AMERICAN UNIVERSITY OF BEIRUT

EVALUATION OF AN *ECHINACEA*-BASED ANTI-COCCIDIAL PREPARATION AGAINST DIFFERENT *EIMERIA* SPP. IN BROILERS

by

DANIELLE BAHIJ AYYASH

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AN ABSTRACT OF THE THESIS OF

Danielle Bahij Ayyash for Master of Science
Major: Poultry Science

Title: Evaluation of an *Echinacea*-based anti-coccidial preparation against different *Eimeria* spp. in broilers

The aim of this thesis is to evaluate an *Echinacea*-based anti-coccidial preparation against different *Eimeria* spp. in broilers.The thesis is divided into three parts namely, Study A, B and C. Study A aimed at establishing a base-line data on intestinal pathogenesis in broilers with a controlled challenge by eight *Eimeria* spp., administered at different ages of 14, 21, 28, and 35 days old. A possible success in reproducing pathogenesis of *Eimeria* spp. infection in broilers by the implemented experimental design could help in evaluating the *Echinacea*-based preparation (EBP) in Studies B and C aiming at reducing mortality, feed conversion, lesion score, oocyst counts and improving the percent weight gain.

Study A proved that birds challenged with the eight non-attenuated *Eimeria* spp., had a reduction of around 10 % in the mean weight gain compared to birds in the control groups. The mean feed conversion ratio increased significantly from 1.5 in unchallenged-controls to 3.1 in challenged groups. The mean frequency of mortality increased in the challenged groups when compared to controls, associated with a significant increase in most lesion scores of the four intestinal organs, and significant presence of intestinal oocyst counts. A trend of decreased mortality was shown when broilers were challenged at older ages; this trend was associated with significant decline in lesion scores in the intestine, along with decreased oocyst counts.

Study B aimed at evaluating a long term administration of EBP and its evaluation of performance in birds challenged at 14, 21, 28, and 35 days of age. Performance was evaluated by determining the % mortality, % weight increase, feed conversion ratio, intestinal lesion scores and oocyst counts. A clear improvement in % weight increase was observed in birds challenged at 14 d. of age and EBP-treated for a period of 14 d.and 21 d. in comparison to positive control birds that were *Eimeria*-challenged but EBP-deprived, with a difference of around 65 %. At this challenge time of 14 days of age, the *Eimeria*-challenged and EBP-treated birds had comparable % weight increase to the corresponding birds in the negative control group that were deprived of both the EBP treatment and *Eimeria*-challenge. In addition, the feed conversion ratio in birds challenged at this age of 14 d. was reduced in the EBP-treated group compared to the corresponding positive control. Unfortunately, the % weight gain

in the EBP groups treated for 1-14 d. and 1-21d. and challenged at 21, 28, and 35 days of age, didn't improve over the corresponding birds of positive control groups that were deprived of the EBP but had the same respective challenges. The long-term administration of EBP (21 days), proved to have a negative effect on the mean feed conversion but not the mean % weight increase of the birds. A peculiar result was obtained in the cecal lesions, showing similar low mean lesion scores in the two groups that were EBP- treated for 1-14 d. and 1-21 d. periods, followed by *Eimeria*-challenge at 14 days of age, which was equivalent respectively to 1.5 and 1.2. The 1.5 and 1.2 mean cecal-lesion scores were apparently lower than the mean cecal-lesion score of 2.4 obtained in the corresponding birds of positive control that were EBP deprived but *Eimeria*-challenged at the same age. This peculiar result was most likely responsible for the birds'decrease in feed conversion ratio, % mortality, and improved % weight increase. There was a consistent improvement in reduction of the oocyst counts in the duodenum, jejunum, ileum and cecum of birds treated by EBP for 1-14 d., and that were challenged at 14 d. of age, compared to counts obtained in corresponding positive control birds that were deprived of EBP but *Eimeria*-challenged at the same age.

The objective of study C was to study the impact of intermittent administration of EBP, at ages of 1-3, 8-10, 15-17 and 22-24 days, of the life of broilers, on production, protection, and immuno-modulation against a challenge at 28 d. with *Eimeria* spp. alone, or with *Eimeria* spp. and *Cl. Perfringens.* The same performance parameters assessed in Study B were also included in Study C, adding assessment of immuno-modulation in the birds by measuring the plasma nitrite levels at 28 d. and 34 d., along with quantitative measurement of interleukins at the mucosal level in the four intestinal organs. Birds that were deprived of the EBP but *Eimeria*-challenged, and those that were *Eimeria* and *Clostridium*- challenged had the lowest average weights at the market age of 34 d (1596.7 and 1608.5 g, respectively) compared to their similarly challenged counter birds that were both administered the EBP (1609.2g and 1660.6g respectively). In addition, an improvement in reduction of the average feed conversion ratio to 1.64 was observed in birds administered EBP and challenged with both the *Eimeria* spp. and *Cl. Perfringens,* compared to a 1.70 ratio obtained by similarly challenged birds that were deprived of EBP. The averages of the oocyst counts and lesion scores were consistently reduced in most intestinal organs of the EBP treated compared to the EBP deprived birds receiving similar challenges. The serum nitrite levels, at the challenge time of 28 days of age in the EBP treated birds, were consistently almost double that obtained by EBP deprived birds, signifying a trend in immuno-modulation of phagocytosis by the EBP's active ingredients. This nitrite level was also the highest (5.91 micromolar) at 6 days post challenge (34 days of age) in birds that were EBP treated and challenged with *Eimeria* species alone, which was correlated with a consistent lowest average oocyst and lesion scores in most of the 4 intestinal organs, compared to that obtained in all other challenged treatments. In addition, there was a significant reduction in transcribed IL-8 at the duodenal level in birds that were EBP treated and challenged with *Eimeria* species alone at 34 days of age. A reduction trend of this chemokine at ileal and cecal levels was observed in birds administered EBP and challenged with *Eimeria* spp. alone or *Eimeria* spp. and *Cl. Perfringens,* compared to similarly challenged birds that were deprived of EBP treatment, which could be the reason behind the lower lesion scores detected in EBP treated birds.

In conclusion, Kock's posulate to reproduce coccidiosis in broilers was achieved in Study A. In Study B, continous adminstration of EBP for the first 21d. of bird's age proved to have a negative effect on performance and we recommended in Study C intermittent adminstration of EBP that had an immunomodulatory effect and protection against *Eimeria* spp. alone or co-infected with *Cl. perfringens*, associated with improved performance especially at 14d. of the bird's age.

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CHAPTER I

INTRODUCTION

Coccidiosis is the most important protozoan disease affecting poultry industry worldwide having an annual loss of more than US $ 4 billion (Williams, 1999a; Shirley *et al.,* 2004). Eighty % losses are due to mortality, reduced weight, and inefficient feed conversion as well as temporary loss of egg production in layers (Dalloul & Lillehoj, 2005). Good management practices can help in controlling the disease by reducing its spread, but still chemoprophylaxis and live-vaccines are the key tool for Coccidiosis control. Feed medication and water treatments result in tremendous costs responsible for this loss.

The etiologic agents of Coccidiosis are various *Eimeria* spp., which invade the lining of the intestine and are transmitted from bird to bird via the ingested sporulated oocysts found in the environment. The life cycle of *Eimeria* is complex, having both sexual and asexual stages. *Eimeria* infections are site specific by which each *Eimeria* spp. infects specific part of the intestine, and host specific, with varying pathogenicity in different poultry breeds (Jeffers *et al.,* 1970; Levine, 1985; Lillehoj, 1988; Lillehoj *et al*., 1989). Seven species of *Eimeria* are considered crucial in pathogenesis namely, *Eimeria acervulina, E. maxima, E. tenella, E. necatrix, E. brunetti, E. mitis* and *E. praecox;* however, *Eimeria hagani* and *E. mivati* validity is under review (Long, 1973b; Shirley *et al.,* 1983; Barta *et al.,* 1997; Tsuji *et al.,* 1997; Vrba *et al.,* 2011).

The pathogenicity of coccidia is dependent on the successful replication of parasites inside the intestine. Theoretically, a single oocyst of virulent *Eimeria tenella*

gives 2,520,000 invasive parasites following the second merogony stage in its life cycle (Levine, 1982).

Coccidia invade the intestinal mucosa causing a certain degree of damage to the epithelial cells, inflammation and villous atrophy (Pout, 1967; Assis *et al.,* 2010). The signs observed during coccidiosis infection depend on extent of the damage caused to the mucosal intestinal lining and inflammation, and may include whitish diarrhea in *Eimeria acervulina* or hemorrhagic fecal material in *E. tenella*, petechial hemorrhages and plenty of orange mucus in *E. maxima* , dehydration and weight loss (Conway and Mckenzie, 2007). The abundant bleeding found in the ceca is a characteristic sign of *E. tenella* infection due to erosion and sloughing of the intestinal mucosa (Witlock *et al.,* 1975).

In general, young chickens are more susceptible to coccidiosis and display the signs of the disease, while older chickens are more resistant (Lillehoj, 1998). Young animals that have recovered from coccidiosis are able to compensate for their lost growth; however, their overall growth might be severely compromised. The magnitude of clinical signs resulting from *Eimeria* infection is influenced to a great extent by the host genetic factors (Lillehoj *et al.,* 1989; Lillehoj *et al.,* 2004).

Infection with *Eimeria* induces protective immunity that last lifelong and is species-specific to that particular *Eimeria* species (Akhtar *et al.,* 2005). Large numbers of oocysts are required to generate a protective immune response against *Eimeria,* but not in case of *E. maxima* that is highly immunogenic, in which lower numbers of oocysts are needed to give almost full immunity. Moreover, the early endogenous stages of the *Eimeria* life cycle are more immunogenic than the later stages (Rose *et al.,* 1984). However, gamete antigens of *E. maxima* were confirmed to be immunogenic (Akhtar *et al.,* 2005).

Since the discovery of the sulfanilamide by Levine in 1939, curing coccidiosis in chickens, many anticoccidial drugs were introduced to the poultry industry all over the world as feed additives (Allen *et al.*, 1998; Peek and Landman, 2011). Currently, polyether ionophorous antibiotics are mainly used. These anti-coccidials are effective for avian coccidiosis but, their continuous use in feed and their misuse have resulted in the emergence of drug-resistant strains (Ruff & Danforth, 1996). Furthermore, there are increased concerns of consumers about drug residues in the poultry products (McDougald & Seibert, 1998). This triggered the need for alternative control of avian coccidiosis. Interactions between poultry coccidiosis and *Clostridium perfringens* that causes necrotic enteritis (NE) occurs and results in increasing health risk to poultry (Al-Sheikhly & Al-Saieg, 1980). NE is controlled by feed Antibiotic Growth Promoters AGPs such as bacitracin, avoparcin, avilamycin, and some ionophores like Narasin and Monensin. (Elwinger *et al.*, 1992; Vissiennonet *et al.*, 2000; Martel *et al.*, 2004). However, the role of AGPs in the emergence of antibiotic resistance in humans has been questioned and the European Union decided to ban AGPs. An important example of antibiotic resistance in humans is that observed in *Campylobacter jejuni* loss of susceptibility to Quinolones family (Ciprofloxacin) (Wieczorek *et al.*, 2013). Hence, the potential for interactions between Coccidia and Clostridia in the chicken host is becoming greater nowadays, due to this ban of AGPs, and stressing the need for investing in research for alternative products (Van Immerseel *et al.*, 2009).

Eimeria spp. induces immunity and vaccination programs, on a commercial scale, have proved to be of limited scope. Existing vaccines consist of live virulent or attenuated *Eimeria* strains with limited scope of protection against *Eimeria* due to antigenic variability among the different species (Peek and Landman, 2011). Thus the most common disease control remains largely dependent on routine use of anticoccidial

drugs (Allen & Fetterer, 2002; Williams, 2002a). In the past years, several different live vaccines composed of either virulent or attenuated strains were commercially introduced to the market. Major disadvantages of live vaccines are their high cost due to their *in vivo* production of oocyts, and the addition of multiple *Eimeria* strains in the vaccine (Dalloul & Lillehoj, 2005; 2006). Live vaccines provide limited alternative to prophylactic medication, and there is a need for a recombinant vaccine composed of parasite antigens or antigen-encoding genes (proteins or DNA), that should result in *Eimeria*-specific immunity. Several difficulties are encountered in identifying the protective antigens or genes that are responsible for eliciting protective immune response, and the use of an efficient delivery method to induce protective immune response in birds. The poultry industry is obliged to rely on prophylactic chemotherapy for *Eimeria* control until more efficient vaccines become commercially available (Dalloul & Lillehoj, 2006).

The introduction of alternative prevention and treatment measures, such as herbal immunopotentiators that effectively improve productivity and boost non-specific immunity of birds, maybe of help to limit the use of chemotherapy. In the meantime, the unavailability of efficient recombinant vaccines, and the increasing drug-resistant strains of *Eimeria*, along with growing public worry over the use of chemicals and their residues in poultry products, provide enough justification for the search to find alternative control methods.

CHAPTER II

LITERATURE REVIEW

A. Etiology, diagnosis and pathogenesis of *Eimeria* spp.

1. Apicomplexans characteristics

The coccidia is a spore-forming intracellular parasite belonging to the subkingdom Protozoa of the phylum Apicomplexa, and genus *Eimeria*. Apicomplexans express characteristic organelles at the anterior end of the parasite, the apical complex after which the phylum was named. The apical complex consists of secretory organelles namely the rhoptries and micronemes, as well as the conoid surrounded by polar rings. The apical organelles play a key role in attachment and invasion to host cells (Fig. 1).

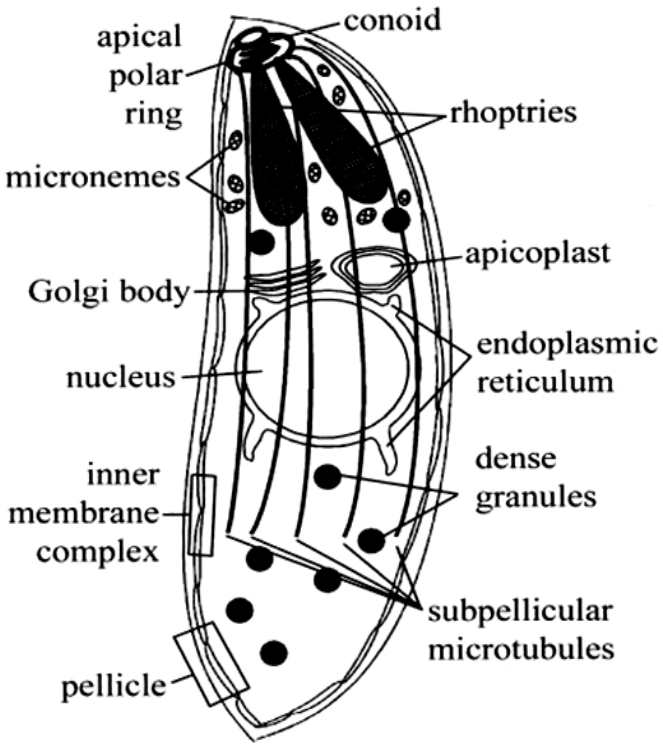


Fig. 1. The morphology of Apicomplexans.

Apicomplexans contains a group of organelles that are distinctive to the phylum.
Source: Morrissette and Sibley (2002).

Invasion of the parasite to host cells is an important stage of its life cycle. Extracellular parasites do not multiply outside the cells of the host, thus they need a

parasitophorous vacuole (PVM) inside host cells to grow and replicate, making them obligate intracellular parasites (Morrissette & Sibley, 2002). The PVM occurs by invagination of the host cell membrane after contact of the parasite with the host cell (Shirley, 1992). During contact there are some secretions from the micronemes, rhoptries and dense granules. Micronemes secrete their contents first and are involved in host attachment, motility and recognition followed by rhoptry protein secretions which aid in the movement of the parasite into the host and formation of PVM. Finally, the dense granules secrete their proteins inside PVM which aid in the remodeling of the PVM (Shirley, 1992; Bromley *et al.*, 2003).

The inner membrane complex (IMC) (Figure 1) lies directly under the parasite plasma membrane and is closely associated with it, producing a three-layered pellicle typical of the Apicomplexa. Most Apicomplexia are motile by actin- and myosin-based machine situated at the pellicle (Frénal *et al.*, 2010), and their motility is coupled with their invasion. Motility and invasion is accompanied by the discharge of soluble proteins (MICs) from the micronemes (Soldati *et al.*, 2001). MICs encode adhesive motifs (Tomley & Soldati, 2001), and are important for motility and attachment; compounds that interfere with their secretions blocks the parasite motility, invasion, and attachment (Wiersma *et al.*, 2004). TRAP (thrombospondin-related anonymous protein) possesses multi- adhesive domains, stored in the micronemes and is exposed at the sporozoite anterior tip when parasite comes in contact with host cells (Akhouri *et al.*, 2008). The TRAP family is a trans-membrane protein of the microneme involved in attachment to the host and gliding locomotion and is conserved among Apicomplexian (Kappe *et al.*, 1999; Tomley & Soldati, 2001). Another type of parasite proteins involved in invasion are GPI-linked surface antigens (glycosylphosphatidylinositol-anchored variant surface proteins) (SAGs) found on the surface of sporozoites and

merozoites (Tabares *et al.*, 2004; Jahn *et al.*, 2009). Obviously, the apical complex and invasion protein gives valuable source of antigens to regard as components of a recombinant vaccine to coccidiosis.

2. Coccidiosis

Coccidiosis is a major parasitic disease affecting poultry industry worldwide. Coccidiosis is referred to adequate number of coccidia to produce clinical signs while Coccidiasis is referred to mild infections without any clinical signs. Coccidiasis is more common than coccidiosis. All species of *Eimeria* invade the lining of the intestine. Seven species of *Eimeria* are considered valid these are *Eimeria acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. brunetti*, *E. mitis* and *E. praecox;* however, *Eimeria hagani* and *E. mivati* validity is under review (Long, 1973b; Shirley *et al.*, 1983; Barta *et al.*, 1997; Tsuji *et al.*, 1997; Vrba *et al.*, 2011). Simultaneous infection with two or more species of *Eimeria* is common, and each specie causes a separate and recognizable disease independent of the other species. Coccidia is transmitted to the host through fecal-oral route. Development of the parasite *Eimeria* includes both exogenous stages where oocysts sporulation takes place in the environment to become infective and endogenous phase of asexual and sexual stages of development and lysis of the host intestinal tissue. A generalized life cycle sketch of *Eimeria* spp. in chickens can be found in Conway &Mckenzie (2007).

3. A generalized life cycle

Fantham (1910) was the first to describe the life cycle of *Eimeria*, later Tyzzer (1929) and Tyzzer *et al.* (1932) described the life cycle stages of various *Eimeria* species. The life cycle was well documented more recently by many authors (Long &

Reid, 1982; Fernando, 1990; McDougald, 2003). Infection starts by ingestion of sporulated oocysts from the litter by susceptible birds. The sporulated oocyst contains four sporocysts each containing two sporozoites. The grinding action of the gizzard and trypsin activity (Britton *et al.,* 1964) leads to the release of the sporozoites. The liberated sporozoites search for specific region of the intestine depending on the species invading the epithelial cells. They penetrate the epithelial cells and changes to trophozoite. The trophozoites enlarge, and go through asexual multiplication known as schizogony (merogony), and are known as a schizonts or meronts. The schizonts rupture when mature, releasing the merozoites. The Merozoites enter other epithelial cells and repeat the process through the trophozoite and schizogonous stages. Minimum of two generations of asexual reproduction are needed to reach the sexual phase depending on the species. The sexual phase starts by development of male (microgametocytes) or female (macrogametocytes) gametocytes. The male gametocyte matures and releases a large number of biflagellate microgametes. The macrogametocyte grows to a macrogamete. Macrogamete is fertilized by a microgamete to form a zygot and a thickened wall is formed then. The zygot is the immature oocyst that ruptures the intestinal cells when matured and passes in the droppings (Conway & Mckenzie, 2007).

4. Pathogenicity and Gross lesions of Eimeria species

a. *Eimeria acervulina* (Tyzzer, 1929)

Eimeria acervulina infects the duodenal loop of the intestine; heavy infections may expand down the intestine. *E. acervulina* causes shortening of villi and reduction in the absorptive area of the intestine (Assis *et al.,* 2010), resulting in reduced broiler growth. Heavy infection (10^6oocysts) of *E. acervulina* causes marked reduction in growth rate. Suppression of weight again may appear 3-4 weeks after infection but is

most evident at one week after infection (Reid & Johnson, 1970). According to Reid & Johnson (1970), gross lesions scoring scale from 0 to 4 was used to determine the pathogenicity of *Eimeria* species. Score +1 and Score +2 (Figs. 2 & 3) show mild infection of *E. acervulina*. The mucosa is covered with white plaques resembling a leader form. Scraping of these white lesions when viewed under the microscope reveals unsporoulated oosysts and gametocytes. These mild lesions might cause a little loss of skin pigmentation and very little or no effect on weight gain and feed conversion. Score +3 and Score +4 (Figs. 4 & 5) show the intestine that looks pale and containing watery fluid attributable to mucous secretions causing diarrhea. The lesions show more coalescing causing thickening of the intestinal wall. Definitely weight gain and feed conversion efficiency are depressed, besides pigment loss in skin (Conway & Mckenzie, 2007).

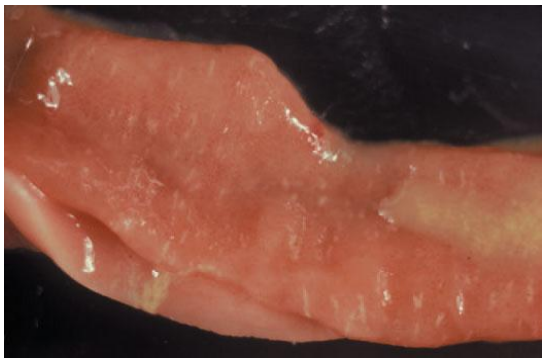


Fig. 2. *E. acervulina* Lesion Score +1
Conway & McKenzie (2007)

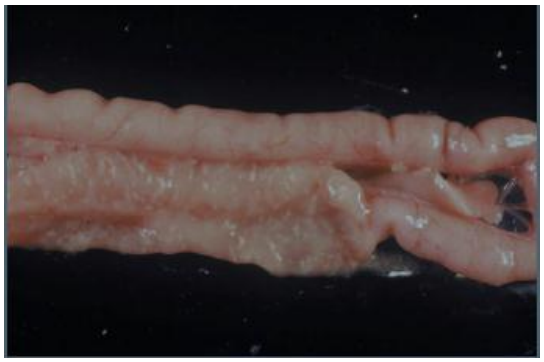


Fig. 3. *E. acervulina* Lesion Score +2
Conway & McKenzie (2007)

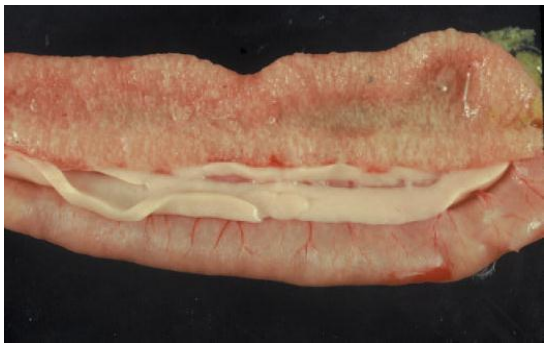


Fig. 4. *E. acervulina* Lesion Score +3
Conway & McKenzie (2007)

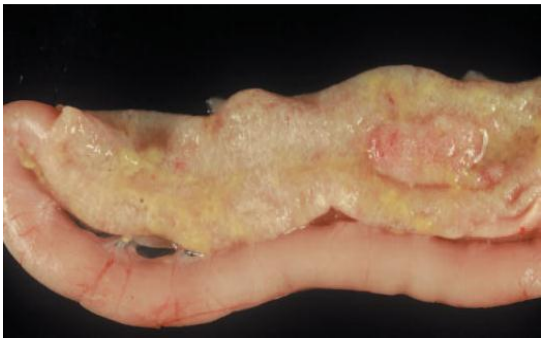


Fig. 5. *E. acervulina* Lesion Score +4
Conway & McKenzie (2007)

b. *Eimeria mivati* (Edgar and Siebold, 1964)

This specie was first recognized as a strain of *E. acervulina* or mixture of *E. acervulina* and *E. mitis* and later named as separate specie (Long1973b; Shirley *et al.,* 1983; Barta *et al.,* 1997; Vrba *et al*., 2011). *E. mivati* moves down the intestine more than *E. acervulina* as the infection progress. *E. mivati* endogenous stages are most numerous in the lower small intestine and proximal ceca (Norton & Joyner, 1980). In light infections lesions looks like *E. acervulina* but are more circular in shape. These lesions are colonies of gametocytes and oocysts, and may be seen from the serosal surface of the intestine. Score +1 and Score +2 (Figs. 6 & 7) may cause no or mild weight loss, and skin pigment loss. Score +3 and Score +4 (Fig 8 & 9) reveal more coalescing of lesions and the intestinal wall is thickened. Weight loss occurs at these lesion scores.

