > 2 bloody droppings per chick.

Table 5. Faecal score after challenge with *Eimeria tenella*.

Group	Faecal score*			
	5	6	7	8
V_1I	+	+	+	-
V_2I	+	+	+	-
NVI	+	++	++	++
NVNI	-	-	-	-

* Days after challenge. The evaluation of faecal score was applied according to the method suggested by Williams (1997): (-) = no bloody droppings seen; 1 bloody dropping per chick; (+) $\leq$2; bloody droppings per chick; (++) = > 2 bloody droppings per chick.