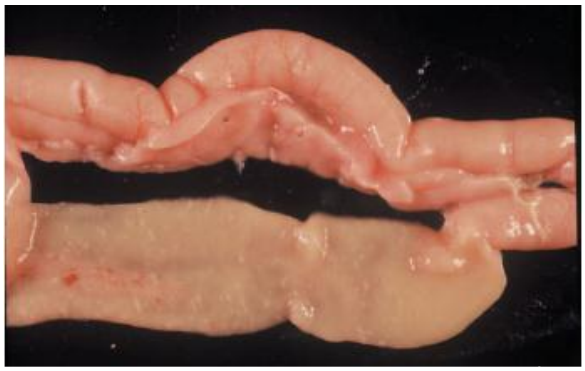


Fig. 6. *E. mivati* Lesion Score +1
Conway and McKenzie (2007)

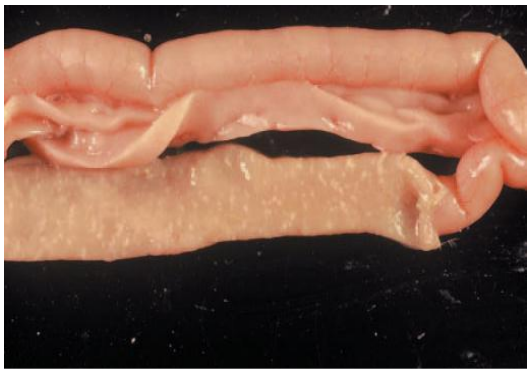


Fig. 7. *E. mivati* Lesion Score +2
Conway and McKenzie (2007)

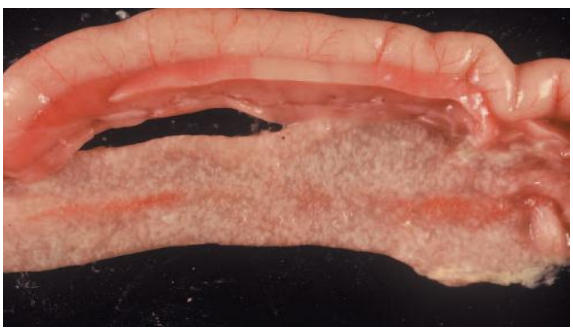


Fig. 8. *E. mivati* Lesion Score +3
Conway and McKenzie (2007)

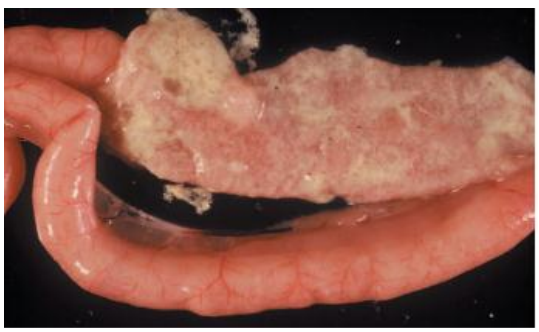


Fig. 9. *E. mivati* Lesion Score +4
Conway and McKenzie (2007)

c. *Eimeria praecox* (Johnson, 1930)

E. praecox often infect the duodenum (Fig. 10) and is overlooked sometimes in the field because no typical gross lesions exist. The basis of low pathogenicity may be due to short prepatent period (83 hours), but there may be watery or mucoid droppings, loss of pigmentation, loss of weight, and depressed feed conversion (Williams *et al.*, 2009). Pathogenicity of *E. praecox* and its effect on performance is debatable. Lowest administered dose ($5x10^3$oocysts per bird) had a significant impact on weight gain (Répérant *et al.*, 2012). Jenkins *et al.* (2008) did not notice any significant effect of *E. praecox* on weight gain with an infective dose of 10^4oocysts, although Répérant *et al.* (2012) observed a significant effect with a dose of 5×10^3. This conflict can be explained by variations in pathogenicity of the different strains of this specie (Répérant *et al.*, 2012).

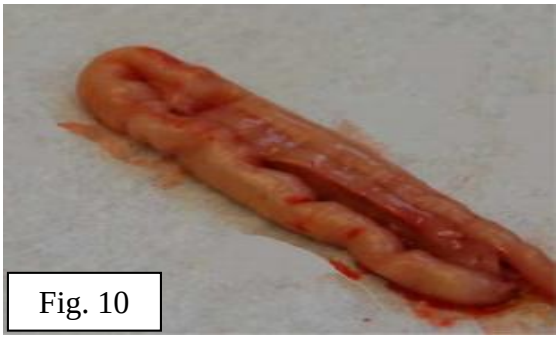


Fig. 10. Birds infected with 10^6 sporulated oocyts of *E. praecox* showing wrinkled duodenum Williams *et al.* (2009).

d. *Eimeria hagani* (Levine, 1938)

This specie is of low pathogenicity and infects the anterior part of the small intestine; it may produce mucosal inflammation and watery contents in the intestine.

e. *Eimeria necatrix* (Johnson, 1930)

E. necatrix is very well known specie by poultry producers because of the well-known lesions in the intestine. The lesions are found in mid intestinal area as that caused by *E. maxima*; however developing oocysts are found in the cecum. This feature

is valuable in the diagnosis of this specie. The oocysts that are located in the cecum are near in size to oocysts of *E. tenella.* This highly pathogenic specie typical signs include high morbidity, mortality, loss of skin pigmentation, and reduced growth that are associated with hemorrhagic enteritis. Layer pullets (7-20 weeks) infected with *E. necatrix* may suffer decreased flock uniformity, and decreased egg-laying capacity. *Eimeria tenella* and *E. necatrix* maximum damage occur during the asexual phase when large schizonts rupture. The range of $2x10^4$-$8x10^4$ oocysts of *E. necatrix* were enough to cause severe weight loss, morbidity, and mortality (Hein, 1971). Chickens that were inoculated orally with *C. perfringens* after *E. necatrix* inoculation, had significantly increased numbers of *C. perfringens* especially in the jejunum and ileum where the endogenous stages of *E. necatrix* take place (Baba *et al.*, 1997). Score +1 and Score +2 (Figs. 11 & 12) show petechiae and white plaques on the serosal surface - salt and pepper appearance - associated with ballooning and increased mucus secretion. Score +3 and Score +4 (Figs. 13 & 14) show more packed petechiae and white plaques on the serosal surface, intestinal mucosa is thickened and contents are tinged with blood and mucus while contents with *E. maxima* may be orange. Definitely weight loss and poor feed conversion occur, and birds do not eat or drink. Microscopic examination on day 4-5 post innoculation may show many aggregates of large schizonts (66µm) (Figs. 15 & 16), with hundreds of merozoites. These aggregates are found deep in the mucosa and submucosa causing damage to the layers of smooth muscle and blood vessels.

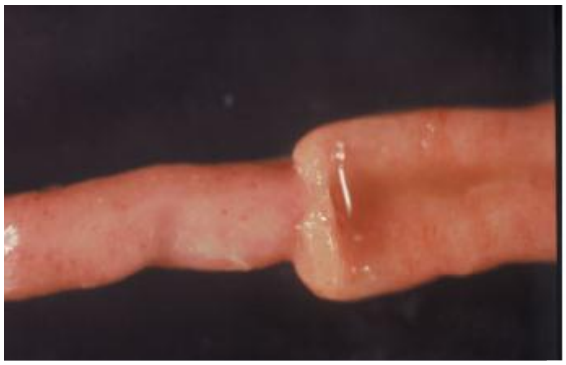


Fig. 11. *E. necatrix* Lesion Score +1
Conway and McKenzie (2007)

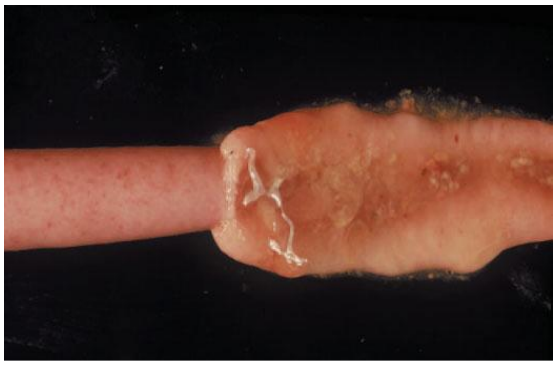


Fig. 12. *E. necatrix* Lesion Score +2
Conway and McKenzie (2007)

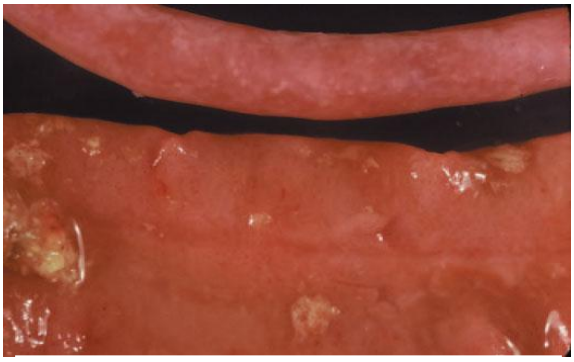


Fig. 13. *E. necatrix* Lesion Score +3
Conway and McKenzie (2007)

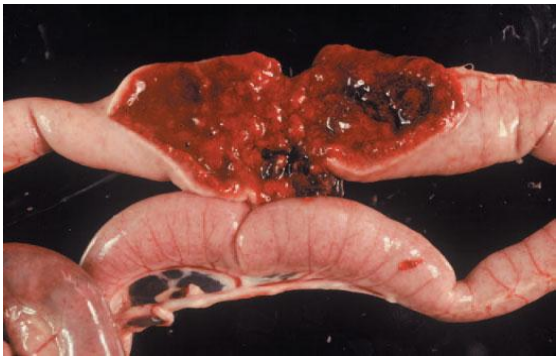


Fig. 14. *E. necatrix* Lesion Score +4
Conway and McKenzie (2007)

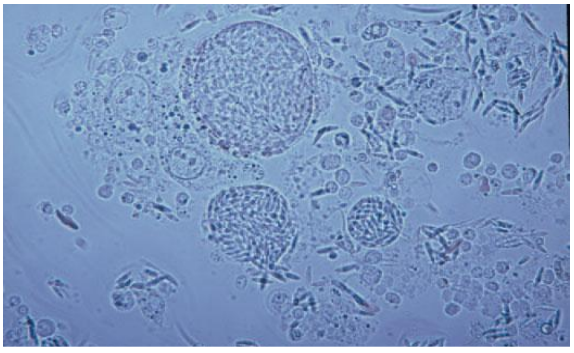


Fig. 15. *E. necatrix* schizonts (low power).
Conway and McKenzie (2007)

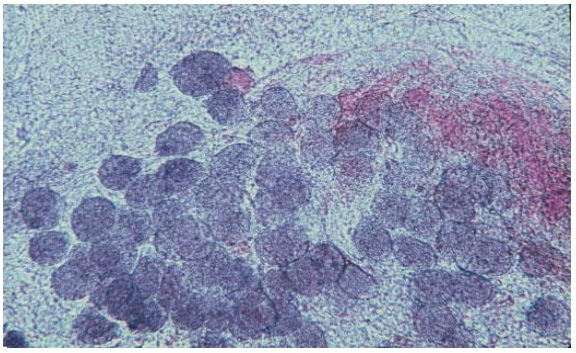


Fig. 16. *E. necatrix* schizonts (high power). Conway and McKenzie (2007)

f. *Eimeria maxima* (Tyzzer, 1929)

E. maxima as *Eimeria necatrix* is often found in the mid intestinal area. In heavy infections, the lesions may extend throughout the small intestine. *E. maxima* is easy to recognize because of the characteristic large oocysts (21-42 x 16-30µm), and was named for its large oocysts. This specie can be differentiated from *E. necatrix* by the absence of large schizonts. *Eimeria maxima* is a moderately pathogenic specie.

Infections with $5x10^4$-$20x10^5$oocysts cause morbidity, mortality, diarrhea, loss of skin pigment, anorexia and weight loss (Schnitzler & Shirley, 1999). Poultry producers concerned about good skin color are also concerned about subclinical infections of this specie that causes significant effect on skin color due to decreased absorption of xanthophyll and carotenoid pigments in the midgut. The majority of tissue damage occurs with sexual stages (oocysts) of *E. maxima.* Score +1 and Score +2 (Figs. 17 & 18) rated as mild infections, showing few petechiae on the serosal surface. Intestinal contents are slightly orange. These lesion scores are accompanied by some weight and pigment loss from the blood and skin. Score +3 and Score +4 (Figs. 19 & 20) show thickening of the intestinal wall and ballooning that may occur with moderate and severe infections.

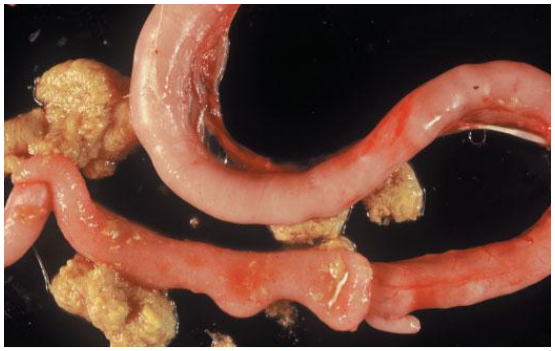


Fig. 17. *E. maxima* Lesion Score +1
Conway and McKenzie (2007)

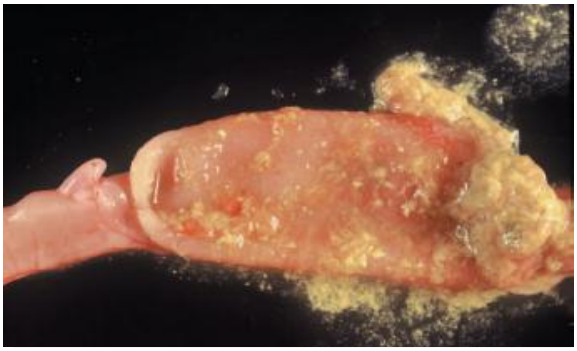


Fig. 18. *E. maxima* Lesion Score +2
Conway and McKenzie (2007)

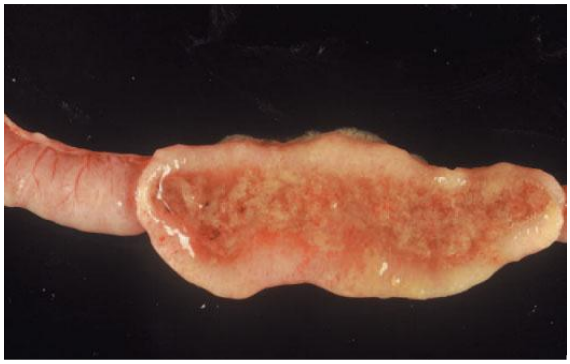


Fig. 19. *E. maxima* Lesion Score +3
Conway and McKenzie (2007)

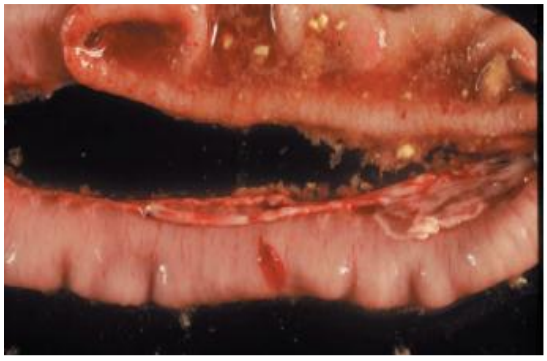


Fig. 20. *E. maxima* Lesion Score +4
Conway and McKenzie (2007)

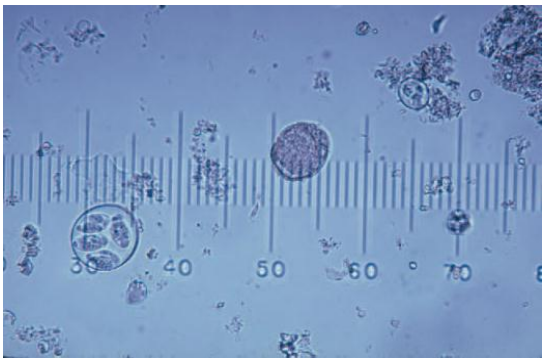


Fig. 21. The center of the figure shows large oocyst of *E. maxima* and its characteristic brownish red color. Sporulated oocyst of *E. maxima* to the left and greatly smaller sporulated oocyst characteristic of *E. mivati* or *E. mitis* are found to the right. Source: Conway and McKenzie (2007)

g. *Eimeria brunetti* (Levine, 1942)

This specie is found in the lower small intestine, and more to the large intestine and ceca in severe cases. Field infections are hard to recognize because of no typical lesion. *E. brunetti* is less pathogenic than *E. tenella* or *E. necatrix*. Two weeks old chicks inoculated with $8x10^5$oocysts of *E. brunetti* can show morbidity, loss of weight and mortality of about 30% (Hein, 1974). Score +1 and Score +2 (Figs. 22 & 23) show few petechiae on the serosal surface and roughened mucosal surface in the lower small intestine that might be detected by feeling it more than by sight. Score +3 (Fig. 24) shows hemorrhagic bands and coagulated materials that were sloughed off the mucosa and mixed with intestinal contents. Drying up of the cecal contents may occur on days 6 and 7 of *E. brunetti* infection. Weight loss and feed conversion efficiency is reduced at this stage. Score +4 (Fig. 25) shows the mucosa that is badly damaged, and the whole mucosal membrane is eroded due to heavy infection. A core (cottage cheese like) may form from this coagulated material, and may obstruct the intestine resulting in death. Score +4, is rare in the field. Mild and moderate infections are more common and are mostly overlooked.

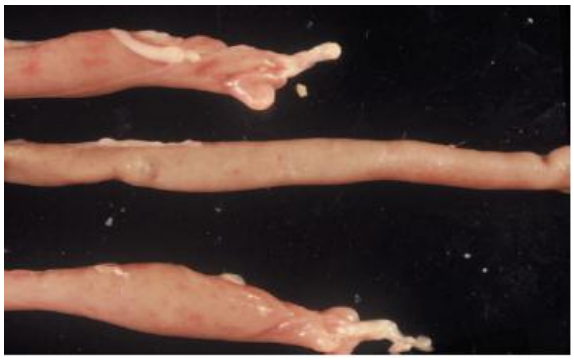


Fig. 22. *E. brunetti* Lesion Score +1
Conway and McKenzie (2007)

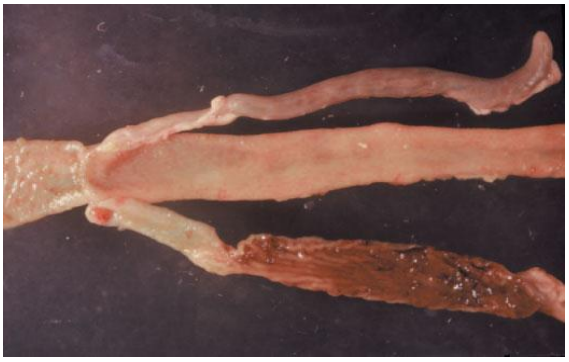


Fig. 23. *E. brunetti* Lesion Score +2
Conway and McKenzie (2007)

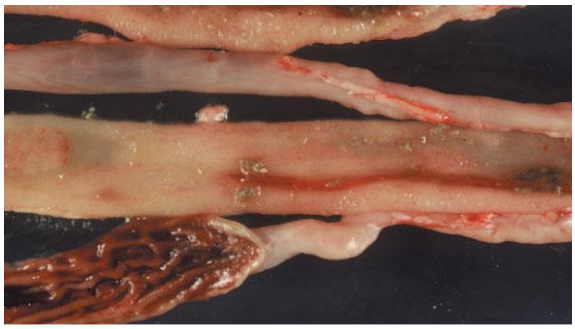


Fig. 24. *E. brunetti* Lesion Score +3
Conway and McKenzie (2007)

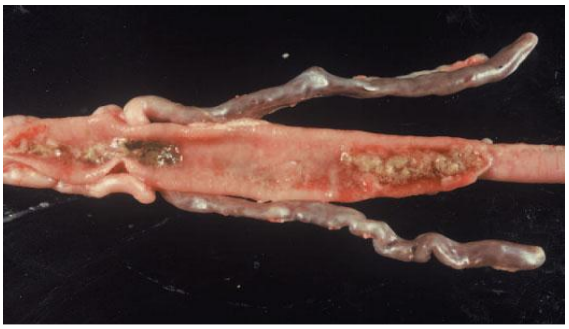


Fig. 25. *E. brunetti* Lesion Score +4
Conway and McKenzie (2007)

h. *Eimeria tenella* (Railliet & Lucet, 1891)

E.tenella is one of the famous species among poultry producers due to the high mortality and characteristic cecal lesions. *E. tenella* invades the epithelium of the ceca, and causes mortality, morbidity, loss of weight, loss of skin pigment, and bleeding. The cecal cores show clear blood that often forms firm bloody cores. Large schizonts and oocysts are often found in the cecal lesions. Inoculation with 10^4oocysts is enough to cause mortality, morbidity, and severe weight loss making it one of the most pathogenic species. *E. tenella* as *E. necatrix* produces large second generation schizonts at day 4 post infection that are the most pathogenic stage. Hematocrite value and erythrocytes count are reduced by 50 % due to blood loss. Score +1 (Fig. 26) shows few scattered petechiae that are seen on the opened and unopened cecum. Cecal contents (not shown) and cecal wall thickening are normal. Score +2 (Fig. 27) shows more petechiae on the

serosal surface of the ceca and more hemorrhage on the mucosal surface. Thickening of the mucosal surface is slight, and clinical signs at this stage are evident in infected chicks. Score +3 and Score +4 (Figs. 28 & 29) show more bleeding and clotting, this clot will harden joining the sloughed mucosa. Clinical signs include bloody droppings.

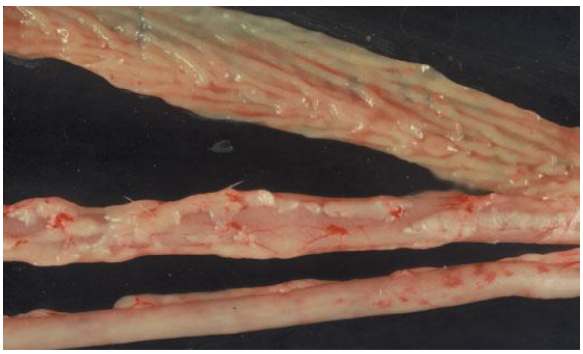


Fig. 26. *E. tenella* Lesion Score +1 , Conway and McKenzie (2007)

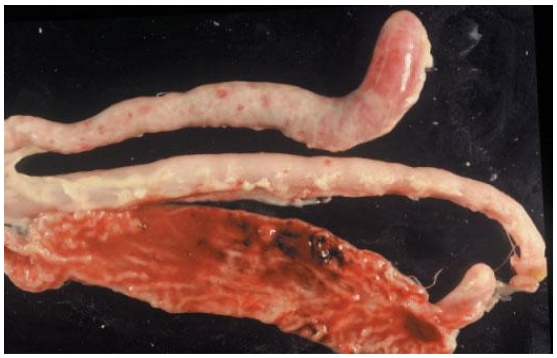


Fig. 27. *E. tenella* Lesion Score +2, Conway and McKenzie (2007)

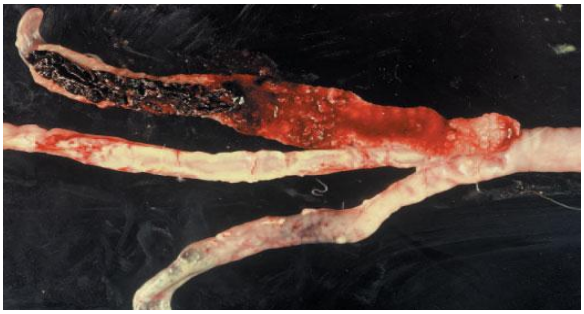


Fig. 28. *E. tenella* Lesion Score +3, Conway and McKenzie (2007)

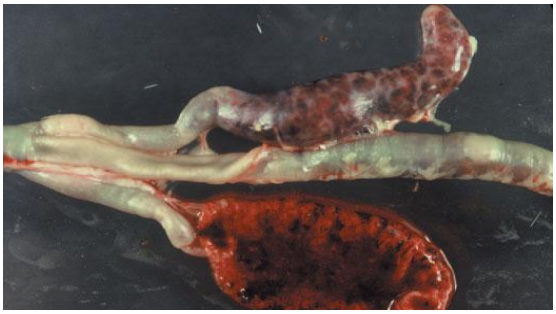


Fig. 29. *E. tenella* Lesion Score +4, Gangrene is seen in purple area. Conway & McKenzie (2007)

i. *Eimeria mitis* (Tyzzer, 1929)

Eimeria mitis is found in the lower small intestine, from the yolk sac diverticulum to the ileocecal junction. No typical lesions are found, but *E. mitis* has a negative impact on weight gain, feed conversion, morbidity, and pigment absorption was recently indicated (Fitz-Coy & Edgar, 1992). Average of $5x10^5$ -$1.5x10^6$ oocysts of *E. mitis* will cause weight loss, morbidity and loss of skin pigment. This specie is overlooked sometimes especially in mild infections due to indistinctive lesions. Table 1 summarizes *Eimeria* species, pathogenicity, oocysts morphology and size besides location in the intestine.

Table 1- Oocyst, habitat, morphology and pathogenicity of *Eimeria* spp. Levine (1985)

Species	Host Habitat	Oocysts size (µm) length	width	Shape	pathogenicity	Reference
E. acervulina	Small intestine	12-23	19-17	Ovoid	Low	Tyzzer (1929)
E. brunetti	Small intestine, rectum, ceca, cloaca	14-34	12-26	Ovoid	Moderate	Levine (1942)
E. hegani	Small intestine	16-21	14-19	Ovoid	Low	Levine (1938)
E. maxima	Small intestine	21-42	16-30	Ovoid	Low to moderate	Tyzzer (1929)
E. mitis	Small intestine	10-21	9-18	Subspheric	Low	Tyzzer (1929)
E. mivati	Small intestine, large intestine	11-20	12-17	Ellipsoid or ovoid	Low to moderate	Edgar & Siebold (1964)
E. necatrix	Small intestine, ceca	12-29	11-24	Ovoid	High	Johnson (1930)
E. praecox	Small intestine	20-25	16-20	Ovoid	No	Johnson (1930)
E. tenella	Ceca	14-31	9-25	Ovoid	High	Raillet & Lucet (1891)

5. *Parameters used in measurement of Pathogenicity*

Various parameters were used to measure the pathogenicity caused by coccidiosis. These parameters include mortality rate (Levine, 1942; Allen *et al.*, 1973); body weight gain (Hein, 1968a, b; Waletzky, 1970); feed conversion ratio (FCR) (Hein, 1976); blood carotenoids (Ruff *et al.*, 1974); changed plasma levels of electrolytes (sodium, potassium, chloride), declined plasma proteins and packed cell volume (Allen *et al.*, 1973); intestinal gross lesion scores (GLS) (Johnson & Reid, 1970) ; dropping score and estimation of dropping appearance deviation from normal (Morehouse & Barron, 1970); plasma NO2- + NO3- (Allen and Lillehoj, 1998); serum decoloration

that is measurement of serum coloration at 480 nm (Yvore *et al.*, 1993); and changes in serum alkaline phosphatase which is a sensitive marker of certain coccidiosis spp., and was directly proportional to weight gains of *E. acervulina*, *E. maxima* and *E. tenella* infected birds (Kogut & Powell, 1993).

GLS provides a measure of the parasite injury and associated gross lesions; and they do not reflect the degree of pathophysiological changes. High lesion scores attributable to *E. acervulina*, *E. maxima* and *E. tenella* in medicated birds maybe coupled with minimal changes in weight gain, whereas the same lesion score in non medicated birds may be coupled with severe changes in weight gain (Conway *et al.*, 1990). GLS alone is not very dependable method to evaluate flock performance to vaccine efficiency, since the majority of flock GLS (68%) that was vaccinated with attenuated lines showed few or no endogenous parasite in the gross lesions when challenged with virulent *Eimeria*. These gross lesions were due to host immune response and not the parasite reproduction. I.e. assessment of a vaccine should be associated with another parameter as weight gains (Williams, 2003). Lesion score is done using a scoring system on a scale from 0 to + 4 , Scoring is best made "blindly" by one individual with no awareness of the treatment used (Johnson & Reid, 1970). Lesion scoring requires a large number of birds to compensate for individual difference, replicates of cages or pens in treatments is recommended (Holdsworth *et al.*, 2004). Based upon severity of the lesions, a score of 0 (no lesions), 1 (mild lesions), 2 (moderate lesions), 3 (severe lesions) or 4 (extremely severe lesions or death due to coccidiosis). Identification of each spp. alone in each bird is impossible and not necessary in mixed infections. In mixed infections four regions are usually examined. The serosal surface is first examined then the intestine is cut to observe the mucosal

surface. Scoring is applied to upper intestine (U), the middle intestine (M), the lower intestine (L), and the ceca (C) (Conway and Mckenzie, 2007).

Oocysts needed to generate a certain lesion score was studied by McKenzie *et al.* (1989 a, b, c and d). The relationship between oocysts dose and lesion score, weight gain, feed conversion, and plasma constituents in infections with *E. acervulina*, *E. maxima*, and *E. tenella* shows that the Coefficients of determination (R^2) is highest for carotenoids, followed by lipids , weight gain, feed conversion ratio, protein and packed cell volume (Conway *et al.*, 1993). Table 2 gives a summary of the data obtained. Carotenoids look like the most sensitive to the inoculation dose of each species, and showed a decline at relatively low dose of inoculation.

Table 2. Effects of *E. acervulina*, *E. maxima*, and *E. tenella* on plasma constituents, chicken performance, and lesion scores. Source: Conway *et al.* (1993).

	Plasma Constituents Means					
oocysts per bird	carotenoids (μg/ml)	lipids (mg/100 ml)	proteins (mg/ml)	mean weight gain (g)	mean feed gain (g)	mean lesion score
E.acervulina:						
0	9.0^{a}	334.7^{a}	24.5^{a}	220.8^{a}	1.30^{ab}	0.03^{a}
10^2	8.4^{b}	303.2^{b}	23.4^{a}	223.8^{a}	1.29^{ab}	0.72^{b}
10^3	7.1^{c}	282.1^{b}	23.5^{a}	214.3^{a}	1.28^{a}	1.00^{b}
10^4	5.1^{d}	210.6^{c}	20.5^{b}	212.2^{a}	1.28^{a}	2.10^{c}
10^5	3.1^{e}	169.0^{d}	18.3^{c}	197.9^{b}	1.32^{b}	2.63^{d}
10^6	2.6^{e}	170.0^{d}	118.3^{c}	161.9^{c}	1.47^{c}	3.50^{e}
R^2	0.977	0.943	0.901	0.889	0.893	ND
E.maxima:						
0	10.4^{a}	343.6^{a}	21.0^{a}	282.6^{a}	1.30^{ab}	0.10^{a}
$6.7X10^1$	10.1^{a}	331.5^{a}	20.9^{a}	289.8^{a}	1.30^{ab}	0.33^{a}
$6.7X10^2$	8.2^{b}	284.2^{b}	20.0^{a}	290.7^{a}	1.28^{a}	0.89^{a}
$6.7X10^3$	3.5^{c}	204.9^{c}	18.5^{b}	262.5^{b}	1.34^{b}	1.50^{b}
$6.7X10^4$	2.4^{d}	204.0^{c}	18.6^{b}	215.0^{c}	1.52^{c}	1.62^{b}
R^2	0.986	0.927	0.647	0.895	0.9	ND

E.tenella						
0	9.7^{a}	336.5^{a}	27.2^{a}	284.9^{a}	1.28^{a}	0.00^{a}
10^2	9.6^{ab}	325.8^{a}	26.1^{b}	284.7^{a}	1.28^{a}	0.20^{ab}
10^3	9.0^{b}	287.6^{b}	25.0^{c}	279.4^{a}	1.34^{a}	1.62^{bc}
10^4	6.4^{c}	265.4^{c}	22.7^{d}	253.4^{b}	1.41^{b}	2.87^{cd}
10^5	4.6^{d}	211.7^{d}	21.4^{e}	202.4^{c}	1.61^{c}	3.28^{d}
R^2	0.965	0.965	0.919	0.908	0.908	ND

R^2 = coefficient of determination for each response variable; ND = not determined. For each species, means that do not share a common superscript element (^{abcde}) within a column are significantly different ($P \leq 0.05$). R^2 is most often seen as a number between 0 and 1.0, used to describe a regression line and prediction of future outcomes.

6. ***Oocyst counts technique***

Oocysts counting is one of the most extensively used parameters of Eimerian infection in chickens (Holdsworth *et al.,* 2004). An oocyst count is done using McMaster chamber method, this method is used in intestinal, litter and fecal oocyst counts. The % sporulation and oocysts dimensions are not essential for this method. Variations of this method have been described by Long & Rowell (1958); Hodgson (1970); Williams (1973, 1995); Long *et al.* (1976); Ministry of Agriculture, Fisheries and Food (1986); Eckert *et al.* (1995); Peek & Landman (2003); Haug *et al.* (2006) and Conway & Mckenzie (2007). Williams *et al.* (2001) developed a new method for counting eimerian oocysts at very low concentrations in aqueous suspensions. Methods used for counting eimerian oocysts either by isolation from intestine, litter or fecal samples (salt-flotation, McMaster chamber method) include a dilution step due to high number of oocysts needed to be counted. Multiplication factor to determine number of oocysts /ml may be no less than one thousand depending on the degree of dilution. This is undoubtedly unsatisfactory level of sensitivity if the sample contains as low as a few tens of oocysts/ ml.

7. *Diagnosis and identification of Eimeria species*

Identication of different *Eimeria* spp. is dependent on traditional (Conway and Mckenzie, 2007) and molecular methods as:

- zone parasitized in intestine
- gross lesions
- oocyst morphology
- minimum prepatent period determined experimentally
- schizont size and location
- minimum sporulation time
- parasite location in the host intestinal epithelium
- immunogenicity tests (cross-immunization tests)
- molecular techniques.

Due to overlapping of morphological characteristics of *Eimeria* spp, identification was recently improved by classification through computer examination using microscope digital images. Different species of *Eimeria* oosyts vary in size, contour, thickness and color of the oocyst wall (Castañón César *et al.*, 2007 & Fig. 30).

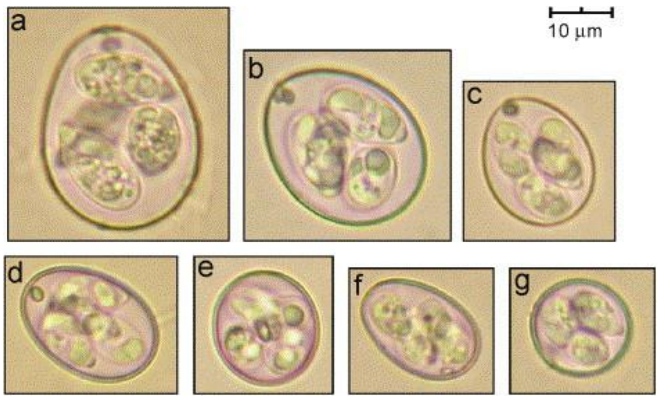


Fig. 30. Photomicrographs of oocysts of seven *Eimeria* species of chicken. *(a) E.maxima, (b) E. brunetti, (c) E. tenella, (d) E. necatrix, (e) E. praecox, (f) E. acervulina*, and (*g) E. mitis.* Source: Castañón, César *et al*. (2007).

Molecular tools for identification of *Eimeria* spp. is of importance. PCR procedure can provide a sensitive means for diagnosis based upon the internal transcribed spacer-1 (ITS-1) regions of ribosomal DNA (rDNA) that were sequenced and regions of distinctive sequences identified (Table 3 ; Schnitzler *et al.,* 1998; 1999). This test was used as a base for molecular diagnosis of *Eimeria* spp., and to study intra-strain variation of chicken *Eimeria* (Lew *et al.,* 2003; Su *et al.,* 2003). However due to the polymorphic nature of ITS-1 of *E. maxima* strains and occurrence of variability in amplification, species RAPD-derived markers, the SCAR (sequenced –characterized amplified region) which can stand for species-specific or strain-specific genetic DNA markers was found (Paran & Michelmore, 1993).

Table 3. DNA sequence, annealing temperatures of ITS1 primers and predicted size of amplification products derived from ITS1-PCR amplification of *Eimeria* spp. DNA. Source: Jenkins *et al*. (2006)

Eimeria species	Primer	PCR product sequence (5′–3′)	Annealing temperature (C)	Amplicon size
E. acervulina	EAF[A]	GGCTTGGATGATGTTTGCTG	60	321
	EAR[A]	CGAACGCAATAACACACGCT		
E. brunetti	EBF[A]	GATCAGTTTGAGCAAACCTTCG	45	310
	EBR[A]	TGGTCTTCCGTACGTCGGAT		
E. maxima	EMAF[B]	CGTTGTGAGAARACTGRAAGGG	51	144
	EMAR[B]	GCGGTTTCATCATCCATCATCG		
E. mitis	EMIF[C]	TATTTCCTGTCGTCGTCTCGC	54	306
	EMIR[C]	GTATGCAAGAGAGAATCGGGA		
E. necatrix	ENF[A]	TACATCCCAATCTTTGAATCG	44	285
	ENR[A]	GGCATACTAGCTTCGAGCAAC		
E. praecox	EPF[C]	CATCATCGGAATGGCTTTTTGA	54	368
	EPR[C]	AATAAATAGCGCAAAATTAAGCA		
E. tenella	ETF[A]	AATTTAGTCCATCGCAACCCT	60	271
	ETR[A]	CGAGCGCTCTGCATACGACA		

Denaturation: 1 cycle—95 C, 7 min; 35 cycles—95 C, 20 sec.
Annealing: 44–60 C, 30 sec; 72 C, 1 min.
Extension: 1 cycle—72 C, 5 min.
[A] *E. acervulina, E. brunetti, E. necatrix, E. tenella* ITS1 primer sequences as indicated by Schnitzler *et al.* (1998)
[B] *E. maxima* ITS1 primer sequence as indicated by Lew *et al.* (2003)

[C] *E. mitis, E. praecox* ITS1 primer sequences as indicated by Schnitzler *et al.* (1999)

8. *Host and site specificity of Eimeria*

Host specificity was first indicated by Johnson (1923) when *Eimeria* infection from chicken was not passed to turkeys. Cross-transmission studies of *Eimeria* was performed in many experiments including chicken and turkey (Levine & Ivens, 1988; McLoughlin, 1969), and showed cross-transmission of some *Eimeria* species (eg: successful transmit from turkeys to chickens). However, most species of *Eimeria* are considered homoxenous –complete their life cycle in one host- and stenoxenous –each specie parasitize single host- but this is not absolute (Fayer, 1980). Sporozoites from various *Eimeria* spp. are able to invade intestinal mucosa of foreign host therefore, excystation of sporozoites is not specific in most foreign hosts (Kogut & Long, 1984; Sundermann *et al.*, 1987), but are not able to complete their life cycle (Marquardt, 1981; Rose & Millard, 1985).

Factors which govern host specificity may be linked to host immune system and the parasite. Avian *Eimeria* with most species display a high level of site specificity invading narrow distinctive areas of the intestine in natural and foreign host eg: *E. tenella* invading the ceca of the turkeys successfully as it does in chicken, similarly with *Eimeria acervulina* invading the upper part of the intestine in foreign hosts (Augustine & Danforth, 1990). Host cells provide characteristics for sporozoites to identify and interact with them. Epithelial cells receptors for attachment and invasion are among these characteristics. 22, 31, and 37 kDa antigens, surface membrane glycoconjugates, which are possible receptors for the lectins found in parasite (sporozoites) (lectin-carbohydrate binding). Thrombospondins protein from microneme may also bind to glycoconjugates (Augustine, 2001). Evidence of the participation of

proteinases that overlay the gut lining in the invasion process and susceptibility of the parasite to proteinases inhibitors was reported (Coombs & Müller, 2002).

B. Control of Coccidiosis: Poultry house management and vaccination

1. *Poultry house management*

Eimeria has a large reproductive potential, so in practice it is very difficult to keep the environment around the chicken free of these organisms. Control of chicken coccidia in the Middle East is important since flocks are kept at a high stocking density in a warm environment favorable to the coccidial parasite. Furthermore, coccidial oocysts wall that protects the parasite from desiccation and chemical disinfectants will ensure long term survival of the parasite that can be easily disseminated throughout the poultry house through many ways as personnel, equipment, rodents, insects,… etc. Research workers emphasized the enhanced risk of coccidiosis due to management and environmental factors including contamination due to visitors, presence of feeders and drinking systems that are difficult to clean, and *Eimeria* species from the previous flocks (Belli *et al.,* 2006; Graat *et al.,* 1998).

Coccidial oocysts can be easily damaged by bacteria and ammonia found in the litter and after 3 weeks their viability will decrease (Williams, 1995). Many producers remove the litter, expose the house to fresh air (2-3 weeks), and add the new fresh litter before introducing a new flock. Other producers may apply a complete cleaning to the house and materials between flocks, and this husbandry practice is becoming more widespread with the decreasing efficacy of anti-coccidial drugs and the increasing use of live vaccines (Allen & Fetterer, 2002). Thus, biocontrol and biosecurity measures to reduce the number of infective oocysts within the house and to prevent its introduction to the farm are of importance. Environmental factors cannot be used to decrease the rate of sporulation since favorable environmental conditions of the parasite are also those of the chicken. The degree of sporulation and infective oocysts found in the litter will

determine the level of challenge and the course of the disease in chicken, thus influencing epidemiology of *Eimeria*.

Factors determining degree of sporulation are temperature, humidity and aeration (Kheysin, 1972). Ambient temperature of 25C and high relative humidity > 60% will favor the oocysts sporulation and survivability (Anderson *et al.*, 1976; Razmi, & Kalideri, 2000.). Chicken coccidia % sporulation is higher in dry litter and not wet litter probably due to build up of bacteria, ammonia and poor aeration in wet litter resulting in loss of viable oocysts (Waldenstedt *et al.,* 2001; Graat *et al.,* 1994; Williams, 1995). However, increasing litter humidity is not recommended for skin burns and footpad lesions causes. In addition, proper ventilation is recommended for better performance of the bird which favors also sporulation. Reducing bird density might be the proper management for decreasing oocysts accumulation in the litter and chances for clinical coccidiosis (Chapman *et al.*, 2002).

2. Vaccination

Immunity to coccidiosis can be an active or passive immunity response, and is defined as the resistance in the face of *Eimeria* challenge. The degree of resistance and reduction of pathogenic effects are observed in less gross lesions, oocyst output and increased performance of the birds. The first reported resistance to a challenge by a homologues strain of *E. tenella* was by Beach & Corl (1925). Twenty seven years after, the first commercial vaccine Coccivac® was registered in the U.S.A (Edgar & King, 1952). During the last 20 years various reports describing coccidial vaccines in poultry have been published (Shirley, 1988; Williams, 1992a; 1996; 1998; 1999b; Chapman, 2000; Williams, 2002a and b; Dalloul & Lillehoj, 2006; Shirley *et al.,* 2007).

a. Live vaccines

Live vaccines contain either several species of *Eimeria* or all the species. The success of this live vaccine depends on the administration of small number of oocysts of each component of the vaccine (each *Eimeria* species) in a way that insure uniform exposure of all birds .i.e. each bird or the majority of the birds have received the same number of oocysts at the same time. Protective immunity is achieved by single high dose or multiple low doses (trickle infections) of the vaccinal parasite (Joyner & Norton, 1973; 1976; Long *et al.,* 1986). It is of importance when using a drug-sensitive strain live vaccine to withdraw anti-coccidial drugs from the feed, otherwise vaccination will fail. Live vaccines are associated with increased sensitivity to anticoccidial drugs (Jeffers, 1976; Mathis & McDougald, 1989; Chapman, 1994, 1996; Newman & Danforth, 2000; Mathis & Broussard, 2006; Peek & Landman, 2006). Live vaccines can reduce resistance to an anticoccidial drug and virulence of a field strain through interbreeding of drug –sensitive attenuated strains in the vaccine with wild-type strains in the local population (Williams, 1998). For fifty years the use of live vaccine was limited. Their primarily use was for layers and breeders (Shirley *et al.,* 1995) however, their use in broiler flocks is increasing (Williams, 2002a). Live vaccines contain strains that are either attenuated (precocious strains) or nonattenuated (virulent, wild- type strains). Commercially available vaccines (Shirley *et al.,* 2005; Williams, 2002a; Conway & Mckenzie, 2007; Peek & Landman, 2011) are shown in Table 4.

Table 4- Overview of some commercially registered vaccines. Source: Peek & Landman (2011).

Vaccine (manufacturer)	*Eimeria* species[a]	Attenuation	Bird type	Administration route	First registration
ADVENT®[b] (Novus International)	*Eac*, *Emax*, *Eten*	Non-attenuated	Broilers	Hatchery spray, water or feed spray	2002 (USA)
CocciVac®-B (Schering Plough Animal Health)	*Eac*, *Emax*, *Emiv*, *Eten*	Non-attenuated	Broilers	Ocular, hatchery spray, water or feed spray	1952 (USA)
CocciVac®-D (Schering Plough Animal Health)	*Eac*, *Ebr*, *Eha*, *Emax*, *Emiv*, *Enec*, *Epra*, *Eten*	Non-attenuated	Breeders/layers	Ocular, hatchery spray, water or feed spray	1951 (USA)
CoxAbic® (Abic Biological Laboratories)		Killed antigen of *Emax* gametocytes	Breeders (to protect hatchlings)	Killed antigen, one species, intramuscular	2002 (Israel)
Eimerivac® Plus (Guangdong Academy of Agricultural Sciences)	*Eac*, *Emax*, *Eten*	Attenuated	Breeders/layers/broilers	Oral	Expected (China)
Eimeriavax® 4m (Bioproperties Pty)	*Eac*, *Emax*, *Enec*, *Eten*	Attenuated (precocious)	Breeders/layers/broilers	Eye-drop application	2003 (Australia)
Hipracox® Broilers (Laboratorios Hipra, SA)	*Eac*, *Emax*, *Emit*, *Epra*, *Eten*	Attenuated	Broilers	Drinking water	2007 (Spain)
Immucox®C_1 (Vetech Laboratories)	*Eac*, *Emax*, *Enec*, *Eten*	Non-attenuated	Broilers	Water or gel	1985 (Canada)
Immucox®C_2 (Vetech Laboratories)	*Eac*, *Ebr*, *Emax*, *Enec*, *Eten*	Non-attenuated	Breeders/layers	Water or gel	1985 (Canada)
Inmuner® Gel-Coc (Vacunas Inmuner)	*Eac*, *Ebr*, *Emax*, *Eten*	Attenuated	Breeders/layers/broilers	Oral	2005 (Argentina)
Inovocox® (Embrex Inc. and Pfizer)	*Eac*, *Emax* ×2, *Eten*	Non-attenuated	Broilers	*In ovo* injection with the Inovoject® system	2006 (USA)
Livacox® Q (BioPharm)	*Eac*, *Emax*, *Enec*, *Eten*	Attenuated (precocious, except *Eten* (embryo-adapted))	Breeders/layers	Hatchery spray, water or feed spray	1992 (Czech Republic)
Livacox® T (BioPharm)	*Eac*, *Emax*, *Eten*	Attenuated (precocious, except *Eten* (embryo-adapted))	Broilers	Hatchery spray, water or feed spray	1992 (Czech Republic)
Paracox®-8 (Schering Plough Animal Health)	*Eac*, *Ebr*, *Emax* ×2, *Emit*, *Enec*, *Epra*, *Eten*	Attenuated (precocious)	Breeders/layers	Water or feed spray	1989 (UK)
Paracox®-5 (Schering Plough Animal Health)	*Eac*, *Emax* ×2, *Emit*, *Eten*	Attenuated (precocious)	Broilers	Hatchery spray, water or feed spray	1989 (UK)
Supercox® (Qilu Animal Pharmaceutical Company)	*Eac*, *Emax*, *Eten*	Attenuated (precocious: *Eten*) non-attenuated (*Eac* and *Emax*)	Broilers	Oral	2005 (China)

Notes: [a]*Eac, E. acervulina; Ebr, E. brunetti; Eha, E. hagani; Emax, E. maxima* x2, two antigenically different strains of *E. maxima; Emit, E. mitis; Emiv, E. mivati; Enec, E. necatrix; Epra, E. praecox;* and *Eten, E tenella.*

Coccivac® and Immucox® are used for broiler breeders, and there was a dislike to use them in broilers because of reduced weight gain and feed conversion compared to medicated broilers with anticoccidial drugs. However, other studies had shown that Immucox® can provide equal or superior weight gain and performance when given in gel form at 1 day of age to chicks. Gel form will ensure concurrent exposure of all birds to small uniform number of oocysts (Danforth, 1998; Danforth *et al.*, 1997a, b).

i. Non-attenuated vaccines

- Coccivac® (Schering-Plough Animal Health; first registration in 1952 U.S.A): contains a mixture of live, wild-type strains of *Eimeria* species that are sensitive to anticoccidial drugs. Vaccination is through Ocular, hatchery spray, water or feed spray at an age of 1-14 days in a single dose.

  - Coccivac-D® (layers and breeders): contains a mixture of *E. acervulina, E. brunetti, E. hagani, E. maxima, E. mivati, E. necatrix, E. praecox,* and *E. tenella*.

  - Coccivac-B® (broilers): is a mixture of *E. acervulina, E. maxima, E. mivati*, and *E. tenella.*

- Immucox (Vetech Laboratories) : Immucox is a live vaccine composed of wild-type *Eimeria* species that are drug-sensitive. Immucox is administered by water or by an edible gum (gel) at the hatchery in a single dose at 1 to 4 days of age.

  - Immucox® for Chickens l (broilers and roasters): contains a mixture of *E. acervulina, E. maxima, E. necatrix,* and *E. tenella*.

  - Immucox® for Chickens 2 (breeders and egg layers): contains a mixture of the above species plus *E. brunetti*.

- Nobilis COX ATM® (Intervet); (broilers): this vaccine contains a mixture of wild-type of *Eimeria* species. These strains are ionophore tolerant or resistant strains of *E. acervulina, E. tenella,* and *E. maxima* (two antigenically different strains). These ionophore-tolerant strains permit the use of an ionophore anticoccidial drug in the feed during the first 3 to 4 weeks to allow the immunity to develop and to prevent outbreaks (Vermeulen *et al.,* 2001). Nobilis COX ATM® could be of importance if the ionophore is required to control *Clostridium perfringens*.

- Advent® (Novus International) (broilers): is a microbiologically sterile product used in hatchery spray cabinet or feed spray or in water. Advent differs from other live vaccines through an *in vitro* assessment of parasite viability (viacyst^{SM}) that stains non- viable sporocysts with ethidium bromide allowing better control of viable oocysts (Dibner *et al.,* 2003). It contains a mixture of *E. acervulina, E. maxima, and E. tenella*. These strains are sensitive to commonly used anti-coccidial drugs.

- Inovocox® (Embrex Inc. and Pyizer) (broilers): is a live oocyst vaccine containing a mixture of *E. acervulina, E. tenella,* and 2 strains of *E. maxima* administered *in ovo* on day 18 of incubated eggs by using an automated *in ovo* injection device (Inovoject System) (Weber and Evans, 2003; Weber *et al.,* 2004). The strains of coccidia used are sensitive to commonly used anticoccidial drugs.

ii. Attenuated vaccines

- Paracox®

  - Paracox-8® (Schering Plough Animal Health);

  (Breeders and layers): consists of precocious species of *E. acervulina, E. maxima* (2 strains), *E. mitis, E. tenella, E. brunetti, E. praecox* and *E.*

necatrix that have the characteristic for their short life cycle and reduced pathogenicity. Strains are drug sensitive. Paracox-8 is administered as single dose on 1-9 days of age through water orfeed spray.

- Paracox-5® (Broilers): contains species of *E. acervulina, E. maxima* (2 strains), *E. mitis,* and *E. tenella.* Paracox-5 is administered as single dose on 1-3 days of age through hatchery spray, water or feed spray.

- Livacox®

-Livacox Q® (Biopharma) (layers and breeders): contains a mixture of *E. acervulina, E. maxima, E.nectrix* and *E. tenella* (embryo-adapted).

-Livacox T® (broilers) contains a mixture of *E. acervulina E. maxima,* and *E. tenella* (embryo-adapted *E. tenella*). Livacox Q® and Livacox T® are administered through hatchery spray, water or feed spray in single dose at 1 to 10 days of age.

Since live vaccines contain more than one species their efficacy test differ from that of anticoccidials , a new protocol to asses efficacy of live vaccines based on growth rates after virulent challenge as the primary criterion , and feed conversion ratio as the secondary criterion and that avoids oocyst production and lesion score (Williams and Catchpole, 2000). The attenuated vaccine (Paracox®) may cause mild lesions in occasional birds but are not associated with clinical disease or poor performance. In assessment of a vaccine, weight gains and feed conversion ratio are the primary consideration that is commercially important.

Antigenicity of the *Eimeria* strains varies with the geographical location (Fitz-Coy, 1992; Martin *et al.,* 1997). Therefore a vaccine must be tested against local strains

before introducing it to flocks, and there is a risk of adding to the environment an unwanted *Eimeria* species. Immunovariant strains can be isolated and included in the vaccine to provide what is called a region-specific vaccine (Danforth, 1998).

The precocious lines are characterized by the depletion of the last one or two schizogonous stages-abbreviation of the life cycle and short prepatent period (e.g. McDougald and Jeffers, 1976; McDonald *et al.,* 1982, 1986b, 1986c; McDonald and Shirley, 1984; Shirley and Bellatti, 1984, 1988; Shirley *et al.,* 1984, 1986). This loss of schizogonous stages will have an effect on the number of gametes and oocysts produced during an infection (Shirley, 1989). In conclusion, the reproductive potential of the precocious line is reduced. The attenuation of virulence is also reduced in comparison to the parent strain (Shirley, 1989; Montes *et al*., 1998). The precocious line has few pathological effects on the host compared to an infection with the wild-type strain, even though this precocious strain is originating from a highly virulent species as *E. necatrix* (Shirley and Bellatti, 1984). In spite of the termination of the asexual phases of the parasite, the parasite immunogenicity will remain similar to the virulent parent strain (McDonald.*et al.*, 1986b; Shirley and Millard, 1986; Shirley *et al.,* 1986).

Adequate parasite reproduction in the host is important to the efficacy of live vaccines, this is especially true in attenuated vaccines, wild type strains tend to have a higher reproductive potential. Factors involved in the reproduction potential are summarized in Table.5, (Chapman *et al.*, 2002).

Table 5. Some factors that influence the reproduction of *Eimeria* under field conditions. Source: Chapman *et al*. (2002).

Factor	Effect	Oocyst accumulation trend
Bird oocyts burden in litter	Large number of initially present in the litter results in earlier and higher number of oocysts accumulating in litter	↗
Chick density	Higher densities increase the build-up of faeces and result in an increase in number of oocysts in the litter	↗
Litter condition	Higher ammonia and lower oxygen levels eventually reduce oocyst survival. Moisture content affects the sporulation of oocysts (see text)	↘
Management	Chick dispersion spreads faeces and increase litter oocyst levels	↗
Excystation	Increased Excystation rates increase the number of oocysts produced	↗
Crowding	High fecundity results in "crowding " that reduces the reproductive potential of the parasite	↘
Interactions	Interaction between species may result in a reduction of reproductive potential	↘
Immunogenicity	Higher parasite immunogenicity reduces oocysts yield more rapidly	↘
Susceptibility of birds	Greater innate susceptibility of birds to *Eimeria* increases oocysts yields	↗

Attenuated vaccines develop faster than the non-attenuated vaccines due to short life cycle of the attenuated lines, but their fecundity or reproductive potential is lower (Table 6). Groups of 15 chickens were challenged with the same dose of either the parent strain or the precocious strain. Using salt-flotation technique, the mean fecal or intestinal oocysts output was measured for the two groups, and the reproductive potential of the precocious strain challenged group was calculated as a percentage of their number of passed oocysts relevant to that challenged with parent strain (Shirley *et al.*, 1984, 1986, McDonald, 1986c; Shirley and Bedrnik, 1997; Shirley, 2005) (Table 6).

For example the vaccine Paracox® strains has 2-18 % less oocysts production than the parent strain (Chapman *et al.,* 2002).

Table 6. Reproduction of precocious lines in comparison to that of wild-type parent
Source: Shirley *et al.* (2005)

Species	Strain	Outputs of oocysts (millions)	Percentage output compared to relevant parent strain
E. acervulina	HP	1.1	7.7
	H (parent)	14.3	100
E. brunetti	HP	3.5	8.4
	H (parent)	41.8	100
E. maxima	CP	1.6	4.1
	C (parent)	39.3	100
E. maxima	MFP	1.9	8.8
	MF (parent)	21.6	100
E. mitis	HP	0.7	2.6
	H (parent)	27.0	100
E. necatrix	HP	2.1	24.7
	H (parent)	6.5	100
E. praecox	HP	5.2	8.5
	H (parent)	61.1	100
E. tenella	HP	28.2	27.9
	H (parent)	101.1	100

Note: Doses of oocysts given were 2×10^2 for all species except *E. brunetti* (1×10^2) and *E. tenella* (5×10^2). Codes 'H'; 'MF' and 'C' refer to parent, virulent strains and addition of 'P' suffix refers to precocious lines.

Crowding effect in non-attenuated lines due to limited epithelial cells and effect of the cell mediated immunity will result in reduced reproduction especially at high doses. Therefore, the less fecundity of the attenuated lines is better for reproduction and immunity development. Attenuated egg adapted lines of *E. tenella* where used in Livacox® and proved to be efficient (Shirley & Bedrnik, 1997). The life cycle of some *Eimeria* spp. can be completed within the chorioallantoic membrane of embryonating chicken eggs.

Competition between the wild–type and the attenuated vaccine may occur, when introducing an attenuated vaccine for the first time, and having wild-type strains in the flock-even after disinfecting the house and the materials- an outbreak may occur since the reproductive potential of the wild-type is higher than that of attenuated, and this will cause an outbreak before the immunity is established, but this is of rare occurrence (Chapman *et al*., 2002).

The marked immunological diversity found among *E. maxima* species raises concerns about the efficacy of these vaccines. Vaccinating against one strain of *E. maxima* may not protect against other *E. maxima* strain. A range of immunological relevance has been demonstrated experimentally with cross protection between strains ranging from 10-15% to 70 % based on oocyst output and lesion score (Long, 1974; Norton & Hein, 1976; Long & Millard, 1979; Martin *et al.*, 1997; Barta *et al.*, 1998; Smith et *al.*, 2002). Inclusion of two strains of *Eimeria maxima* representing extreme immunological diversity is needed (Shirley & Bellatti, 1988).

b. Subunit vaccines and recombinant vaccines

Subunit vaccines are proteins derived from the virulent *Eimeria* parasite. Identifying antigens specific to parasite life cycle inducing protective immunity is a necessary step in subunit vaccine development. Recombinant proteins of both parasite surface antigens and internal antigens were examined as candidates to vaccines (Jenkins, 1998; Vermeulen, 1998; Min *et al.,* 2004; Schaap *et al.,* 2004), including surface proteins of invasive stages, sporozoites, merozoites and gametocytes. These recombinant proteins induced good level of humoral and CMI immunity, but despite this no commercial product except CoxAbic® is marketed till today. CoxAbic® (maternal immunization transmission blocking immunity; breeders; Wallach, 1997) is an oil emulsion vaccine that induce maternally derived antibodies to protect broiler progeny (Michael, 2003, 2007; Finger & Michael, 2005; Ziomko *et al.,* 2005), and is the only vaccine found commercially that transmit passive immunity (antibodies are transferred through the egg yolk to the progeny) through administration of purified gametocyte antigens (230kDa, 82KDa, 56KDa) of *E. maxima* that are injected intramuscularly to the breeders (Pugatsch *et al.,* 1989; Wallach *et al*.,1990, 1995; Wallach, 1997). Immunization of the antigenic determinants to the breeders will protect the chicks from challenges of *E. acervulina, E. maxima,* and *E. tenella* with 60-80% less oocysts output than controls. Oocyst counts were determined every week following the challenge when samples of litter were taken from each pen and oocysts counting was performed (Wallach, 1997).

A major limiting factor in the development of the recombinant vaccine was that until now none of the antigens responsible for the potent protective immunity in natural infections has been isolated. Further studies on the parasite-host interaction on the cellular and molecular level need to be completed. *E.tenella* genome project may be

helpful in indentifying the protective antigens in the parasite and their interaction with host immune system (Shirley *et al.*, 2007).

c. DNA-based vaccines

These vaccines use genes encoding immunogenic proteins of the parasite, rather than the protein itself. These are administered in conjunction with promoters and enhancers. Vectors for DNA are used in order to have efficient expression of the antigenic proteins (Dalloul & Lillehoj, 2006). In conclusion, research is focusing on finding recombinant vaccines since live vaccines carries disadvantages, but also have advantages that can be summarized as follows:

3. ***Advantage and disadvantage of live vaccines***

a. Advantage

- Live vaccines give a practical and important alternative to the exclusive use of anticoccidial drugs for:
    - A number of studies indicated that vaccines give a comparable level of coccidiosis protection to the broilers as anticoccidial programs when given in gel form at 1 day to ensure synchronous exposure.
    - The use of live vaccines, (exception of Nobilis COX ATM®) leads to a replacement of the drug- resistant strains of coccidia in the broiler house with drug-susceptible coccidial strains carried over from the vaccine. This will result in increasing the efficacy of anticoccidial drugs i.e. is used effectively in rotation programs with vaccines.
- Live vaccines permit gradual buildup of solid immunity, and protection

against subsequent coccidial challenges with high safety and minimal tissue damage especially with the use of attenuated vaccines (Williams, 1994).

b. Disadvantage

- Loss of oocysts infectivity with time affecting their expiry date (Jeston *et al*, 2002).
- High production costs since oocyts are propagated in chickens.
- Antigenicity of the coccidial strains can vary with different geographical Areas, i.e. it is important to screen the local population of the coccidial parasite against the vaccinal one. Moreover, there is a risk of introducing unwanted *Eimeria* species into the environment.
- Live vaccines carry the risk of unintended infection under the immunosuppressive conditions.
- The way of vaccine administration may result in asynchronous exposure of all birds to small uniform numbers of oocysts, thus leaving susceptible birds (Not immunized) in the flock and increasing possibility of outbreaks, or insufficient immune response in case of low doses of the attenuated lines.
- Adding anti-coccidial drugs by error to feed of vaccinated flocks with sensitive strains.

C. Control of coccidiosis: Prophylactic coccidiostatic drugs and their mode of action

Coccidiosis has been a major cause of poor performance and loss of productivity in poultry industry. Early recommendations to control coccidiosis were established through improvements in management and hygiene. This management practice is still found till today but the intensive nature of the poultry industry ensures the continuous presence of coccidia. Attempts to eliminate the disease were ineffective.

The report about sulfonamide effect to control *Eimeria* infections led to research about properties of these compounds (Levine, 1939). At that point in time it was thought that sulfonamides were just used to treat sick birds. Later, a continuous in-feed low concentration of sulfaquinoxaline to control *Eimeria* was reported by Grumbles *et al.* (1948). These workers showed for the first time that it was achievable to control coccidiosis by the continuous inclusion of a low level of a drug in the feed of chickens (prophylaxis or prevention). In the same year sulfaquinoxaline was introduced

as a commercial product and the age of chemotherapy started. Horton Smith (1951) wrote that "we believe that continuous feeding of any drug, even at a low level, as a means of preventing disease is unwise because of our ignorance of the possible long-term effects on the bird itself". He pointed out that resistance to *Eimeria* may develop due to inclusion of low concentrations of the drug.

The use of suboptimal levels of the anticoccidial drugs may increase the probability of selecting drug-resistant strains (Chapman, 1984). Shortly after introduction of Sulphaquinoxaline and Nitrofurazone resistance had been reported (Cuckler & Malanga, 1955; Waletzky *et al.*, 1954). Since that time, many reports concerning anticoccidial drugs resistance were reported (Table 7), (Chapman, 1997). It is therefore possible that in medicated birds adequate parasite multiplication may occur and permit immunity development, a fact that permitted producers to increase the duration of the withdrawal period (less cost or less food residues), with a risk of susceptibility to coccidiosis and outbreaks (McDougald & Reid, 1971). From a practical point of view, it is important not to withdraw anticoccidial drugs ahead of time, since birds may not have developed solid immunity. Solid immunity in medicated birds did not develop until birds were 6 to 7 weeks of age (Chapman, 1999a, b).

Table 7. Reports of resistance of *Eimeria* field strains to anti-coccidial drugs. Chapman (1997).

Drug	Year introduced	Country	Resistance described	Year	Species[a]
Sulphaquinoxaline	1948	USA	Waletzky	1954	T
Nitrofurazone	1948	USA	Cuckler & Malanga	1955	Not given
Nicarbazin	1955	Britain	Hemsley	1964	T
Dinitolmide	1960	Britain	Hemsley	1964	T, N
Amprolium	1960	Britain	Hemsley	1964	B
Clopidol	1966	Britain	Williams	1969	A, M, T
Buquinolate	1967	USA	McManas	1968	Not given
Methyl benzoquate	1967	Britain	Millard	1970	T
Decoquinate	1967	Britain	Millard	1970	T
Monensin	1971	USA	Jeffers	1974	M
Robenidine	1972	USA	Jeffers	1974	M
Halofuginone	1975	France	Hamet	1986	A, T
Lasalocid	1976	USA	Weppelman *et al.*	1977	A
Arprinocid	1980	Britain	Chapman	1982	T
Salinomycin	1983	USA	Jeffers	1984	Various
Narasin	1988	USA	Weppelman *et al.*	1977	A, M, T
Maduramicin	1989	USA	McDougald *et al.*	1987	Various
Diclazuril	1990	Brazil	Kawazoe & Fabio	1994	A, M, T
Toltrazuril	1986	Netherlands	Vertommen & Peek	1993	Not given

[a]A: *Eimeria acervulina*; M: *E. maxima*; T: *E. tenella;* B: *E. brunetti;* N: *E. nectarix*

1. *Classification*

Anticoccidial drugs are classified into the following groups (a, b and c) mentioned below according to Chapman (1999 a, b); Allen & Fetterer (2002); Conway & Mckenzie (2007); Peek & Landman (2011).

a. Synthetic drugs (chemicals)

These are created by chemical synthesis, and have definite modes of action in face of parasite metabolism.

b. Polyether antibiotic (ionophores)

These are created by fermentation of Streptomyces species or Actinomadura species. Ionophores work throughout common mechanisms of shifting ion transport and

disturbing osmotoic balance. These drugs are today the basis of coccidiosis control (Jeffers, 1997). Ionophores are classified into the following groups:

i. Monovalent ionophores

- monensin, narasin and salinomycin

ii. Monovalent glycosidic ionophores

- maduramicin and semduramycin

iii. Divalent ionophores

- lasalocid

c. Mixed products

- Maxiban® (nicarbazine/narasin)
- Lerbek® (Meticlorpindol/methylbenzoquate)

Anticoccidial drugs used in feed for the prevention of coccidiosis in chickens are listed in Table 8, while the anticoccidial products used for therapeutic treatment are listed in Table 9.

Table 8. Contemporary anticoccidial drugs used for the prevention (prophylactic) of coccidiosis in chicken

Chemical name	Poultry category	Concentration in feed (ppm)
Chemicals		
Amprolium	Broiler, rearing	125–250
Amprolium + ethopabate	Broiler, rearing	125–250 + 4
Aprinocid	Broiler	60
Clopidol	Broiler, rearing	125
Decoquinate	Broiler	30
Diclazuril	Broiler, rearing	1
Dinitolmide (zoalene)	Broiler, rearing	125
Halofuginone	Broiler, rearing	3
Nequinate (methyl benzoquate)	Broiler, rearing	20
Nicarbazin	Broiler	125
Robenidine	Broiler	33
Polyether ionophores		
Lasalocid	Broiler	75–125
Maduramicin	Broiler	5–6
Monensin	Broiler, rearing	100–120
Narasin	Broiler	60–80
Narasin + nicarbazin	Broiler	54–90 (of both drugs)
Salinomycin	Broiler, rearing	44–66

Note: Products listed are those known to be used with some frequency in Europe, Latin America, Asia/Pacific region, and/or North America. Source: Conway & Mckenzie (2007); Peek & Landman (2011).

Table 9. Anticoccidial products and doses for therapeutic treatment of coccidiosis in chickens. Source: Conway & McKenzie (2007).

Chemical name	Application form	Used concentration	Treatment schedule
Amprolium	Feed	250 ppm	2 wks
	Water	0.006%	1-2 weeks
	Water	0.012-0.24%	3-5 days
Sulfadimetoxine	Water	0,05%	6 days
Sulfaguanidine	Feed	10,000-15,000 ppm	5-7 days
Sulfamethazine	Feed	4,000 ppm	3-5 days
	Water	0.1%	2 days
	Water	0.05%	4 days
Sulfaquinoxaline	Feed	1,000 ppm	2-3 days on,3 days off; then 500 ppm for 2 days on
	Feed	500 ppm	3 days on, 3 days off, 3 days on
	Water	0.04%	2-3 days on, 3 days plain water and then 2 days 0.025%
Sulfaquinoxaline + pyrimethamine	Water	0.005% + 0.0015%	2-3 days on, 3 off and 2 days on
Furazolidone	Feed	110 ppm	5-7 days on, 2 weeks 55 ppm on
Nitrofurazone	Feed	110 ppm	5 days
	Water	0.0082%	5 days
Toltrazuril	Water	0.0025%	2 days continuous
	Water	0.0075%	6-8 h/day for 2 days

2. *Mode of action*

The investigation for new drugs would be easier if the approach is based upon thorough knowledge of the biochemical pathways found in the parasite and how they vary from those of the host. This may lead to the finding of new enzymes that could be a goal for drug inhibition (Table 10). Important examples of pathways in *Eimeria*, and not in the host are folic acid and purine salvage pathways (*Eimeria* cannot make purines by itself, it has to have the ability to take exogenous purines with the aid of purine-

salvaging enzymes) (Wang, 1978; Wang & Simashkevich, 1981). Recently companies did not introduce new anticoccidial drugs to the market. However, two drugs might have this capacity, and those target enzymes of mannitol cycle (Allocco *et al.*, 1999, Smith *et al*., 1998) and trophozoite histone deacetylase (Smith *et al*., 1998).

Table 10 gives a brief view of the metabolic processes that are affected by the anticoccidial drug, their mode of action and speed of resistance.

Table 10. Metabolic process affected by anticoccidial drugs, mode of action and speed of resistance. Source: Chapman (1997)

Metabolic process	Drug	Mode of action	Resistance[a]
Membrane function	Ionophores	Cation transport	Slow
Cofactor synthesis	Amprolium	Thiamine uptake	Slow
	Sulphonamides + DHFR inhibitors	Folate synthesis	Slow
Mitochondrial function	Quinolones	Electron transport	Rapid
	Clopidol		Moderate
	Nicarbazin	?	Slow
	Robenidine	?	Moderate
Unknown	Halofuginone	?	Moderate
	Diclazuril	?	Moderate

[a]Resistance has eventually developed to all drugs that have been introduced.
?Mechanism unknown.

b. Products affecting cofactor synthesis

i. Amprolium

Amprolium was used extensively as a prophylactic drug, but with the introduction of ionophores its use has declined. Like the sulphonamides, amprolium is used as water treatment for clinical coccidiosis. Amprolium is a thiamine antagonist. A thiamine antagonist may be defined as a compound that can compete with thiamine or

thiamine derivatives in enzymatic reactions. Amprolium blocks thiamine absorption in coccidia at relatively low levels and have an antagonistic effect on Vitamin B1.

Thiamine is converted to thiamine pyrophosphate in the cell an important coenzyme in carbohydrate metabolism. However, Amprolium due to the lack of hydroxyl group that is found in thiamine it cannot be pyrophosphorylated, thus these reactions does not take place. Thiamine transport in the parasite is more sensitive to Amprolium than that of the host, making Amprolium efficacious against the parasite (Rogers, 1962). Amprolium competitively inhibits the uptake of thiamine by second generation schizonts of *E. tenella* (James, 1980). It is effective against cecal species as *E. tenella* and *E. necatrix* and to a lesser degree against *E. acervulina, E. maxima* and *E. brunetti* (McLoughlin and Gardiner, 1962). Amprolium does not affect immunity development (Karlsson and Reid, 1978). Since amprolium has poor spectrum of activity, it is used in mixtures with the folic acid antagonists as ethopabate and sulfaquinoxaline.

ii. Folate antagonists and inhibitors

According to Conway & Mckenzie (2007), this class includes:

- Ethopabate
- Sulfonamides

  -Sulfadimethoxine, Sulfaguanidine, Sulfamethazine, Sulfanilamide, and Sulfaquinoxaline
- 2, 4 Diaminopyrimidine (DAPs)

  - Diaveridine, pyrimethamine, and ormetoprim that are used in combination with sulfonamides for improved efficacy

Several products with anticoccidial activity act by blocking a biochemical pathway in the parasite through affecting an important cofactor in the pathway (Greif, 2001). Folic acid, or tetrahydrofolic acid, plays a important role in the synthesis of purines and thymidylate, and therefore is a vital cofactor for nucleic acid synthesis and for cellular replication (Bertino and Johns, 1967). Thus, interference with folate metabolism can affect cell growth.

- Ethopabate is a folate antagonist that inhibit folic acid pathway and blocks a step in the synthesis of Para-Amino Benzoic Acid (PABA) (Rogers *et al.*, 1964). Ethopabate affects 2nd generation schizonts and mostly active against *E. maxima* and *E. brunetti* (Reid, 1975). Ethopabate is used in conjunction with Amprolium to improve its efficacy and spectrum of activity.

- Sulphonamides also inhibit the folic acid pathway by inhibiting the enzyme dihydropteroate synthase (not present in the host) that is important in the synthesis of dihydrofolate, blocking the conjugation of pteridine and PABA. Sulphonamides affect 2nd generation schizonts (Reid, 1975). These products are effective against intestinal coccidiosis as *E. brunetti*, *E. maxima*, and *E. acervulina* and to lesser degree against cecal coccidiosis as *E. tenella and E. necatrix* (Ryley & Betts, 1973). An important concern about the use of these sulfonamide drugs is their high ability for toxic effects in chickens at doses close to or within the range of their recommended levels, especially at recommended dose for therapeutic treatment (Peckham, 1978; Julian, 1991). Care in estimating the dose for treatment is very important when given in feed or water. Signs of toxicity may include decreased egg production, loss of eggshell pigment, hemorrhagic syndrome, bone marrow depression, thrombocytopenia and depression of immune system.

- 2, 4 Diaminopyrimidine (DAPs): Another step in the folate pathway is the reduction of dihydrofolate to tetrahydrofolate (Active form of folic acid and important in the synthesis or purines and pyrimidines in coccidial parasite) by the enzyme dihydrofolate reductase (DHFR) and this enzyme is inhibited by DHFR inhibitors as pyrimethamine (DAPs). Pyrimethamine alone has little anticoccidial activity and is able to potentiate the action of sulphonamides and is used in mixture with sulphonamides having a clear synergistic effect (Kendall & Joyner, 1956). Similar to sulphonamides, DAPs affect 2nd generation of schizonts (Reid, 1973).

b. Products affecting mitochondrial function

i. 4-hydroxyquinolone group

Three products under 4-hydroxyquinolone group affect the mitochondrial function namely:

- Buquinolate
- Decoquinate
- Nequinate (methyl benzoquate).

These products show an anticoccidial activity at very low doses, and inhibit respiration of the coccidial parasite (*E. tenella*) by blocking the electron transport system in the mitochondria of this parasite and are shown to inhibit respiration of sporocysts and sporozoites of *E. tenella*, with 100 folds less activity against chicken liver mitochondria (Wang, 1975; Wang, 1976), however quinolones rapidly induce resistance (McManus *et al.*, 1968) that have limited their use in the field. Quinolones arrest sporozoite stage, thus affecting adequate immunity development (Reid, 1973; Yvoré, 1968). These Quinolone drugs are effective against *E. acervulina, E. brunetti, E.*

maxima, and *E. mivati* and to lesser extent *E. tenella* and *E. necatrix* (Ryley & Betts, 1973).

ii. Pyridone group

- Clopidol
- Meticlorpindol

The most important member of the group is Meticlorpindol that acts as quinolones by inhibiting electron transport system in the mitochondria, but at another level due to the fact that cross- resistance with quinolones does not exist. Synergism between Meticlorpindol and 4-hydroxyquinolone drugs was found (Challey & Jeffers, 1973). The known widely used product Lerbek ® (Meticlorpindol/methylbenzoquate) is an example of this Synergism. Similar to Quinolone this pyridone group affects early stages of the life cycle of all *Eimeria* (Reid, 1973; Ryley & Wilson, 1975), thus inhibiting immunity development (Bennejean *et al*., 1970).

iii. Nicarbazine (4, 4'-dinitrocarbanilide)

Studies by Ott *et al.* (1956) and Sherwood *et al*. (1956) indicated that Nicarbazine adversely affect eggshell pigmentation, egg production, and egg hatchability depending on the in-feed dose of nicarbazin. Low level of 50 ppm of nicarbazine given in layers feed resulted in extensive mottling of egg yolks (Polin *et al*., 1957). Studies by Beers *et al.* (1989) indicated that Nicarbazin (125ppm in broiler feed) increases body temperature in heat-stressed birds, which resulted in adverse effect on blood acid-base balance, blood lactate, and heart rate than in control-fed birds. The exact mode of action of Nacarbazine in not known , and is thought to be through

inhibition of succinate-linked nicotinamide adenine dinucleotide (NAD) reduction in mitochondria of beef hearts and the energy-dependent transhydrogenase and calcium accumulation in the rat liver mitochondria (Dougherty, 1974). Nicarbazine affects 2nd generation schizonts but earlier stages are also affected (McLoughlin & Wehr, 1960).

Nicarbazine is mostly effective against *E. tenella*, *E. necatrix* and *E. acervulina* and to a minor extent *E. maxima* and *E. brunetti* (Morrison *et al.,* 1961). Ott *et al.* (1956), and McLoughlin *et al.* (1957) indicated that Nicarbazin allowed immunity development, although Berg *et al.* (1956) indicated that there was no immunity development against *E. necatrix* with Nicarbazin.

iv. Robenidine hydrochloride

The exact mode of action is not known; however in mammals studies showed that Robenidine at high concentrations inhibit oxidative phosphorylation of mitochondria, assuming that it has the same function in the parasite (Wong *et al.,* 1972). Robenidine acts on 1st generation schizonts, allowing development of immunity (Ryley and Wilson, 1971; Karlsson and Reid, 1978). Robenidine is active against all species of *Eimeria* (Kantor *et al*., 1970). Robenidine sensitivity is best managed when used in rotation with other anticoccidial drugs (Chapman, 1989).

v. Toltrazuril

Toiltrazuril is possibly affecting mitochondrial function and belongs to the triazine class of compounds that have a high degree of activity against *Eimeria* species when given in feed or water at relatively low doses of treatment. The anticoccidial action of this class is cidal, not static (Chappel *et al.,* 1974; Ryley *et al.,* 1974; Haberkorn & Stoltefuss, 1987). Harder, & Haberkorn (1989) indicated that the activities

of some enzymes of the respiratory chain, such as succinate-cytochrome C reductase and NADH oxidase and succinate oxidase from mouse liver, were reduced in the presence of toltrazuril. Toltrazuril also showed an inhibitory effect on the dihydroorotate-cytochrome C reductase from mouse liver. Concluding that toltrazuril, affects the respiratory chain. Recently, Hackstein *et al.* (1995) indicated that Toltrazuril targets plastid-like organelles in the parasite. Toltrazuril affects all intracellular stages and is active against *Eimeria tenella, E. acervulina* and *E. maxima* (Mehlhorn *et al.*, 1984, 1988; V´azquez, and V´azquez, 1990; Mathis *et al.,* 2003). Toltrazuril does not affect immunity development (Greif & Haberkorn, 1997; Greif, 2000).

c. Products with effect on the cell membrane

Polyether ionophores: These drugs are not effective for therapeutic treatment in poultry and are able to form lipid-soluble complexes with mono- or divalent cations as Na+, K+, Ca++, and Mg++, and transporting them across cell membranes causing osmotic damage (Pressman, 1976; Smith and Strout, 1979). These later writers found that both narasin and lasalocid caused accumulation of considerable quantities of ionophores in extracellular sporozoite (prior to invasion) of *E. tenella* with no consequences on the host cell. Studies on monensin, salinomycin, and lasalocid showed that these ionophores altered Na-K pump in the cytoplasmic membrane (Smith and Rozengurt, 1978; Austic and Smith, 1980; Smith and Galloway, 1983). The entry of sodium into the parasite exceeded its ability to remove it, and resulted in death. These ionophores allow some degree of infection and immunity development depending upon the *Eimeria* spp, dosage of ionophore used, and the intensity of infection (Jeffers, 1989;

Chapman & Hacker, 1993; Chapman, 1999 a, b). Table 8 gives an overview of all ionophores.

d. Products with unknown mode of action

i. Diclazuril

Diclazuril belongs to the nucleoside analogue group and it is effective at 1 ppm in diet against *E. tenella, E. acervulina, E. necatrix, E. brunetti, E. maxima,* and *E. mitis* (Vanparijs *et al.,* 1989), however its exact mode of action in not known. Diclazuril affected both the asexual and sexual development of *Eimeria tenella,* (Verheyen *et al.,* 1988) resulting in complete degeneration of schizonts and gamonts. The establishment of normal pattern of oocyst wall was completely disturbed, resulting in thickened and incomplete oocyst wall (Verheyen *et al.,* 1989).

ii. Halofuginone

Halofuginone is of unknown mode of action. Christensen *et al.,* (1994) indicated that halofuginone hinders collagen synthesis, and reduced skin strength causing increased incidence of skin tears at slaughter time. Halofuginone was greatly effective against *E. tenella, E. maxima, E. acevulina, E. necatrix, E. brunetti,* or *E. mivati* at 3 ppm in broiler feed, and not effective against *E. acervulina* as other species. The drug was found to be cidal rather than static (Edgar and Flanagan, 1979).

3. *Antimicrobial and growth promoting effect of anticoccidial drugs impact on necrotic enteritis (NE).*

Enteric diseases are important to poultry industry due to decreased production and contamination of the poultry products. Necrotic enteritis caused by Gram-positive spore-forming anaerobe *Clostridium perfringens* was first described by Parish (1961). This disease is common in broilers with economical importance. Yearly worldwide economic loss caused by necrotic enteritis to poultry industry is estimated to be over $2 billion (Van der Sluis, 2000a, b). Antibiotics Growth promoters (AGPs) have been banned in European Union, a fact that resulted in higher incidence of necrotic enteritis (Van Immerseel *et al.,* 2009).

Necrotic enteritis is most common between 2-6 weeks in broilers, and characterized by sudden diarrhea and necrosis of the mucosa caused by the overgrowth of the bacteria in the intestine (Long, 1973a; Dahiya *et al.,* 2006). The disease is fatal and may cause sudden increase in mortality that can rise to 50% (Helmboldt & Bryant, 1971; Wijewanta & Senevirtna, 1971; Riddell & Kong, 1992). Chronic damage to the mucosa causes loss of production due to poor digestion and absorption (Elwinger *et al.,* 1992; Kaldhusdal *et al.,* 2001). In subclinical infection, colonization of the liver by large numbers of *C. perfringens* results in cholangiohepatitis. These livers are enlarged, pale and with white and red foci (Onderka *et al.,* 1990; Løvland & Kaldhusdal, 1999; Sasaki *et al.,* 2000), thus there will be increased condemnations at processing time due to these liver lesions.

C. perfringens is transmitted to humans through food chain, and is frequently isolated in food after *Campylobacter* and *Salmonella* (Buzby & Roberts, 1997). *C. perfringens* is able to produce different toxins and enzymes therefore, based on production of their toxins, *C. perfringens* strains are classified into five toxinotypes (A, B, C, D and E) (Songer, 1996; Petit *et al.,* 1999). *C. perfringens* type A, is the causative

agent of necrotic enteritis clinical and the subclinical form, and to lesser degree type C. Alpha-toxins produced by *C. perfringens* are the major virulence factor in the pathogenesis of necrotic enteritis (Songer & Meer, 1996; Engstrom *et al.*, 2003).

A very important predisposing factor for NE is the mucosal intestinal damage caused by *Eimeria* leading to release of plasma proteins that are growth substrates to *C.perfringens.* These substrates are more than 11 amino acids, various growth factors and vitamins (Boyd *et al.*, 1948; Petit *et al.*, 1999; Dahiya *et al.*, 2006). Feeding *C. perfringens* in feed without *Eimeria* infection did not cause mortality (Al-Sheikhly & Al-Saieg, 1980).

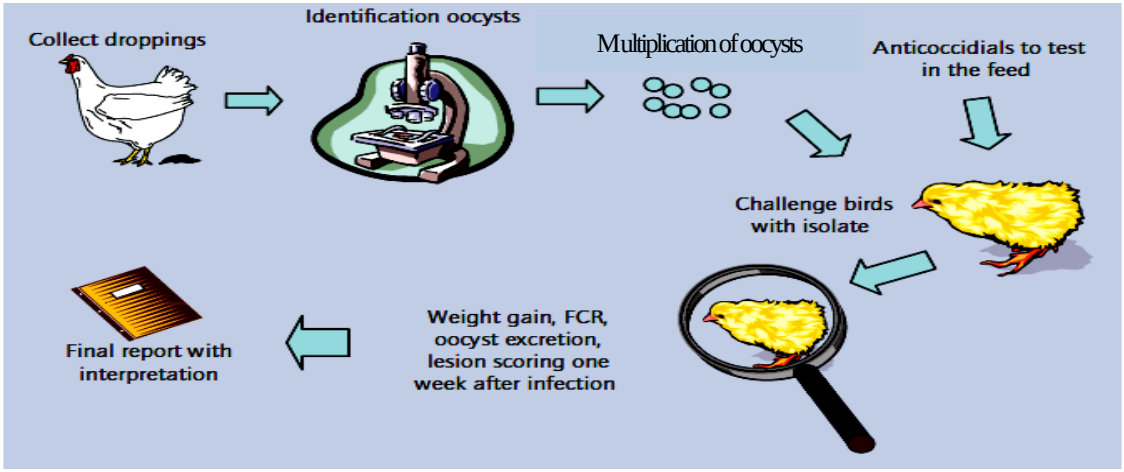
Besides acting against *Eimeria*, ionophores have antimicrobial and growth promoting function as inhibiting G+ bacteria and mycoplasma (Shumard & Callender, 1967; Dutta & Devriese, 1984; Stipkovits *et al.*, 1987). The ionophorous anticoccidial drugs Narasin and Monensin (Elwinger *et al.*, 1992; Vissiennonet *et al.*, 2000; Martel *et al.*, 2004) reduced occurrence of NE and inhibited *Clostridium perfringens* in birds. Supplementing feed with AGPs may not be needed if the ration is supplemented with an anticoccidial drug having antibacterial effects (Elwinger *et al.*, 1998). However, due to developed resistance to AGPs (Devriese *et al.*, 1993; Benno *et al.*, 1988) it is unwise to blindly rely on AGPs or ionophores to control necrotic enteritis.

4. *Preliminary Anticoccidial Sensitivity Tests (AST) for Lebanese poultry industry*

In vivo AST should be done in order to optimize the use of prophylactic medication. This AST is a scientific data on the *Eimeria* species isolated from the field and its drug-sensitivity profile. However the technique is costly and slow since chicken are needed for field strains oocyts multiplication and their subsequent inoculation in

tested chickens for the anticoccidial drug. This AST gives helpful information and it is essential in coccidiosis control (Holdsworth *et al*., 2004; Peek & Landman, 2011; U.S. Department of Health and Human Services, F.D.A., 2012). Figure 31 is a helpful sketch to understand the steps involved in performing an AST.

Fig. 31. The procedure for the Anti-coccidial Sensitivity Test (AST). De Gussem (2008).



A preliminary screening of broiler droppings samples from Jbeil, Koura, Batroun, and Kesrwan area farms were collected in 2011 from Lebanon. The total Samples were only five tested against 7 Anticoccidial drugs. These strains isolated from the Lebanese field are thought to be very pathogenic. UUC = Uninfected Untreated Control and IUC = Infected Untreated Control (Table 11). Maxiban and Lasalocid were recomended for these strains with 56% and 47% improvment in weight gains from IUC respectively.

Table 11. Anti-coccidial Sensitivity Test (AST) against commonly used coccidiostats in Lebanon; De Gussem (2011); *Alpharma Animal Health, Belgium*

Group	Dose ppm	FCR	Ranking	Anticoccidial Improvement	Ranking
UUC	-	1,55			
IUC	-	2,68			
Robenidine	36	2,27	7	9%	7
Maduramicin	6	2,25	6	31%	6
Decoquinate	20	2,17	4	33%	5
Monensin	125	2,09	3	35%	4
Decoquinate	40	2,18	5	37%	3
Lasalocid	125	2,06	1	47%	2
Maxiban	100	2,08	2	56%	1

UUC = Uninfected Untreated Control ; IUC = Infected Untreated Control
FCR: feed conversion ratio; ppm: parts/million or grams/ton

5. ***Advantages and disadvantages of anticoccidial drugs***

a. Advantages

- For 50 years anticoccidial drugs played a key role in the growth of poultry industy. Anticoccidial drugs can control *Eimeria* infections when used in shuttle and rotation programs to decrease the risk of anticoccidial drug resistance in the long term. Different mode of action of anticoccidial drugs should be used in rotation or shuttle programs due to cross-resistance between anticoccidial drugs (McDougald *et al.*, 1981; Peek and Landman, 2011).

- Might permit the development of a natural immunity in medicated birds depending on the anticoccidial drug and its inclusion level besides the level of coccidial challenge occurring (see text)

- May be required in vaccinated flocks if severe coccidial challenge is diagnosed before full immunity development (Table 9)

- Might control *Clostridium perfringens* (Narasin and Monensin) and decrease feed cost when Antibiotic Growth Promoters (AGPs) are not used (see text).

b. Disadvantages

- Increased cost of the feed
- Emergence of resistant strains to the drug after a short period of use (Table 7)
- Residues in meat, eggs and litter that might contaminate the environment eg: Organic arsenicals, Roxarsone® that is used with ionophores to improve efficacy and skin pigmentation .The buildup of arsenals as toxic residues in litter is a growing fear where broilers are raised and requires sometimes long withdrawal periods (McDougald *et al.,* 1981; Bednar *et al.,* 2003; Garbarino *et al.,* 2003; Rutherford *et al.,* 2003)
- Narrow therapeutic index of some drugs increasing toxicity concern (see sulfonamides in the text)
- Toxicity problems under heat stress (see Nicarbazine in the text)
- Ionophores are incompatible with some frequently used drugs like tiamulin, chloramphenicol, erythromycin, some sulfonamides, and some antioxidants (Umemura *et al.,* 1984; Prohaszka *et al*., 1987; Dowling, 1992; Von Wendt *et al*., 1997)
- Lapses in the anticoccidial drug program as a result of restricted feeding, a withdrawal period beyond seven days, low feed consumption due to build up ammonia in the litter , temperatures outside of the acceptable range, and low feed quality control or diseases, increasing the risk of coccidiosis outbreaks, i.e. must be cautiously evaluated
- Incorrect feed dosage mixing, non-uniform mixing and dust losses, leading to outbreaks
- Need for routine Anticoccidial Sensitivity Test (AST) to test for field isolates sensitivity against anticoccidial drugs (see text)

D. Alternative control of *Eimeria*: natural-products and feed additives

Anticoccidial drugs were used effectively over the past 50 years. Their use provided the rapid growth of poultry industry and the affordable poultry products to the

consumers. However, the increasing resistance to *Eimeria* species has stimulated the efforts to search for alternative controls. Before the availability of anticoccidial drugs, formulation of diets for reducing the impact of coccidiosis was used, suggesting that skim milk, butter milk or whey were effective when added to diets (Beach and Corl, 1925; Becker, 1937). More recent reports for diet modifications to control coccidial infections are summarized in the Table 12.

Table 12. View on dietary treatments having beneficial effects on chickens infected with *Eimeria*

Feed component	Mode of action	*Eimeria* spp./clinical effect	References
Maize	High concentration of Vit A and Vit E stimulating immunity, niacin and riboflavin in wheat enhances infection	Reduced mortality from *E. tenella*	Williams (1992b); Warren (1968)
Short term only feeding raw soybean supplement	Affected excystation of sprorozoites (trypsin inhibitor)	Reduced lesion scores from all *Eimeria* species tested	Mathis *et al.*(1995)
Low protein/low amino acids diets	reduced trypsin activity resulting in reduced excystation	Reduction in mortality and increased weight gain from *E. acervulina* and *E. tenella*	Britton *et al.* (1964); Willis and Baker (1981).
Semipurified diets with graded cellulose % vs. natural diets	Unidentified factor in natural diets promoting *E. tenella* lethal effect	Reduced mortality from *E. tenella*	Kolveit (1969)
Restricted feeding	lower trypsin production	Improved resistance to *E. tenella*	Zulkifli *et al.* (1993); Britton *et al*. (1964)
Coconut oil supplement (medium chain saturated fatty acids , MCT)	Improved fat digestibility during coccidiosis	Improved weight gains during *E. acervulina* infections	Adams *et al.* (1996); Babayan (1987)

Fish and flax oils (n-3) FA	Reduced parasite invasion and development by oxidative stress, and immune modulation	Improved weight gains and reduction in lesion score from *E. tenella*	Allen *et al.* (1996 1997a); Korver *et al.* (1997)
Vitamin A supplement	Stimulate intestinal health and immunity	reduced mortality and oocysts shedding from *E. tenella* and *E. acervulina*	Erasmus & Scott (1960); Dalloul *et al.* (2002); Lessard *et al.* (1997)
Vitamin E and selenium supplement (alpha-tocopherol)	Immune stimulation	Improved weight gains, reduced mortality from *E. tenella*	Colnago *et al.* (1984); Gore & Qureshi (1997).
Vitamin E (gamma-tocopherol)	Inactivation of reactive radicals	Improved weight gains and reduced lesion score from *E. maxima* and not *E. tenella*	Cooney *et al*. (1993); Christen.*et al.* (1997); Allen *et al.* (1998)
Vitamin K supplement	influence on blood clotting	Reduced mortality from haemorrhagic species (*E. tenella* and *E. necatrix*)	Harms *et al.* (1962); Davies &Joyner (1963); Ryley & Hardman (1978)
Vitamin C supplement	immune stimulation	Improved weight gains, but no reduction in lesion scores from *E. tenella* or *E. acervulina*	Webber & Frigg (1991)

1. *Artemisinin*

Artemisinin is a Chinese herbal extract from *Artemisia annua* (annual wormwood), and *A. sieberi.* Artemisinin has an antimalarial effect in humans; its mode of action is attributed to the fact that Artemisinin bears an endoperoxide grouping that act against the parasite by generating free oxygen radicals (oxidative stress) (Klayman, 1985; Levander *et al.,* 1989; Krungkrai and Yuthavong, 1987; Yang *et al.,* 1994; Meshnick *et al.,* 1989). Oh, *et al.* (1995) was the first to report that *A. annua* extracts have an anticoccidial activity against *E. tenella* as measured by improved weight gain, improved feed conversion and reduced lesion scores. Dried *Artemisia annua* leaves were tested as feed additives for their anticoccidial activity in poultry; they were fed to birds over a period of 3 weeks at a level of 5% dietary concentration (17ppm pure Artemisinin), and gave significant protection against lesions o *E.tenella* in poultry, when pure Artemisinin was fed for period of 4 weeks at 2, 8.5 and 17 ppm it was able to reduce significantly the oocysts output from single and dual spp. infection of *E. tenella* and *Eimeria acervulina* (Allen *et al.,* 1997b). Similar study by Arab *et al.* (2006) pointing out the protective activity of Artemisinin against *E. tenella* and *E. acervulina* and not *E. maxima.*

2. *Betaine*

The beneficial effect on performance of adding sugar beet (*Beta vulgaris*) to livestock feed was long known. One of the ingredients of sugar beet is betaine a bipolar electrolyte, choline analogue and methyl donor. Betaine protects cells against osmotic stress, stabilize cell membrane, and ensure normal metabolic activity in cells (Rudolph *et al.,* 1986, Ko *et al.,* 1994). Coccidiosis might cause diarrhea and dehaydration

resulting in osmotic stress and this stress is enhanced with ionophorous anticoccidials such as salinomycin. An osmoprotectant might therefore give benefits on performance parameters when used with ionophores. Augustine *et al.* (1997) indicated a positive effect on performance of birds infected with mixture of *E. acervulina, E. maxima* and *E. tenella,* measured by weight gain and feed efficacy and when using salinomycin (66ppm) and betaine (0.15%) in combination.

3. *Echinacea species (purple coneflower, American coneflower)*

All species of *Echinacea* are herbaceous, perennial flowering plants of the family *Asteraceae*, one of the largest families of flowering plants. *Echinacea* is considered one of the top 10 species of herbs sold for its wound healing and resistance boosting properties (Miller and Yu, 2004). *Echinacea* is native to central and southwestern America. *Echinacea* consists of nine spp. three of which- *Echinacea angustifolia*, *Echinacea pallida, and Echinacea purpurea*- have been used medicinally in the United State and Europe and are introduced to other regions due to the increased interest in alternative herbal medicine. Annual sales of *Echinacea* products were estimated to be 300 billion dollars in the USA alone, with hundreds of preparations including juices, dried leaves and flowers mostly used to treat cold without the need for physician prescription.

Echinacea is used in treatment of common cold and upper respiratory system infections in humans reducing the severity and duration of the infection (Barrett, 2003; Islam and Carter , 2005; Caruso and Gwaltney, 2005), and many other pharmacological activities as anti-inflammatory and wound healing properties in rats and mice (Speroni.

et al., 2002; Raso *et al.,* 2002). Furthermore, it has an enhancing immune response against tumor growth in mice (Currier and Miller, 2002).

Echinacea activates innate immune response through macrophage activation in mice and rats (Stimple *et al.*, 1984, Goel *et al.,* 2002), macrophage-derived cytokine and activation of polymorphonuclear leukocytes and natural killer cells in mice and humans (See *et al.,* 1997; Currier and Miller, 2001), thus acting as immunostimulant (Barrett, 2003, Percival, 2000). Phagocytic activity of alveolar macrophages in rats was specifically activated by alkamides component of *Echinacea pupurea* (Goel *et al.*, 2002). Polysaccharide constituent of the plant *Echinacea purpurea* were found to enhance phagocytes' activities and inhibit growth of *Candida albicans* and *Listeria monocytogenes* in mice (Roesler *et al.,* 1991). Moreover, certain standardized preparations extracts of *Echinacea purpurea* have a potent antiviral and antimicrobial activity in humans (Schoop *et al.,* 2006; Hudson, 2012).

The first attempt to establish a dose of 0.147% of *Echinacea pupurea* roots in feed of chicken broilers, inducing immunopotentiation in broilers and resulting in less immune suppression due to IBD (Infectious Bursal Disease), lower infectivity by *Salmonella* Enteritidis, was concluded by Barbour *et al.* (2000). Several studies suggested modulation of adaptive immune response with *Echinacea* treatment in mice (Zhai *et al.,* 2007). Freier Do *et al.* (2003) suggested enhancement of humoral immune responses and innate immune responses in mice with an extract of *E. purpurea*. Other studies in rats and mice (*in vivo*) showed an increased humoral response (Rehman *et al.,* 1999; Bodinet *et al.,* 2002) by increasing antigen-specific immunoglobulin production, but indirectly due to the use of *Echinacea* with other herbs. Morazzoni *et al.* (2005) reported that *E. angustifolia* extract stimulated T-cells function in murine by increased

production of IFN-gamma *in vitro.* However, results concerning its benefits are conflicting since most studies have used different preparations resulting in phytochemical variability (Islam and Carter, 2005; Percival, 2000).

Echinacea species are harvested for their roots, flower heads, seeds, or pressed as juice of the whole plant. The major pharmaceutical components of *Echinacea* are caffeic acid derivatives (cichoric acid and echinacoside), alkamides, polysaccharides, cinnamic acids, glycoproteins, flavonoids, essential oils and phenolic compounds (Bauer, 1996; Liu *et al.,* 2007; Miller and Yu , 2004). The activity is due to a combination of cichoric acid, alkylamides, and polysaccharides, which are present in all three species - *E. angustifolia, E. pallida, and E. purpurea* -, but in different amounts. A purified phytochemical does not give the same immunological activity as whole plant extract, and it depends on several active compounds (Randolph *et al.,* 2005). Additionally using a mixture of *Echinacea* plant extract resulted in greater effect than using one plant alone (Bodinet *et al.,* 2002).

Studies on *Echinacea* toxicity proved non-toxic at relatively high doses (Mengs *et al.,* 1991). Overall adverse effects are rare and mainly are gastrointestinal upsets and skin related problems including nausea, abdominal pain, diarrhea, itch, and rash (Huntley *et al*., 2005).

There are a few reports of *Echinacea* therapy, for horses (O'Neill *et al.,* 2002), swine (Stahl *et al.,* 1990) and cattle (Schuberth *et al.,* 2002), and three been reported on its use in poultry (Barbour *et al.,* 2000; Allen, 2003; Böhmer, 2009). Allen (2003) studied the effect of *Echinacea purpurea* (EP) (0.1% and 0.5% ground root preparations mixed with broiler starter feed and was supplemented for 2 weeks) on the development of immunity with live vaccine (half strength Immucox® at day 1)

following challenge with mixed infection of *E. acervulina, E. maxima, E. tenella* and *E. necatrix.* Weight gains before challenge were improved with EP treatment compared to live-vaccinated alone. EP persisted effect protected challenged broilers at age of 28 days against weight gain suppression and development of gross lesions. Allen, (2003) noted that EP alone without vaccination had little effect on the weight gain against challenge infection, suggesting that its action is dependent on concurrent antigen presence, and that its potential use as an adjuvant is concurrent with the application of live vaccine. Moreover it can be used with its immunostimulant benefits in the presence of coccidia in the litter. Böhmer (2009) reported increased laying hens lymphocytes with treatments of 5 consecutive days of *Echinacea* juice in feed, and elevated Newcastle titers with treatments of 2 consecutive days. Performance was not affected and phagocytic cells were suppressed in the two treatment. Phagocytosis of granulocytes was considerably decreased in hens and was thought to be due to high alkamid concentrations. Alkamids are known to increase phagocytosis, however low dose of *Echinacea* and alkamids could enhance phagocytosis. The author concluded that two days-trickle stimulation of *Echinacea* juice is adequate to increase the immune response of layers. Based on the available information of intermittent application of *Echinacea* juice, EP juices were orally supplemented for 3 consecutive days with 9 days free application , and was repeated 3 times during 35 days rearing period of Ross broilers. The results showed no negative effect on performance and health as well as improved performance and immunity with EP fermented juices as opposed to alcohol based EP juice. Moreover, EP juices can have a positive effect on cardiac muscles and fast growing broilers (Zahid 2009).

4. *Aloe vera*

Aloe vera has been cited in the literature for its curative and therapeutic properties, having more than 75 biologically active compounds. *Aloe vera* immunostimulatory effect in broilers infected with *Eimeria* species was reported by Akhtar *et al.* (2012). *Aloe vera* extracts enhanced humoral and cellular immune responses in chickens. *Aloe vera* stimulated macrophages phagocytic activity and their cytokines production, and induced antibody production. Furthermore, *Aloe vera* reduced oocysts output and lesion scores. The Akhtar study was in agreement with Yim *et al.* (2011) testing *Aloe vera* against *E. maxima,* where oocysts shedding and lesion scores were significantly reduced with the treated groups; however no data were presented in relation to production performance of chicken given the *Aloe vera.*

5. Azadirachta indica (neem)

This plant is commonly found in African and Asian countries, as is known for its therapeutic effect on various infectious diseases including coccidiosis. The neem fruit at a concentration of 150 g/50 kg feed was shown to have an anticoccidial activity against *E. tenella* measured by oocyst output and mortality in broilers (Tipu *et al.,* 2002). Neem leaves water extract have shown to have an anticoccidial activity against *E. tenella* comparable with baycox (Toulah *et al.,* 2010) and against mixed infection comparable with amprolium, enhancing the cellular immune response, and increasing antibody production (Biu *et al.,* 2006; National Research Council, 1992). Further studies on the mechanism of action and dose adverse effect in birds - due to its bitterness - affecting feed intake, should be carried on.

6. *Camellia sinensis*

Camellia sinensis is the specie of plants whose leaves and leaf buds are used to produce the popular beverage tea, and are a rich source of flavonoides with antioxidant properties and anticoccidial effect (Jang *et al.*, 2007; Chen *et al.*, 2008; Quan *et al.*, 2011). *C. sinensis* contains flavonoids, alkaloids, carotenoids, minerals, amino acids (especially L-theanine), carbohydrates, lipids, and volatiles/aroma compounds (Engelhardt, 2010). The effects of green tea (ground leaves 0.5% and 2.0% in diets) on *E. maxima* infection was measured by oocysts output and weight gains. The green tea-based diet significantly reduced oocysts output but didn't improve body weights loss induced by the parasite (Jang *et al.*, 2007). The author concluded that further studies on other parasite spp. must be investigated.

7. *Curcuma longa Linn*

Curcuma longa Linn is a rhizomatous herbaceous perennial plant of the ginger family, widely used and cultivated in the tropical regions. The rhizomes are boiled for several hours and then dried in hot ovens, after which they are ground into a deep orange-yellow powder known as Turmeric (diferuloylmethane), that it is best known as one of the ingredients used to make curry and gives mustard its bright yellow color. Turmeric medical phenolic compound and active ingredient is curcumin that has an antioxidant, anti-inflammatory and anti-tumour activity (Mukhopadhyay et *al.,* 1982; Conney *et al.,* 1991; Ammon *et al.,* 1993; Brouet, and Ohshima, 1995). In developing countries as Pakistan Turmeric is used as a feed additive for broiler chicken (Abbas *et al.,* 2010). Effect of 1% tumeric dietary supplementation on chickens infected with *E. maxima* or *E. tenella* was tested by Allen *et al.* (1998). Improved body weight and

reduced lesion scores was shown with *E. maxima* infected birds but not *E. tenella*. Significant reduction in the plasma NO_2^- and NO_3^- was found in *E. maxima* and curcumin treated birds, which explains the activity of curcumin against this specie. Curcumin inhibits induction of NO synthase (iNOS) by activated macrophages (Brouet & Ohshima, 1995).

8. *Origanum vulgare*

Oregano or *Origanum* are the dried leaves and the flowering tops of any plant of the various perennial herbs belonging to the mint family (lamiaceae), thus the word oregano refers to specific aroma and flavor rather than to a specific plant species (McGraw-Hill, 2006). Origanum species are known for their antiprotozoal (Milhau *et al.,* 1997), antibacterial, antifungal (Shahidi Bonjar *et al.*, 2000; Hammer *et al*., 1999), and antioxidant activity (Alma *et al*., 2003). Essential oil of oregano is obtained by steam-distillation of *Origanum vulgare* species. Major constituents of *Origanum vulgare* are carvacrol and thymol that constitute about 78 – 82% of the total oil (Adam *et al.,* 1998). Oregano essential oil at a level of 300 mg/kg (Giannenas *et al.,* 2003) was shown to have an anticoccidial activity against *E. tenella*, although lower than that of lasalocid at 75 mg/kg. Other studies indicated that the combination of vaccinaction and *Origanum vulgare* is useful in coccidiosis control and that caecal numbers of *Clostridium perfringens* were decreased (Waldenstedt, 2003). Thus, it is recommended to vaccinate against coccidiosis in combination with *Origanum vulgare* as an alternative coccidiosis control in organic production.

9. *Saccharum officinarum (sugarcane extract, SCE)*

One of natural byproducts of sugar cane industry was reported to be an immunostimulant enhancing immune response, and growth in chicken. El-Abasy reported enhanced growth and lower oocyst counts in the cecum upon oral administration of SCE inoculated into the crop (500 mg/kg/day for 3 consecutive days) and challenging with *E. tenella* (El-Abasy *et al.,* 2002, 2003a, and b). *In vivo* studies on the effects of oral administration of a polyphenol-rich fraction (PRF) of SCE or SCE (Hikosaka *et al.,* 2007) showed an immunostimulating effect of PRF of SCE. Recently sugar cane extracts significantly enhanced resistance against coccidiosis by enhancing humoral response and resulted in higher weight gain, reduced oocyst output, and lesion scores (Awais *et al.,* 2011).

10. *Mushrooms and their extract*

Mushroom extracts have been increasingly sold as dietary supplements for their enhancement of the immune system, and ability to promote health (Garcia *et al.,* 1998). Mushroom extracts have anti-tumor properties, however their use carries the possibility that there are toxic levels of metals, including arsenic, lead, cadmium, mercury ,and the presence of radioactive contamination with ^{137}Cs (Borchers *et al.,* 2004). Guo *et al.* (2004) have investigated the positive effects of polysaccharide extracts from two mushrooms, *Lentinus edodes* and *Tremella fuciformis*, and a herb, *Astragalus membranaceus*, on cellular and humoral immunity of chicken infected with *Eimeria tenella*. Guo *et al.* (2005) further investigated the effect of these same polysaccharide extracts supplemented for 1 wk (from 7 to 14 days of age) at a level of 1 g/kg of diet, in conjunction with live- attenuated vaccine, and the results gave better growth during

immunization compared to immunized and not treated with the extract. However, when challenged with *E. tenella*, treated birds with the extract showed similar lesion score but lower oocysts output than the vaccinated only birds. Dalloul *et al.* (2006) reported a novel immunopotentiating effect of a lectin extracted from the mushroom *Fomitella fraxinea*, and injected in 18 day old embryos. The extracted lectin protected broilers inoculated with *E. acervulina* at one week post hatch from weight loss and was associated with significant oocysts ouput reduction compared to non-injected embryos. Dalloul suggested that lectins act as immunopotentiators to innate immune response, and could be used as an alternative control to coccidiosis.

Other botanicals reported to have an anticoccidial activity are summarized in Table 13.

Table 13. Overview of botanicals used in treatment of coccidiosis. Source: Abbas *et al.* (2012)

Botanical name	Active ingredient	Dose	Mode of action	spp. studied	Affected parameters	Reference
Agele marmelos (Bael fruit, Stone apple)	Unknown	Orally at 2 ml b.i.d. for 5 consecutive days	Unknown	Mixed infection	OC↓, FC↑ WG↑	Khan *et al.* (2008)
Ageratum Conyzoides L. (Billygoat weed)	Flavonoids	Orally 500-1000 mg /kg b.w.	Oxidative stress	E.t	OC↓, WG↑ PCV↑, RBC↑	Nweze and Obiwulu (2009)
Carica papaya (Papaw)	Papaine enzyme, Carotene	Dry leaf powder 15% of feed	sporozoites digestion in ceca	E.t	OC↓	Al-Fifi (2007)
Cyamopsis tetragonoloba (Guar beans Meal)	Saponins	5% of feed	Binding with sterol molecule present on protozoal cell membrane	E.t	OC↓, BD↓	Hassan *et al.* (2008)

Eclipta alba (Bhringaraj, False Daisy)	Coumestans	120 ppm in feed	surface activate innate defense mechanism	E.t	OC↓, FC↑ WG↑	Michels *et al.* (2011)
Musa Paradisiaca (banana)	Unknown	Methanolic extract of roots at 1,000 mg/kg b.w.	Unknown	E.t	OC↓, CS↓, PCV↑	Anosa & Okoro (2011)
Olea europaea L. (Olive tree)	Maslinic acid found in leaves and fruits	90 ppm of maslinic acid in feed	Anti-inflammatory & antioxidant properties	E.t	OC↓, LS↓, WG↑	De Pablos *et al.* (2010)
Pinus radiate (Monterey pine)	35% condensed tannins	Oocysts exposed to 500-1000 μg pine bark extract/ml	Damage of cytoplasm (sporont)	E.t; E.m; E.a	Sporulation↓	Molan *et al*. (2009)
Pasum sativum (Pea plant)	Anti-Et. antibody fragments expressed In pea plant	----------	Inhibition of sporozoites reproduction	E.t	Sporozoite infectivity and reproduction↓	Khalafalla and Daugschies (2010)
Sophora Flavescens Aiton (Sophora)	Unknown	6-30g/liter drinking water	unknown	E.t	M↓, LS↓, OC↓, WG↑	Youn and Noh (2001)

Tulbaghia violacea (Society garlic)	Antioxidant compounds as S-(methylthiomethyl) Cysteinesulfoxide (marasmine); bis[(methylthio) methyl] disulphide and various derivatives	Aqueous extract 35 mg/kg feed	Oxidative stress	mixed infection	FC↑, OC↓	Naidoo *et al.* (2008)
Vitis vinifera (Grape seed)	Tannins	Grape seed proanthocyanidin Extract at 10-20 mg/kg of diet	Oxidative stress	E.t	M↓, LS↓, WG↑	Wang *et al.* (2008)
Vernonia amygdalina powder (Vernonia tree)	Vernoside	Dry leaf at 15% of feed	Oxidative stress	E.t	OC↓	Al-Fifi (2007)

↑=improvement/increase/higher; ↓=decrease/lower; E.t=*Eimeria tenella*; E.a=*Eimeria acervulina*; E.m=*Eimeria maxima*; WG=body weight gain; FC=feed conversion ratio; LS =lesion scores; OC=oocyst counts; BD=bloody diarrhea; M=mortality; PCV=packed cell volume; RBC=red blood cells; CS=clinical Signs; b.i.d. = twice daily.

11. *Pre-and Probiotics*

The prebiotic Mannanoligosaccharides (MOS) derived from the cell wall of the yeast *Saccharomyces cerevisae*, was used widely in poultry ration to promote the health of the gut and increase performance of birds. The mode of action of MOS differ from those of prebiotics in that they have the ability to bind and remove the pathogen from the intestinal tract and stimulate the immune response (Spring *et al.*, 2000). El-musharaf *et al.* (2006) showed that dietary MOS at 1g/kg was able to reduce the number of schizonts from an infection of 3500 or 5000 sporulated oocysts of *E. tenella*, but there was no significant decrease in fecal oocyt counts and the severity of cecal lesions besides no significant effect on growth performance. El-musharaf *et al.* (2006) concluded the presence of a protective effect and enhanced immunity through significant decrease in numbers of schizonts in the ceca with unknown reason of unaffected cecal lesion scores. Another Experiment by El-musharaf, *et al.* (2007) showed that at a concentration of 10g/kg diet of MOS, reduction occurred in number of oocysts per gram of feces (OPG) of birds that were infected at day one with a mixture of *E. acervulina, E. maxima* and *E. tenella* at doses of 900, 570 and 170 sporulated oocysts respectively (Fig. 32). In addition, a reduction of *Eimeria acervulina*- induced mean lesion score by 0.33 with p-value < 0.05 was documented; However, this treatment did not reduce the lesions induced by *E. maxima* and *E. tenella*.

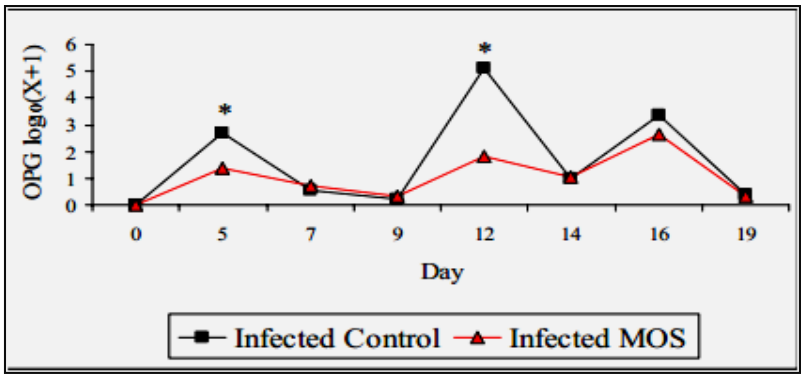


Fig. 32. Oocysts per gram of feces (OPG) of *Eimeria*- infected birds fed diets without or with a Mannanoligosaccharides (MOS) preparation. El-musharaf, *et al.* (2007)

McCann *et al*. (2006) reported that supplementation of MOS at 0.5g/kg and tannins at 0.5 g/kg diet, each one alone or combined did not reduce the severity of infection by *E. acervulina*, *E. maxima* and *E. tenella* at doses of 50,000, 15,000 and 15,000 inoculated sporulated oocysts respectively. Thus, further studies must be carried out to confirm the conflicting results possibly due to different concentration of MOS and different inoculation doses, and to confirm the anticoccidial activity of MOS at high rate of inclusion combined with high doses of inoculation.

The use of probiotics for poultry was proposed in order to competitively exclude the colonization of intestinal pathogens especially after the European Commission banned certain antibiotics used routinely in poultry ration. Probiotics are effective against a variety of pathogens including *Clostridium perfringens* (La Ragione *et al.,* 2004). Probiotics promotes growth, feed efficiency, egg production and egg mass in poultry (Jernigan *et al*., 1985; Jin *et al*., 1997). Probiotics are effective against *Eimeria,* due to reduced invasion and development of *Eimeria,* and enhanced local cell mediated immunity in lactobacillus probiotic supplemented feed given to *E. acervulina* infected broilers (Dalloul *et al.,* 2003a, 2003b, and 2005).

E. Immunity and genetic predisposition to Coccidiosis

1. *Immunity*

Innate immunity is the first line of defense against foreign antigens and it is considered non specific and lack the immunological memory. Cells which are involved in innate immunity are macrophages, dendritic cells, granulocytes (polymorphonuclear lymphocytes, PMNL: neutrophils, eosinophilis and basophils) and NK cells. Innate immune system can detect some structures in the pathogen which are lacking in the self. Innate immune system have pattern recognition receptors (PRR) as toll like receptors present on different types of cells like dendritic cells that can detect microbial products and send signals to the nucleus for cytokines production known as inflammatory cytokines, resulting in initiation of adaptive immune response (Parham, 2009; Medzhitov, 2001).

Adaptive or acquired immunity activates antigen-specific mechanism that includes T cells, B cells, dendritic cells and macrophages and the production of memory cells. Those memory cells will remember the antigen and provides a rapid and strong immune response when this same antigen is encountered a second time. Adaptive immune response includes the cellular immunity and the humoral immunity.

Cell mediated immunity includes both the innate and acquired immune response, including T-cells NK cells and macrophages. The T- cells are composed of two different subpopulations that can be distinguished according to their surface phenotype. Tcytotoxic/suppressor (CD8+) are restricted to Major Histocompatability Complex class I (MHC I). MHCI is found on almost all cells, enabling CD8+ T-cells to destroy the cells that are infected or pathogens that have penetrated the cell. T helper (CD4+) restricted to (MHC II), this complex present segments of proteins which are

phagocytised foreign parts from the pathogens. Cells that have MHC II restriction are APC (antigen presenting cells) like macrophages, dendritic cells and B cells. CD4+ cells T helper are divided into Th1 and Th2 based on their cytokine secretion. Th1 mediate cell mediated immunity and Th2 mediate humoral immunity (McDonald, 1999; Parham, 2009). T-regulatory (T-reg) with suppressive function is $CD4^{+}CD25^{+}$ T-reg cells. These CD4+ CD25+ T cells from mice or humans are capable of suppressing the *in vitro* proliferative response of conventional CD4+ and CD8+T cells. They eliminate T-cytotoxic cells at the site of inflammation, inhibit interlukin-2 (IL-2) production and function in diverse suppressive activities as IL-10 production to terminate the immune response, thus their absence or defect is correlated with autoimmune diseases, and their presence with tolerence (Thornton, 2005; Askenasy *et al.,* 2008). Cytokine production capacity of Th1 and Th2 and their balance is regulated by T-reg through immunoregulatory cytokines such as Transforming Growth Factor- β (TGF-β) and IL-10 (Ozdemir *et al*., 2009). Maternal tolerance to the fetus was explained by immunoregulation of Th1/Th2 cytokine production balance, and predominant Th2 that over-rules Th1 durning pregnancy therefore, protecting the fetus from Th1 attack (Wegmann *et al*., 1993).

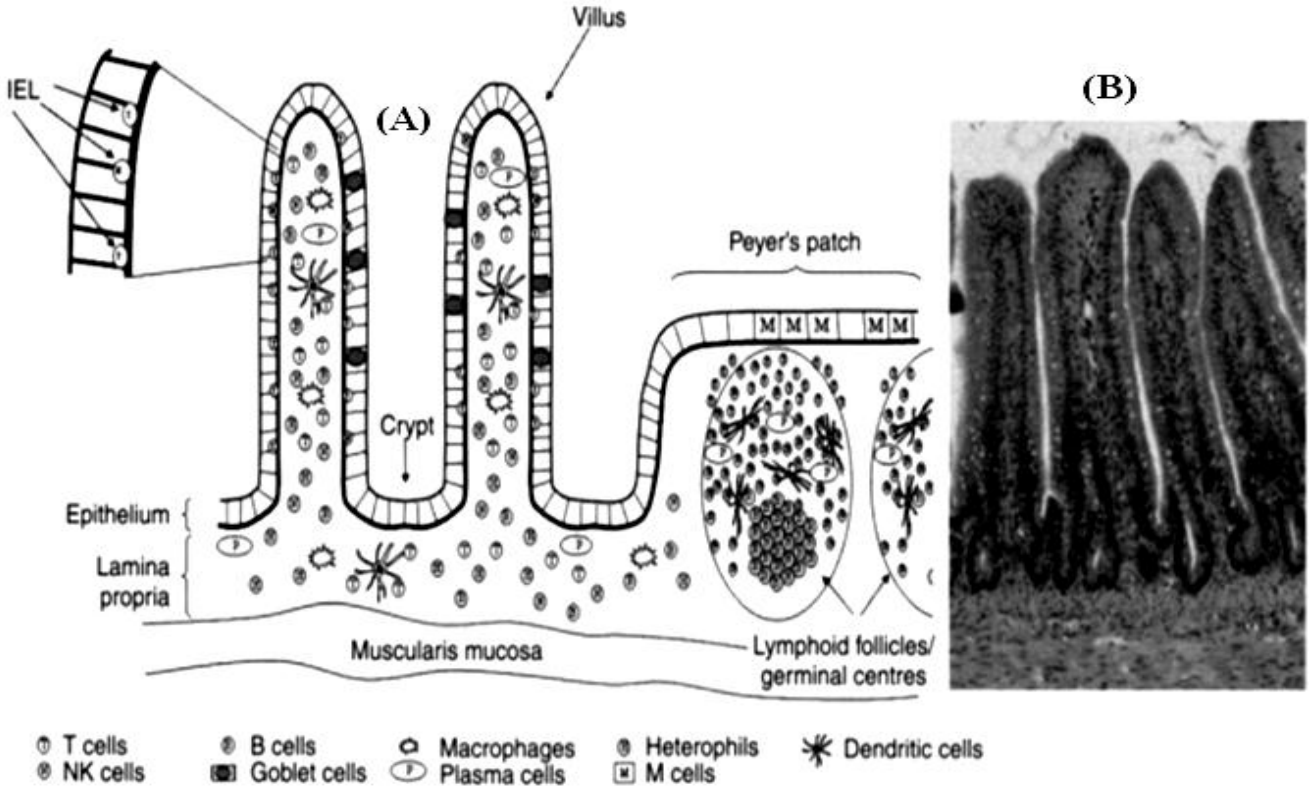
Humoral immunity includes antigen-specific antibodies that circulate in the blood and in the mucosal secretions (Lillehoj and Trout, 1996; Parham, 2009).

a. <u>The Gut Associated Lymphoid Tissue (GALT)</u>

The GALT is a multilayer tissue, exposed constantly to food and pathogens. The GALT have outer epithelial cells and lymphocytes situated above basement membrane (BM); beneath BM is the Lamina propria (Fig. 33), (Yun *et al.,* 1999). The

GALT in the chicken includes specialized lymphoid organs as Bursa of Fabricius, cecal tonsils, and to a large degree is presented by the Peyers Patches and cells including epithelial, lymphoid, antigen representing cells and natural killer cells to defend against intestinal pathogens; other cell types include mast cells and fibroblasts. All these cells secret and respond to cytokines (Yun *et al.*, 1999).

Fig. 33. (A) Schematic organization of immune cells in the intestinal mucosa. (B) photo-micrograph of a chicken ileum showing intestinal villi and crypts (100X magnification). Source: Davison *et al.* (2008)



b. <u>Peyers Patches (PP)</u>

PP are described by their thickened villi, flattened epithelium, absent globlet cells; and they have accumulated lymphocytes in the germinal centers and diffuse lymphoid tissue (Burns, 1982). Payer patches in chicken contains clusters of T & B lymphocytes besides antigen presenting cells (Fig. 33), (Yun *et al.*, 1999; Davison *et al.*,

2008). Antigens are absorbed by M–cells found on PP and transported to the inside to be presented by antigen presenting cells (MHCII-restricted) (Richman *et al.,* 1981) to activate B & T cells. Activated lymphocytes travel to the lamina propria and mucosal epithelium and function as effector cells.

c. Immunoglobulin A (IgA)

Activated B-cells in the lamina propria, i.e. plasma cells, secrete immunoglobulin A (IgA). Secretory IgA (sIgA) is dimeric form of IgA associated with a J-chain and secretory component produced by epithelial cells (Crago and Tomasi, 1988). In birds, The IgA secretory component complex is transported to the external surface (lumen secretions) through vesicles as in mammals (Peppard *et al.,* 1983). In mice, IgA in the lamina propria is accumulated at apical portion at the location of parasite development (Nash and Speer, 1988). Cecal contents of *E. tenella* infected birds contained high levels of sIgA after primary and secondary infections (Davis *et al.*, 1978), while IgG and IgM were significantly lower.

d. Intestinal epithelial cells (IECs)

The epithelial cells act as selective barrier, allowing nutrients to be absorbed and resisting the entrance of pathogens and normal bacterial flora (Figure 34).

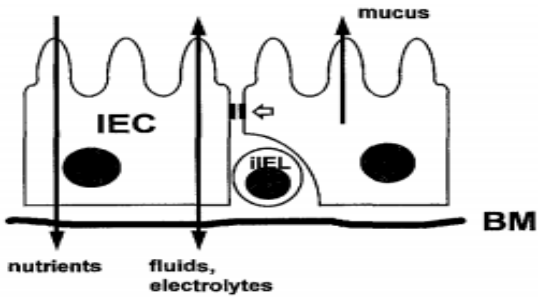


Fig. 34. Intestinal epithelial cells (IECs).

Intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs) are situated above the basal membrane (BM)**.** IECs are barrier against pathogens that is sealed by tight junction *(open arrow)*, while allowing nutrient uptake. Mucus is secreted by IECs, and these later regulate fluid and electrolyte balance.

Beside their passive role as physical barrier, IECs secret intestinal trefoil factor (ITF) into the mucus, that has an important role in wound healing, providing innate resistance to mucosal injury. Furthermore, they secrete cryptdins that are bactericidials (Christ & Blumberg, 1997). IECs act as a communication network secreting high levels of chemoattractants that can attract polymorphonuclear lymphocytes (PMNL) and monocytes/macrophages, and are early sensors for invasive and noninvasive pathogens (Kagnoff, 1996; Kagnoff and Eckmann, 1997; Strober, 1998). IECs are important source for a number of cytokines that modulate the immune response at the mucosal level (McGee *et al*., 1993; Panja *et al.,* 1995; Reinecker *et al.,* 1996; Strober, 1998). In avian coccidiosis there is limited information concerning function of IECs in immunity to *Eimeria* (Yun *et al.,* 1999).

e. Intestinal intraepithelial lymphocytes (IELs)

The term IELs is used to describe lymphocytes occupying intraepithelial space of the mucosal epithelium (Fig. 34). The IELs mainly are T-cells bearing CD8+ antigen (Mcdonald, 1999). These T-cells in IELs have a T-cell receptor TCR gamma-delta expression (Bucy *et al.,* 1988). An important characteristic of IELs T- cells is the presence of high percentage of TCR gamma-delta, and mostly express CD8+ (cytotoxic/suppressor) with very small percentage of CD 4+ (Selby *et al.,* 1984). IELs T-cells are known to secret many cytokines upon stimulation, including Th1- interferon gamma, and interleukin 2 (IL-2); besides Th2- IL-4 and IL-5 (Lundqvist *et al*., 1996; Abe *et al.,* 1998, Fan *et al.,* 1998; Mcdonald, 1999), i.e. they are immunologically active, however the stimulation level is poor compared to peripheral T-cell lymphocytes.

f. Lamina Propria Lymphoctes (LPLs)

These are activated T-cells with slight B-cells and plasma cells. CD8+ T- cells are found in both epithelium and lamina propria whereas CD4+ T-cells are almost exclusively located in lamia propria (Rothwell *et al.,* 1995). LPLs T- cells posses mainly TCR- alpha-beta (Bucy *et al.,* 1988). After the sporozoites go through the villus epithelium of the ceca, they travel to crypt epithelium through the lamina propria for asexual and sexual stages to occur. Immunized chicken showed increased number of leukocytes in the lamina propria than naïve birds after *E. tenella* infection. In naive chickens, most infiltrated leucocytes were macrophages and T cell of phenotype CD4+ were more than CD8+, suggesting that CD4+ cells and macrophages are involved in protective immune response. In immune chickens, higher number of CD8+ than CD4+ infiltrated the lamina propria, suggesting these cells are involved in effective immune response (Vervelde *et al.,* 1996). Jeurissen *et al.* (1996) suggested three criteria that are important for immunity:

- Occurrence of the parasite in the Lamina Propria
- Sporozoites are the most essential stage for immunity
- Cytotoxic T-cells for inhibition of the parasite development

Concluding that , future research for vaccine development against coccidiosis should be directed to introduce sporozoite antigens into the lamina propria in a way that will specifically stimulate CD8+ T cells (Jeurissen *et al.,* 1996).

g. Natural killer cells (NK cells)

NK cells are also known as natural killer lymphocytes. These are non-T, non-B, and non-macrophages mononuclear cells lacking immunological memory, cytotoxic

in activity with no MHC restriction. NK cells belong to the innate immune system and are found in IELs and LPLs of murine (Tagliabue *et al.,* 1982; Bucy *et al.,* 1990). These NK cells were found to express TCR0 (Bucy *et al.,* 1990), and are also present in the splenic lymphocytes of birds (Cheung and Lillehoj, 1991). NK cell activity increased with age and peaked at 6 weeks after hatching to full potential (Lillehoj and Chai, 1988). Chicken IELs NK and splenic NK cells are important in coccidiosis control. This role is by cytolysis of the infected cells or by secreting interferon-gamma that initiate adaptive immune response (Lillehoj, 1989). Additionally NK cells in genetically different chicken were positively linked with resistance to coccidiosis (Lillehoj, 1989).

h. Macrophages and dentritic cells

These are mononuclear phagocytes, and are antigen presenting cells (APC). Macrophages are found in primary and secondary lymphoid tissue (Dalloul and Lillehoj, 2006). Vervelde *et al.* (1996) has shown that macrophages are primarily involved in primary infections of *Eimeria*, and in antigen presentation to CD4+ cells and production of cytokines. *In vitro* activated Macrophages by interferon-gamma produced NO that was toxic to *E. tenella* replication **(**Dimier-Poisson *et al.,* 1999). Plasma NO_2^- and NO_3^- were increased significantly at 6 days PI in primary infections of *E. acervulina, E. tenella* and *E. maxima* (Allen, 1996, 1997a; Allen and Lillehoj, 1998; Allen & Fetterer, 2002), but not in immune birds suggesting a role of NO_2^- and NO_3^- in innate resistance rather than acquired immunity to coccidiosis. Furthermore, there is a link between genetic resistance to *E. tenella* and NO production (Allen and Lillehoj, 1998).

i. Cell mediated immunity

Chicken infected with *E. acervulina* and were depleted from CD4+ in primary and challenge infections showed no increased oocyst output although oocyst counts was less in secondary infection than primary. Depletion of CD4+ in *E. tenella* infections increased oocyst output in primary infections and not secondary, indicating an important role of CD+4 in resistance in primary infection of *E. tenella* (Trout & Lillehoj, 1996). Depletion of CD8+ in *E. tenella* and *E. acervulina* infections decreased oocyst output in primary infections, indicating a role of CD8+ in transport of sporozoites. In challenge infections with *E. acervulina* and *E. tenella*, depletion of CD8+ resulted in higher oocyst output, indicating an important role in resistance of CD8 + in challenge infections of both *E. acervulina* and *E. tenella* (Lillehoj & Trout, 1994 ; Trout & Lillehoj, 1996). Thus different involvement of CD8 and CD4 in resistance to *Eimeria* was reliant on *Eimeria* species and whether it is primary or secondary infection. Therefore, different mechanisms of immunity are activated depending on *Eimeria* species.

After primary infection with *E. acervulina* congenic lines differing at the Major Histocompatibility B-Complex (MHC) locus showed a positive relation between resistance of the strain to *Eimeria* and CD8+ increase (Lillehoj & Bacon, 1991). Similar study was conducted with White Leghorn SC (B^2B^2) and TK ($B^{15}B^{21}$) lines (Two inbred Hy-line chicken strains that differ at the MHC II that is restricted to T-helper cells and is carried by B-cells.Those SC and TK lines have different levels of susceptibility to coccidiosis and SC being more resistant than TK) in *E. acervulina* infection, showing a significant increase in CD8+ IEL in SC chicken in primary and secondary infections, and reflecting the presence of an enhanced immune status of SC chicken (Lillehoj,

1994), concluding that the dynamics of different T cell population after *Eimeria* infection is a sign of genetic resistance to different *Eimeria* spp.

T-Cells from *E. acervulina*-immune birds responded well to both *E. acervulina* and *E. tenella* antigens (Prowse, 1991), suggesting that some *E. acervulina* antigens are cross-species reactive. Birds immune to one species of *Eimeria* are not able to resist infection with heterologous species (Rose, 1987). The question remains, why birds immune to *E. acervulina* are susceptible to *E. tenella* infection (Prowse, 1991). The answer might be that adaptive immune response recognizes many parasite antigens, but cross reactive epitopes are non-protective.

The studies with suppressive drugs that severely depress T-cells and cell mediated immunity (Lillehoj, 1987; Isobe & Lillehoj, 1993), besides thymectomized rats that were highly susceptible to primary and secondary infections of *Eimeria* (Rose & Hesketh, 1979) have been used to study the role of T-cells in *Eimeria* infections. These studies concluded that cell mediated immunity (CMI) is the main line of defense against *Eimeria* infections (Lillehoj and Trout, 1993, 1994, 1996).

j. Humoral immunity

Humoral immunity is part of the adaptive immune response through which production of antibodies occur by activated B cells; these B cells originate from the bursa. The main chicken immunoglobulins (Ig) are IgA, IgM and IgY; IgY resembles IgG in mammals (Lillehoj *et al.*, 2004). Ig binds to extracellular pathogens and destroys it through antibody-dependent cell mediated cytotoxicity (ADCC).

Following infection with *Eimeria,* specific antibodies to coccidia are found in the circulating blood, bile and intestinal mucosa. IgY (IgG) is concentrated in the egg

yolk and transported to the embryo through a mechanism called passive immunity (Dalloul and Lillehoj, 2006), but protective levels of antibody cannot be maintained for long time. After infection with *Eimeria,* chicken produced large amounts of IgA, IgM and IgY that peaked at 9-20 days post infection in sera, bile and intestinal secretions (Trees *et al.,* 1989). Early studies indicated that the removal of the bursa did not affect protective immunity after challenge (Pierce and Long, 1965), Early studies also indicated that immunized laying hens against *Eimeria* passively transfered IgY to the progeny and protected it in a primary infection with the same *Eimeria* spp. (Rose, 1972). Furthermore, a correlation exists between antibody titer and protection to *Eimeria,* specifically IgY since IgA and IgM are nearly absent in egg yolk and albumin (Smith *et al.,* 1994).

Recently, it was shown that hens immunized with gametocyte antigens of *E. maxima* passively transfered IgY via egg yolk and gave partial protection to progeny against infections with *E. maxima*, *E. tenella* and *E. acervulina*. Three major gametocyte antigens of *Eimeria maxima* gave protection of 60-80% less oocysts shedding in the progeny (Wallach *et al.,* 1995; Wallach, 1997). *E. tenella* gametocyte antigens also protected the progeny from homologues challenge with 70 % less oocysts shedding (Hafeez *et al.,* 2007). More recent studies showed that *E. tenella* gametocyte antigens protected birds against mixed infections (Anwar *et al.,* 2008).

In short, humoral immunity may have a role in *Eimeria* infections and is in debate in literature that has concluded that humoral immunity plays a minor role. Wallach (2010) pointed out that this scenario of minor role has been concluded because

- there is no need for humoral immunity and titers to *Eimeria* in development of a protective immunity since CMI alone can give good protective immunity.

- research is focused on long lasting protection from a vaccine that replaces live vaccine, and this is not attainable using passive immunity alone.

k. Cytokines

Lymphocytes, macrophages, and other effector cells in the intestine secrete cytokines to induce CMI to terminate the invading parasite, and to induce memory responses. Although the importance of cytokines in mediating innate and acquired immunity has been well documented, their function in the avian immunity and disease development needs more understanding and characterization (Lillehoj *et al.,* 2003).

i. TNF-alpha

Tumor Necrosis like factor alpha production was detected *in vitro* from macrophages of *Eimeria* infected chickens. TNF-alpha production showed biphasic pattern with the first peak associated with pathogenesis of the disease and the second peak with protective immunity development (Byrnes *et al.,* 1993a; Zhang *et al.,* 1995a). Excessive production of TNF-alpha in SC (B^2B^2) birds (Inbred Hy-line chicken strain that has Major Histocompatibility B-Complex Class II restricted to T-helper and defined as B^2B^2 homozygous haplotypes. SC lines are known to be resistant to coccidiosis) in primary infection has a role in enhanced weight loss and the pathophysiology of *Eimeria* infection (Zhang *et al.,* 1995b).

ii. TGF- beta

Transforming growth factor-beta is an anti-inflammatoy cytokine that plays a role in intestinal healing (Robinson *et al.,* 2000). Lymphocytes secret TGF- beta and down regulate the inflammatory response in the intestinal mucosa (Strober *et al.,* 1997).

After infection with *E. acevulina*, expression of Transforming growth factor-beta 4 (TGF- β4) mRNA increased 5-8 times in IELs and 2.5 times in splenic cells (Jakelow *et al.,* 1997), suggesting a role in *Eimeria* infection.

iii. IFN-gamma

Interferon-gamma (IFN- γ) is used to measure T-cell response and CMI to *Eimeria* infections (Byrnes *et al.,* 1993b; Martin *et al.,* 1994). IFN-gamma is produced by CD4+ T-helper cells and it enhances macrophages to express MHC II (Kaspers *et al.,* 1994). Difference between the chicken lines in IFN-gamma production was noted (Martin *et al.,* 1994). IFN-gamma has a role in limiting intracellular parasite development (Kogut & Lange, 1989). Rose *et al.,* (1991) indicated that inhibition of the parasite development was mediated via the host cells. Recently chicken IFN-gamma gene was cloned (Digby & Lowenthal, 1995). Parental administration of chicken recombinant interferon-gamma reduced weight loss and oocyst outputs in *E. acervulina* infected birds (Lowenthal *et al.,* 1997; Lillehoj & Choi, 1998). Macrophages, fibroblasts and epithelial cells are activated by interferon-gamma, thus they limit the infection by inhibiting *Eimeria* development (Hughes *et al.,* 1987; Rose *et al.,* 1989; Woodman *et al.,* 1991; Hériveau *et al.,* 2000). In short, interferon-gamma plays a key role in limiting *Eimeria* infection in chicken.

iv. Interleukin -6, Interleukin -8, Interleukin -12, and Interleukin -15 (IL-6, IL-8, IL-12, and IL-15)

- Interleukin-8

IL-8 is a potent stimulator of neutrophil activation and chemotactic cytokine with high selectivity to neutrophils within the intestinal mucosa. IL-8 is associated with numerous acute and chronic inflammatory reactions (Baggiolini *et al.*, 1989; Sturm *et al.*, 2005). After primary infection *of Eimeria maxima,* IL-8 and IL-6 were significantly up-regulated with similar kinetics (Hong *et al.*, 2006c).

- Interleukin-6

IL-6 is a Pro-inflammatory cytokine that induce the maturation of B cells into antibody-producing plasma cells and plays a role in regulating acute phase reactions (Narazaki & Kishimoto, 1994; Schneider *et al.*, 2001). Studies indicate production of interleukin-6 during *Eimeria* infections; IL-6 activity was detected during the first few hours post *Eimeria* infection, indicating an influence on the developing immune response and a possible role of this cytokine in the development of acquired immunity (Lynagh *et al.*, 2000).

- Interleukin -12 and Interleukin -15

IL-12 is a Pro-inflamatory cytokine produced by phagocytes and other cells (monocytes macrophages or neutophils). IL-12 activates Natural Killer (NK) cells, induces Th cells to become Th1 cells, and increases cytotoxic T cell (Ma *et al.,* 1996). IL-15 is a Pro-inflamatory cytokine produced by mononuclear phagocytes and other cells in response to viral or parasitic infections. Chicken IL-15 is structurally homologous to mammalian IL-2 and stimulates proliferation of NK cells (Sundick and Gill-Dixon, 1997; Lillehoj *et al.*, 2001). IL-12 and IL-15 were highly increased in intestinal IELs following *E. maxima* primary infection (Hong *et al.,* 2006c).

l. The influence of the host /*Eimeria* interaction in the immune response nature of the chickens

Chickens produce an immune response in order to develop protective immunity to homologous re-infection. Seven species of *Eimeria* are considered valid, these species have different niche in the intestine, and different immunogenicity resulting in different host immunity development to re-infection. The time of development of full protective immunity in the broilers under continuous exposure to *Eimeria tenella, E. maxima, and E. acervulina* was within 25, 24, and 16 days respectively apart from the age of the broilers (Stiff & Bafundo, 1993). Protective immune response is species-specific and even strain-specific (Basak *et al.*, 2006; Blake *et al.*, 2005, 2006). *E. maxima* genome encodes various antigens that stimulate the immune response with cross-protection against more than one strain, but are strain-specific. Full protective immune response against a heterogonous strain of *E. maxima* was never found. Furthermore, Dalloul *et al.* (2007) found unique avian macrophages response to different *Eimeria* species. Chemokine and cytokine expression by macrophages was different with different *Eimeria* species. Thus infection history of a flock is an important factor in resistance to re-infection.

Different stages of development of the *Eimeria* have different antigen epitopes which add to the complexity of the immune response (Tomley, 1994). The infecting oocysts dose is also of significance to immunity development. Different doses of *Eimeria* show dose-related effect on the balance between Th1 and Th 2 responses during primary infection and sensitivity of the host to re- infections (Blake *et al.*, 2005). This difference in host response might also be due to overcrowding effect above which the reproductive potential of *Eimeria* decreases (Williams, 2001). Increasing oocysts

dose will result in increased oocysts outputs to a point known as ‘crowding threshold' after which the oocysts output will start to decrease due to limited epithelial cells. Therefore, older birds can shed at the same infecting dose more oocysts compared to young birds (Krassner, 1963; Rose, 1967), and in *vivo* production of oocyst stocks must use the appropriate infective dose according to the age of birds and the infective dose (Williams, 2001).

The genetic background of the host is also of significance in immunity outcome (Lillehoj, 1986; Li *et al.,* 2002). Finally, birds infected at different ages resulted in different susceptibility to *Eimeria*, although it is well known that the immune system (T and B lymphocytes) in newly hatched chicks is not mature (Bar-Shira *et al.,* 2003), difference in susceptibility of host to *Eimeria* due to age is not reported yet (Ramsburg *et al.,* 2003; Chapman *et al.,* 2005).

m. The relation of *Eimeria* endogenous phases and induction of protective immune response

During the endogenous phase of the parasite, it expresses a wide range of antigens that are presented to the immune system. (Rose & Hesketh, 1976; McDonald *et al.,* 1988; Tomely, 1994). Many studies showed that the early stages of *Eimeria* development are mainly essential for protective immunity development (eg: Rose and Hesketh, 1976). Rose and Hesketh (1976) indicated that the second generation schizogony of *E. maxima* is responsible for protective immunity development and that sexual stages are most susceptible to immune inhibition. Furthermore, the effective immunogenicity of *E. maxima* lines were characterized by lack of terminal schizogonous stages (McDonald *et al.,* 1986b, Shirley & Bellatti, 1988; Schnitzler &

Shirley, 1999). McDonald *et al*. (1986a) indicated that *E. tenella* first schizogonous stages are greatly immunogenic. Moreover, the radiated oocysts that permit the sporozoites invasion but stop schizogony are able to induce full protection against *E. maxima* challenge (Jenkins *et al.,* 1993), and *E. acervulina* (Jenkins *et al.,* 1991). Thus immunoprotective antigens are in the early stages of development of *Eimeria*, and mostly are sporozoites or antigens presented by them.

n. Transfer of sporozoites and extra-intestinal stage

Infection in the intestine is started by sporozoites that attack villus enterocytes. *E. brunetti* and *E. preacox* complete their whole development in the enterocytes of the villus, while the remaining five species starts in the enterocytes of the villus and go on with their life cycle in the crypts of lieberkuhn. The means by which these sporozoites reach the crypts is not very clear. Early reports showed that macrophages (Van Doorninck & Becker, 1956; Doran, 1966) or intraepithelial lymphocytes (IELs) (Al-Attar & Fernando, 1987; Fernando *et al.,* 1987; Lawn & Rose, 1982) were in charge for sporozoite transport; however, the identity of the cells was not confirmed. Fernando *et al.* (1987) indicated that sporozoites invaded the mucosa very shortly after infection and some of them went to the liver and spleen and later left to enter the gut mucosal epithelium. Sporozoites were found within intraepithelial lymphocytes but not macrophages. Trout & Lillehoj (1993, 1995) indicated that sporozoites were in 28% of CD8 + cells, 28% in macrophages, and 12% in CD4+ cells, concluding that sporozoite transport occurs in T lymphocytes and macrophages. Vervelde *et al.* (1995) indicated that *E. tenella* sporozoites seldom enter intraepithelial leukocytes (B & T cells and macrophages) and therefore they believed that the function in transport of

sporozoites through the lamina propria is still uncertain. Aside from this transport means, it is achievable to pass on infection to naïve chicken through blood, spleen and liver whether the development was in the villus or in the crypts. Infections transferred through blood and splenic homogenates was not occurring at all times. Transfers that were made within a short time of the inoculation were more successful in producing obvious infections (Fernando *et al.*, 1987; Perry & Long, 1987). Riley & Fernando (1988) indicated that livers and spleens of *Eimeria* infected birds resulted in obvious infection when they were fed to susceptible birds, and infections are transferred till 72 hours after inoculation. This occurrence of *Eimeria* in liver and spleen was referred to in literature as extra –intestinal phase which is common to all species of *Eimeria*.

2. *Effect of avian genetics on immunity to Eimeria*

The use of naturally resistant birds to coccidiosis is one of the attractive alternatives to control coccidiosis. Evidences that resistance and susceptibility to avian coccidiosis are linked with inheritance were given by Rosenberg *et al.* (1953). Increasing resistance to coccidiosis was probable through selection (Johnson & Edgar, 1986) however, for economical and ethical reasons it cannot be a routine practice for commercial lines. Pinard-Van Der Laan *et al.* (1998) compared 5 out-bred lines for resistance to *E. tenella* that were challenged at 4 wks of age. This resistance was measured by parameters as lesion score, mortality, and body weight gain at slaughter, as well as plasma coloration 4 days post infection. Resistant lines showed no mortality, reduced gross lesion scores and significantly less reduction in growth, although no effect of major histocompatibility complex MHC- linked genes (B-complex) was detected. In contrast to other studies, the mechanism responsible for the resistance

differences to infection involves MHC-linked genes and their importance on acquired immunity (Bumstead et *al.,* 1995; Clare & Danforth, 1989; Clare *et al.,* 1985; Nakai *et al.,* 1993). These later studies confirmed that particular B-complex haplotypes are responsible for immunocompetence of birds.

Congenic lines differing at MHC were studied by Lillehoj & Bacon (1991); changes in intestinal IELs T- lymphocyte subpopulations were examined and suggested a significant increase in the duodenal CD8+ IEL after *E .acervulina* primary infection may reflect an enhanced acquired immunity of B^2B^2 and not B^5B^5 chicken lines. Cytokine production was studied in SC (B^2B^2) and TK ($B^{15}B^{21}$) chicken by Choi *et al.* (1999).The SC and TK are two inbred Hy-line chicken strains that differ at the Major Histocompatibility B-complex that is restricted toT-helper cells and is carried by macrophages and B- cells.Those SC and TK lines have different levels of susceptibility to coccidiosis and SC being more resistant than TK. IFN-gamma and (TGF) Beta-4 mRNA expression in caecal tonsils and splenic lymphocytes were in general higher in SC compared to TK chickens following *E. acervulina* infection, thus influencing CMI to coccidiosis.

Because chickens show different degrees of disease manifestation after an infection with *Eimeria* spp., resistance or susceptibility to *Eimeria* can be thought of as a quantitatively inherited trait and therefore controlled by multiple genes. Quantitative Trait Loci (QTL) related to this resistance was mapped with DNA marker technology. Resistance to avian coccidiosis QTL has been recognized near the two microsatellite markers LEI0071 and LEI0101 on chromosome 1 (Zhu *et al.,* 2003; Kim *et al.,* 2006). Single nucleotide polymorphisms (SNP), which are most frequent DNA difference in the genome, are single base changes between individuals that occur within or between

genes. Hong *et al.* (2009) verified that SNP in chicken genes encoding the zyxin (located between LEI0071 and LEI0101) is a candidate gene potentially linked to increased resistance to avian *Eimeria*. Additionally, SNP in the myeloid leukemia factor 2 (MLF2) gene located on chromosome 1 can be one of the markers to select for coccidiosis resistance in chickens (Kim *et al.,* 2010).

Relationships between subclinical infections of *Eimeria* species and various risk factors were studied by Shirzad *et al.* (2011). Breeds as Cobb and Ross hold the same risk factor to coccidiosis, while Lohmann was at slightly higher risk. The emergence of anticoccidial drug resistance and the ban of Anticoccidial Growth Promototrs (AGPs) is driving coccidiosis research to vaccine development and breeding of more resistant types of birds (Williams, 2006). Broiler breeds are bred for economically important traits as growth rates and feed conversion ratio, however no specific selection on disease resistance has been made (Leshchinsky & Klasing, 2001). Selection of poultry for fast growth rate is often linked to reduction in immunological responses or increased disease susceptibility (Han and Smyth, 1972; Saif *et al.,* 1984; Sacco *et al.,* 1991, 1994; Tsai *et al.,* 1992; Qureshi & Havenstein, 1994). The reciprocal situation of selection for enhanced immunological response has been shown to result in decreased body weight (Siegel & Gross, 1980; Siegel *et al.,* 1982; van der Zijpp, 1983; Okada *et al.,* 1988; Martin *et al.,* 1990; Afraz *et al.,* 1994).

3. ***q-RT-rtPCR: Reverse transcription - real time polymerase chain reaction in uncovering immune response mechanism***

qRT-rtPCR has become an essential tool in most molecular biology laboratories to measure the quantity of gene expression by measuring the mRNA levels

(Hong *et al.*, 2006 b, c). The mRNA template is first converted into a copy DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR and DNA polymerase. qPCR involves serial dilution of known quantities of the cDNA and the use of fluorescence (eg: ethidium bromide staining to cDNA) to detect the threshold cycle (Ct). This Ct value is responsible for the accurate quantization by qPCR. The Threshold Cycle (Ct) reflects the cycle number at which the fluorescence, generated within a reaction, crosses the threshold. The Ct value assigned to a particular well thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated in that well to be at a statistically significant point above the baseline. The software then displays the standard curves that are used to determine unknown quantities. The qPCR technology helped to uncover mechanisms of immune responses to allergens and microbes, as reviewed recently by Barbour *et al.* (2013).

CHAPTER III

MATERIALS AND METHODS

A. Production and pathogenesis in broilers subjected to differently-timed *Eimeria* spp. challenge.

1. *Introduction*

Eimeria spp. infection in chicken is the most economic disease requiring a continuous supplementation of coccidostat in their feed, causing emergence of serious resistance in *Eimeria* to drugs (Stephen *et al.,* 1997). In addition, broilers raised in developing countries are not following the proper withdrawal periods of drugs from the feed before slaughter, resulting in significant residues of these coccidiostat, even in chicken carcasses following a respected withdrawal period (Mortier *et al*., 2005). Infections are caused by eight *Eimeria* spp. namely: *E. acervulina, E. mivati, E. maxima, E. necatrix, E. hagani, E. praecox, E. tenella,* and *E. brunetti.* The species *E. acervulina, E. tenella, E. maxima,* and *E. necatrix*, are the most frequent *Eimeria* spp. infections in broilers (Lee *et al.,* 2011). The optimization of reproduction of the intestinal pathogenesis by a controlled challenge, using the previously mentioned four species of *Eimeria* is of paramount importance, before evaluation of any drug or immunopotentiator against such infection (Elmusharaf *et al.,* 2010). The multiplication and pathogenesis of *Eimeria* spp. is related to innate immunity of different chicken breeds (Lillehoj, 1994); thus, the inclusion of a certain broiler breed in the *Eimeria* spp. challenge, requires a detailed optimization to reproduce the pathogenesis of such protozoa. To our knowledge, no previous research did establish any base-line data

related to intestinal pathogenesis in broilers challenged at different ages with $1.064x10^6$ of sporulated oocysts per ml of the eight *Eimeria* spp. present in non-attenuated live vaccine.

2. *Purpose of the study*

The purpose of Study A is to establish a base-line data on intestinal pathogenesis in broilers with four *Eimeria* spp.-controlled challenge at different ages namely 14, 21, 28, and 35 days.

3. *Experimental design*

- Eighty day-old chicks were reared on same basal diet, and were divided into five groups of 10 chicks in each of the first four groups and 40 in the last control group. Chicks were placed in separate rooms, with floors covered by corrugated paper.
- The corrugated papers were changed every 24 hours of rearing. However, at challenge time assigned to each of the four groups, a new paper was used and was left in the cage for 6 days post challenge to ensure the establishment of the first life cycle of *Eimeria* spp. infection. The controls were left with no challenge, changing the floor paper every 6 days.
- Each group of the 10 birds assigned for the challenge at a certain age, was weighed, challenged, and kept for 6 days before scarification. A parallel control group of 10 birds, at the same age, was weighed and also sacrificed after 6 days. Weights of the challenged groups and controls were taken at the same scarification day, and the percent increase in weight of each group, during the 6 days-period following challenge was calculated.

- The chickens were sacrificed after 6 days of each challenge, thus those that were challenged at 14, 21, 28 and 35 days are sacrificed at respective ages of 20, 27, 34, and 41 days. The controls were sacrificed in groups of 10 at 20, 27, 34, and 41 days of age to allow for comparison of performance and lesions frequencies with challenged groups.

- Feed consumption during the 6 days-incubation period of *Eimeria* spp. was recorded for challenged and control groups.

- At scarification day, by the end of the 6 days-incubation period, scores were given to gross lesions present in the duodenum, jejunum, ileum and cecum of each sacrificed bird. The scores were 0 (no gross lesions), 1 (mild inflammation), 2 (moderate inflammation) and 3 (severe inflammation) (Fig. 35). The mean score for each treatment was calculated for statistical comparison between controls and challenged groups.

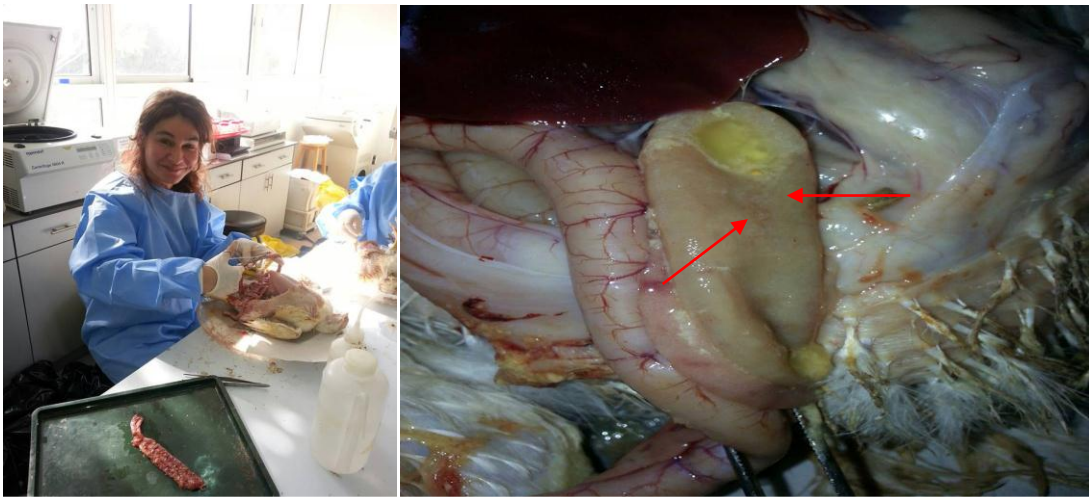


Fig. 35. Left photo: Animal and Veterinary Science Laboratory- American University of Beirut - Gross Lesions scoring of intestinal parts (duodenum, jejunum, ileum,and cecum). Right photo: duodenum of Lesion Score +0 (no Gross Lesions).

- Mortality frequency in the different treatments was calculated during the 6

days-incubation period of *Eimeria* infection in challenged compared to control-unchallenged groups.

- The oocyst counts in each part of the intestine (duodenum, jejunum, ileum, and cecum) was calculated per gram of sample (Conway and Mckenzie, 2007), and the mean count for each treatment was calculated for statistical comparison between challenged and control groups.
- The SPSS V.20 statistical computing program was used for analysis of data. Means of percent weight increase during the 6 days-incubation period of *Eimeria* infection, means of feed conversion, means of lesion scores, and means of oocyst counts were compared between controls and challenged groups by Analysis of Variance ANOVA followed by conserved Tukey's test. The comparison of the mean frequency of mortality in controls and challenged groups was done by Fisher Exact Test. Significant difference in means of frequencies of examined parameters between controls and challenged groups were reported at 95 % confidence limit ($p<0.05$).

4. *Eimeria inocula*

Each vial of non-attenuated strains of Coccivac-D, designed to vaccinate 1000 birds (Intervet Inc., Summit, NJ 07901, USA), and containing eight chicken *Eimeria* spp. (*E. acervulina, E. mivati, E. maxima, E. necatrix, E. hagani, E. praecox, E. tenella,* and *E. brunetti*) was diluted with sterile saline to a volume of 10ml. Each bird at challenge time was inoculated intra-esophageally with 1 ml of the prepared inoculum which is equivalent to 100 times the vaccine dose/bird, and adjusted to $1.76x10^5$ sporulated oocysts of *Eimeria* of all eight spp. per bird (Hong *et al*., 2006c; Danforth *et al*., 1997a; Danforth, 1998).

5. *Diet formulation*

Basal diet was formulated according to NRC (1982), and provided to us by AREC (Agriculture Research and Education Center) in Bekaa Valley, Lebanon. The respective protein and energy in the diet were 22 % and 3100 KCal/Kg. The basal diet was deprived of coccidostat supplementation and fed *ad libitum* all through the rearing period.

6. *Broiler chicks*

Day-old Ross 308 broiler chicks were obtained from Nahas Farms (Bekaa valley, Lebanon) and were apparently healthy.

7. *Oocyst counts technique*

- About 1cm pieces of each of duodenum, jejunum, ileum, and cecum were taken from 9 birds of each sacrificed group.
- Pool of 3 pieces from 3 birds of each of the duodenum, jejunum, ileum, and cecum were made.
- Fecal material was washed out with tap water.
- The weight of the pooled samples was taken.
- The pooled samples were homogenized in 10 ml of tap water.
- Pooled samples were shaken vigorously by vortex mixer and were filtered through single thickness muslin.
- Nine ml of the filtrate were obtained in a 10 ml graduated tube.
- The filtrates were centrifuged at maximum speed for 5 minutes to form oocysts pellets.

- The supernatants were discarded and 2ml of 35 % NaCl solution were added to the pellet then the graduated tube was shaken gently.
- Another 3ml of the saline solution (35% NaCl) were added and mixed gently by inverting the tube several times upside down.
- The tubes were stored at $4^{o}C$ for the next day.
- Mc Master Chamber was filled from each of prepared sample using sterile micropipette tips, and oocysts were counted.
- Number of oocyts/g of intestine was obtained using the following formula :

Number of oocyts/g of intestine= N/0.1 x volume of sample x 1000/wt of sample, in which

N = Average number of counted oocysts of the five counted squares of the McMaster Chamber.

0.1 ml = the volume of the sample filling the Mc Master counting chambers.

Volume of sample = 5 ml of 35% saline added over the 3 pooled organ of the intestine.

Wt of sample = total weight of the 3 pooled samples.

B. Production and pathogenesis in long term-supplementations of *Echinacea*-based preparation (EBP) to broilers subjected to differently timed *Eimeria* spp. challenge.

1. *Introduction*

The possible success in establishing a base-line data from the conclusion of Study A related to the reproduction of the coccidiosis lesions by the controlled challenge applied to broilers at different ages could allow the performance of Study B. Study B aims at evaluation of a water extracted from the herb *Echinacea purpurea*, adjusted to Alkamides level of 95.9 mg/liter. Many *Echinacea purpurea* preperations are sold over-the-counter in developed countries for stimulating the immune system of humans, especially against cold, without the need for a physician prescription (Barret *et al.,* 2002; Turner *et al.,* 2005; Schoop *et al.,* 2006; Blumenthal *et al.,* 2007). This practice is followed due to the absence of any toxicity effect in extracts of this herb (Mengs *et al*., 1991), and the presence of active ingredients that are proven to have immune-enhancer effects against a wide spectrum of microbes (Bergner, 1997; Wichtl, 2004; Canlas *et al.,* 2010). The first reported attempt for establishing the dose of *Echinacea purpurea* for inducing immunopotentiation in chicken was concluded by Barbour *et al.,* (2000). The inclusion of 0.147% level of this herb in feed of broilers resulted in highest growth, absence of systemic *Salmonella* Enteritidis (SE) infection in challenged birds, in obtaining the highest vaccine titer specific to immunosuppressive Infectious Bursal Disease Virus, and in obtaining the highest bursal weight index. Three years after the publication of this work, Allen in (2003) proved that such inclusion of *Echinacea purpurea* in feed of broilers improves growth, and reduces the lesions caused by coccidia. However, the challenge by coccidia was fixed at one age of the broilers

namely, four weeks of age, which doesn't reflect the unexpected time of challenge that these broilers might be faced under the field conditions. In addition, the period of administering the herb was not used as a variable.

2. *Purpose of the study*

The purpose of Study B is to search for the proper long term period for administering the EBP (*Echinacea*- based preparation) extract in drinking water (ages of 1-14 days versus ages of 1-21 days) that could result in better growth and alleviation of the intestinal lesions frequency and severity, and in possible reduction of the *Eimeria* multiplication in broilers subjected to differently timed *Eimeria* spp challenge.

3. *Experimental design*

- EBP was administered at varied time, in which group C14 birds received the EBP in drinking water, from d1-d14, while group C21 birds received it from d1-d21. Positive control groups, (PC) were challenged and deprived of EBP; Negative control groups (NC) were not challenged and deprived of the EBP.
- A total of 160 day-old birds were divided into four groups (NC, PC, C14, and C21), with 40 birds/group.
- Birds were weighed individually at one-day old and distributed into differently treated groups.
- Ten birds from each of the PC, C14, and C21 were challenged with unattenuated strains of Coccivac-D at different times namely, at 14, 21, 28, and 35 days of age. The Negative controls (NC) were left without a challenge.
- The corrugated papers were changed at challenge time assigned to each of

the 3 groups, PC, C14, and C21, and were left for 6 days post challenge to ensure the establishment of the first life cycle of *Eimeria* spp. infection. The NC groups corrugated papers were also changed at the same day.

- Each group of 10 birds from PC, C14, and C21 assigned for the challenge at a certain age, was weighed, challenged, and kept for 6 days before scarification. A parallel NC group of 10 birds, at the same age, was weighed and also sacrificed after 6 days. Individual weights (Fig. 36) of the birds in NC, PC, C14, and C21 were taken at the same scarification day, and the percent increase in weight of each group, during the 6 days-period following challenge was calculated.

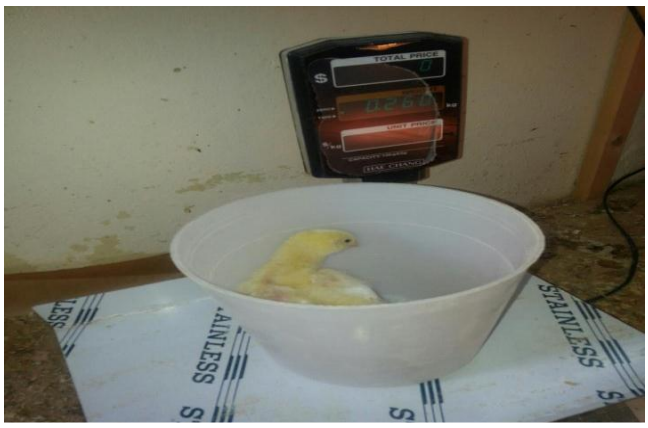


Fig.36. Individual weighing of birds - Animal house – Campus of the American University of Beirut

- Feed consumption during the 6 days-incubation period of *Eimeria* spp. was recorded for NC, PC, C14, and C21 groups.

4. *Echinacea-based preparation (EBP) treatment*

EBP was administered at a level that is comparable to the successful level of dried roots of *Echinacea purpurea* administration established in chicken by Barbour *et al.,* (2000) and by Allen, (2003) that is equivalent to 4.6 ml EBP/Liter of drinking water.

This calculated dose was on the basis of alkamide concentration (0.44mg alkamides/liter of water).

5. ***Parameters used in measurement of Eimeria pathogenicity***

- Mean percentage of weight increase in each broiler group in the 6 days period following the challenges.
- Mean feed conversion ratio in each group deduced during the 6 days period following each of the 4 challenges.
- Mean percent mortality in each broiler group during the 6 days period following each of the 4 challenges.
- Mean score of lesions in each broiler group located in each of the 4 intestinal organs at the end of the 6 days period following each of the 4 challenges.
- Mean *Eimeria* oocyst counts in each broiler group per gram of each of the intestinal organs, observed at the end of the 6 days period following each of the 4 challenges.
- The mean of the above parameters were compared by ANOVA followed by Tukey's test reporting significant difference in means at $P<0.05$.

Note that Feed formulation, *Eimeria* inocula, Broiler chicks, and oocyst counts technique respectively are the same as used in Study A.

C. Protection, production, and immuno-modulation in broilers with intermittent administraion of *Echinacea*-based preparation (EBP) against challenges with *Emeria* spp. alone and *Eimeria* spp. plus *Clostridium perfringens*

1. *Introduction*

The possible success in study B in maintenance of production in *Eimeria* spp. challenged broilers administered EBP between 1-14, and the poor performance results observed when the EBP treatment was extended in a continuous daily administration beyond the 14 days of age, urged us to reduce the EBP-treatment to an intermittent schedule in the first 4 weeks of the life of broilers. The EBP was administered in the first 3 days of each of the first 4 weeks of the broilers' life (Böhmer, 2009) and the challenge was fixed at the 28 days of age, giving an incubation period of 6 days post challenge, with an anticipated allowance of approximately one life cycle of *Eimeria* spp. The possible success in establishment of baseline data on intestinal pathology by the controlled challenges of *Eimeria* spp. at different ages of the broilers (Study A), could help to include the same model of challenge in this Study C. *Clostridium perfringens* was included with or without *Eimeria* challenges, since this bacterium is nowadays considered as an important etiology of an economic disease in poultry causing necrotic enteritis (Van Immerseel *et al.*, 2004). The absence from literature of the study of immunity caused by *Echinacea purpurea* extract against coccidiosis and/or *Clostridium perfringens* requires the inclusion of immuno-modulation assessment for helping to interpret the observations related to pathology and growth of the broilers that are differently treated. Many researchers are moving away from the use of anti-coccidial drugs against coccidiosis due to drug resistance build up in these protozoa, and due to consumer's awareness of drug residues in poultry products. Accordingly, researchers in

the last decade directed their research towards manipulation of the chicken immunity (Lillehoj, 1994; Caron *et al.*, 1997; Pinard-Van der Laan *et al.*, 1998; Yun *et al.*, 2000; Dalloul and Lillehoj, 2006). The humoral immunity against *Eimeria* spp. infections seems to play a minor role in protection, while the cell-mediated responses is proved to confer resistance to this protozoan disease (Lillehoj and trout, 1996). The accomplishment of the chicken genome project helped in uncovering new genes expressing vital cytokines and chemokines that are involved in significant immune responses and inflammatory reactions (Hughes and Bumstead, 2000; Kaiser *et al.*, 2005; Hong *et al.*, 2006a). This led workers to study the immune-related gene expression following *Eimeria* spp. infection of chickens (Hong *et al.*, 2006c). The most prominent increase in gene expressions against *Eimeria* spp. infection were seen in pro-inflammatory increase of IL-12, and IL-15 (Th1 and Th2 cytokine responses), increasing post primary infection with coccidia (Hong *et al.*, 2006c), followed by an increase in IL-8 chemokine, a mediator of cell migration during inflammation caused by *Eimeria* infection.

2. *Purpose of the study*

The objective of Study C was to investigate the impact of intermittent administration of EPB in the first three days of each of the first 4 weeks of the life of broilers and its role in alleviation of pathologic effects. This pathogenic effect was deduced from weight gains, feed conversion, score of intestinal lesions, and dynamics of immuno-modulation at 34 days of age, by measuring IL-6, IL-8, IL-12, and IL-15 in differently treated groups of chickens that were challenged or unchallenged at 28 days.

3. *Experimental design*

- A total of 100 day-old Cobb birds were divided into five groups: treatment #1, treatment #2, treatment #3, treatment #4, & treatment #5, these treatments included 20 birds per group.

Treatment #1: EBP treated at ages of 1-3, 8-10, 15-17, and 22-24 days, and no challenge. Expected hypothesis of Treatment #1: Best performance among the 5 treatments

Treatment #2: EBP treated at ages of 1-3, 8-10, 15-17, and 22-24 days, followed by a challenge with *Eimeria* spp. only at 28 d. Expected hypothesis of Treatment #2: This treatment might prove if the intermittent administration of EBP leads to protection in broilers against a challenge with *Eimeria* spp. at the critical age of 28 days.

Treatment #3: EBP treatment at ages of 1-3, 8-10, 15-17, & 22-24 d., followed by a double challenge with *Eimeria* spp. and *Cl. Perfringens* at 28 d. of age.
Expected hypothesis of Treatment #3: This treatment might prove that intermittent administration of EBP leads to protection in broilers against double challenge by *Eimeria* spp. and the secondary associated *Cl. Perfringens* at the critical age of 28 days.

Treatment #4: Deprived of EBP treatment and challenged at 28 d. with *Eimeria* spp. only. Expected hypothesis of Treatment #4: This Treatment #4 might prove that the deprivation from EBP in *Eimeria* spp.-challenged birds will lead to lower performance than EBP-treated birds that are challenged similarly (Birds in Treatment #2)

Treatment #5: Deprived of EBP treatment and challenged at 28 d. of age by *Eimeria* spp. + *Clostridium perfringens*. Expected hypothesis of Treatment #5: This Treatment #5 is needed to compare to Treatment #3 that had the same bivalent challenge, but

administered the EBP, with expectation of better performance in birds of Treatment #3 compared to those of Treatment #5.

- Birds were weighed individually at one-day old and distributed into 5 treatments of 20 birds each, in a way to obtain similar weights of birds in all pens.
- The corrugated papers of the five treatments were not changed after they were inoculated with *Eimeria spp.* and *Cl. perfringens* at the age of 28d, allowing the shedding of oocysts in the fecal material and resembling field conditions.
- Feed consumption at ages of 14, 28, and 34 days was recorded for all 5 groups.
- Hundred birds were weighed individually at the ages of 14, 28, and 34 days, to allow obtaining percent weight increase and feed conversion ratio
- Mortality in each broiler group during the rearing period was recorded at the age of 14, 28, and 34.
- Blood samples were taken from 3 birds of each of the 5 treatments at the ages of 28 and 34 days and were centrifuged to obtain the serum for plasma NO_2^- analysis (Allen & Teasdale, 1994a) (Fig. 37). This analysis reveals the impact of EBP on immuno-modulation of phagocytosis, an important Cell-Mediated Immune component that could reduce the multiplication of *Eimeria* spp. and consequently its related lesions in the intestine. The used Nitrite Kit is called "Griess Reagent Kit" for Nitrite Determination, by Invitrogen Molecular Probes Co., Eugene, Oregon, USA.

Fig. 37. Left: Blood samples were taken from brachial veins of 3 birds of each of the 5 treatments. Right: blood serum was collected and stored in 2 ml Eppendorff tube after centrifugation

- Birds of all groups were sacrificed at the age of 34 days and lesion scores were assigned to gross lesions present in the duodenum, jejunum, ileum and cecum of each sacrificed bird. The scores were 0 (no gross lesions), 1 (mild inflammation), 2 (moderate inflammation) and 3 (severe inflammation).

- Average oocyst counts of *Eimeria* spp in each of the 4 intestinal organs the (duodenum, jejunum, ileum and cecum) of differently treated broilers at 34 days of age was determined confirming the presence of oocysts in the challenged groups and their absence from the unchallenged group. These 4 intestinal organs were taken from 3 birds out of 20 birds found in each treated broiler group. In parallel, another 4 intestinal organs were taken from the same 3 birds for collection of the intestinal intraepithelial cells. Details of this collection will follow.

- qRT-rt PCR oligonucleotide primers for cytokines, chemokines, and Glyceraldehyde 3-phosphate dehydrogenase (GADPH) control was applied on intestinal intraepithelial lymphocytes (IEL) at 34 days of age. The targeted quantitation of cytokines and chemokines included:

- Pro-inflammatory cytokine measurement namely, IL-6.
- Th1 and Th2 cytokine response by measuring IL-12 and IL-15.
- Chemokine measurement of IL-8.

- Details of optimization of qRT-rtPCR will follow.
- The mean of the above parameters were compared by ANOVA followed by Tukey's test reporting significant difference in means at $P<0.05$. The chi-square test was used to compare % mortality among differently treated groups.

4. *Eimeria inocula*

The *Eimeria* spp. used in this challenge contained 4 species that are involved in major poultry coccidiosis outbreaks. The Oocyst counts of each specie delivered to each bird was as follows: *Eimeria acervulina* (7.5×10^4), *Eimeria tenella* (7.5×10^4), *Eimeria maxima* (7.5×10^4), and *Eimeria necatrix* (1.5×10^4).

5. *Clostridium perfringens inocula*

a. <u>*Clostridum pefringens* 3 % transmittance suspenion preparation</u>

- Incubate 5ml of Thioglycollate broth with a loop-full amount of *Cl. Perfringens* suspension and inoculate anaerobically at 37 °C for 8 hours. At Log phase transfer these 5ml of *Cl. perfringens* culture into 200 ml of Thioglycollate broth. At log phase, harvest the *Cl. Perfringens* by the following steps:
  - Centrifuge aliquots of 50 ml. suspension at 5000 rpm for 10 minutes.
  - Remove supernatant and wash the pellets with 20 ml sterile saline (0.85 % NaCl).

- Repeat centrifugation and washing for two additional times
- Re-suspend the pellets in 3 ml sterile saline and pool them in one aliquot
- Adjust transmittance of the bacterial suspension to 3% at 540 nm using a blank saline.

b. *Clostridum perfringens* culture adjustment to 10^6/ml

- Perform plate counting using TSN (Tripticase Sulfate Neomycine) agar (Anaerobic medium) as per the below protocol:
  - The 3 % transmittance bacterial suspension is serially diluted in sterile saline in a dilution of 1/10 up until 10^{-10} dilution.
  - Add 0.1 ml of each dilution to a corresponding empty petri dish.
  - Pour around 25ml of TSN of over the bacterial suspension and mix gently, using the letter “8”movement.
  - Let the agar solidify for around 15 minutes at room temperature.
  - Incubate under anaerobic conditions for 24 hours at 37°C
  - Count the plate that has between 30-300 cfu (colony forming unit) (Fig. 38).
- Adjust the 3 % trasmittance culture to 10^6 cfu per ml accordingly.
  - Determine the count in the original 3% trasmittance culture (C1) by the formula:
  - C1= Counted plated dilution (cfu) x dilution factor x 10
  - Using the formula C1V1=C2V2 formula to determine (V1), in which

- C_1 = Count of the original 3 % transmittance culture
- C_2 = final targetted count of the culture inoculated to each birds (adjusted to 10^6 cfu)
- V_1 = volume of the original 3 % transmittance culture to be diluted (unkown).
- V_2 = final volume after dilution that is given to each bird (1ml)

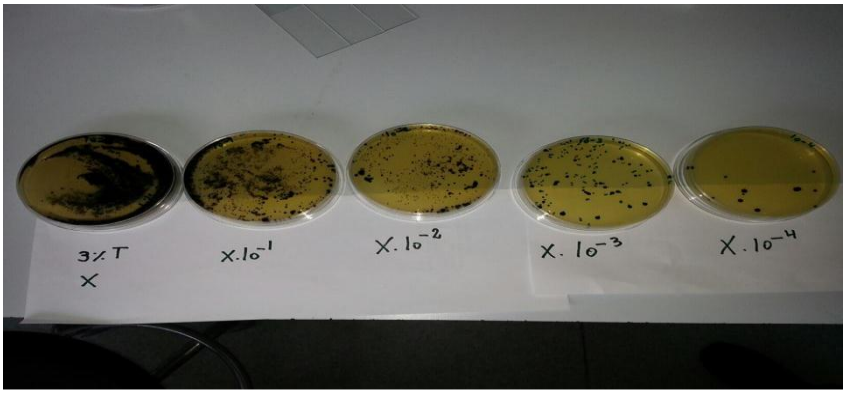


Fig. 38. *Clostridum perfringens* colonies are shown in black colonies, with entire edges

6. ***Echinacea-based preparation (EBP) treatment***

Intermittent administration of EBP at 4.6 milliliter per liter of drinking water, administered *ad libitum* at the assigned days.

Note that Feed formulation, Broiler chicks, and oocysts counting technique respectively are the same as described under Study A.

7. ***Plasma Nitrite (NO_2^-) analysis***

- Mix together equal volumes of N-(1-naphthyl) ethylenediamine and sulfanilic acid to form the Griess Reagent.
- Mix the following:

- An amount of 100 µL Griess Reagent
- An amount of 300 µL of the sample for determination of its nitrite content
- An amount of 2.6 ml of deionized water

Note: Nitrate concentrations in the samples should fall within the linear range of the assay approximately (1- 100 µM).

- Incubate the mixture for 30 minutes at room temperature.
- Prepare a photometric reference blank sample by mixing 100 µL of Griess Reagent and 2.9 ml of deionized water
- Measure the absorbance of the sample for determination of its nitrite at 548nm relative to the reference blank sample.
- Convert absorbance reading to nitrite concentrations, using the calibrated standard curve.
- Calibrated Standard curve
  - Prepare sodium nitrite dilutions with concentrations between 1-100 µM. Use deionized water to dilute the nitrite standard solution as described under step 2 above. Read the absorbance of the different dilutions.
  - Plot a standard curve of nitrite concentration (x-axis) against absorbance (y-axis)
  - Read the nitrite concentrations of the experimental samples from the standard curve.

8. ***Intestinal intraepithelial lymphocytes (IELs) collection***

Each of the four intestinal parts, namely the Duodenum, Jejunum, Ileum and Cecum were removed from three chickens in each group, cut longitudinally, and washed three times with ice-cold Hank's balanced salt solution (HBSS) containing 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma, St. Louis, MO). The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM Ethylene-Diamine-Tetraacetic Acid (EDTA) and 5% fetal calf serum (FCS) with active enzymes and incubated for 20 min at 37 °C with constant swirling. Cells released into the supernatant were pooled, passed through nylon wool (Robbins Scientific, Sunnyvale, CA) to remove dead cells and cell aggregates and washed twice with 1 ml of HBSS. IELs were purified on a 5 ml Discontinuous Percoll Density Gradient by centrifugation at 600 × g for 25 min at 24 °C (Chai and Lillehoj, 1988). IEL layer was collected using a micropipette and transferred into 1.5 ml Eppendorff tube. The tube and its contents were snap frozen using liquid nitrogen and kept at -80°C for PCR analysis.

9. *qRT-rt PCR for quantitation of cytokines and chemokines in IELs*

a. RNA extraction and Reverse transcription

IELs count was standardized to $1.5x10^6$ cell/ml using Giemsa stain and McMaster chamber method. Total RNA was extracted from IELs using TRIzol (Sigma, St. Louis, MO) as recommended by the manufacturer. The RNA was reverse-transcribed using Enhanced Avian Reverse Transcriptase (Sigma, St. Louis, MO) according to the manufacturer's recommendations. Briefly, 8.3 µL of RNA extract was combined with one microliter of 5mM of each dNTP, and 0.7 microliter of Anchored oligo $(dT)_{23}$ (50 mM). The mixture is placed in the thermal cycler at 70°C for 10

minutes and cooled to room temperature. Twenty units of Enhanced Avian Reverse Transcriptase, 2 microliters of 10x AMV-RTbuffer, and 6 microliters of water were added; the mixture was incubated at 42 °C for 50 minutes, and the reaction was stopped by heating at 94 °C for 4 min.

b. Real-time PCR

The primer sets used for the amplification of the Housekeeping gene (GAPDH) and the Four target genes (IL-6, IL-8, IL12, and IL 15) are listed in Table 14.

Table 14. Sequence of the oligonucleotide primers used in Real-time PCR

RNA target	Primer sequences		Size for PCR product (bp)
	Forward	Reverse	
GAPDH	5'GGTGGTGCTAAGCGTGTTAT3'	5'ACCTCTGTCATCTCTCCACA3'	264
IL-6	5'CAAGGTGACGGAGGAGGAC3'	5'TGGCGAGGAGGAGGGATTTCT3'	254
IL-8	5'GGCTTGCTAGGGGAAATGA3'	5'AGCTGACTCTGACTAGGAAACTGT3'	200
IL-12	5'AGACTCCAATGGGCAAATGA3'	5'CTCTTCGGCAAATGGACAGT3'	274
IL-15	5'TCTGTTCTTCTGTTCTGAGTGATG3'	5'AGTGATTTGCTTCTGTCTTTGGTA3'	243

Real time PCR was performed using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, St Louis, MO). Briefly 2.5 microliters of the cDNA, 2 microliters of the forward (9μM) and the reverse (3μM) primers, 1microliter of 25mM $MgCl_2$, 12.5 microliters of 2X JumpStart Ready Mix were mixed together and the volume was completed to 25 microliters with PCR reagent water. The PCR amplification was performed using C1000Touch Thermocycler (BioRad, 2000 Alfred Nobel Drive, Hercules, CA). The cycling conditions for the housekeeping and each of the target genes are presented in Table 15.

Table 15. Cycling conditions of the Real-time PCR for the amplification of GAPDH and target genes of four interleukins

RNA target	Cycling conditions
GAPDH	95°C for 3 min, then 40 cycles of: 95°C for 10s, 55°C for 30s
IL-6	95°C for 3 min, then 40 cycles of: 95°C for 10s, 59.8°C for 30s
IL-8	95°C for 3 min, then 40 cycles of: 95°C for 10s, 57.9°C for 30s
IL-12	95°C for 3 min, then 40 cycles of: 95°C for 10s, 59.3°C for 30s
IL-15	95°C for 3 min, then 40 cycles of: 95°C for 10s, 57.6°C for 30s

The $2^{-\Delta\Delta Ct}$ method was adopted as a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR (Livak & Scmittgen, 2001; Schmittgen & Livak, 2008). Relative quantification relates the PCR signal of the target transcript in a treatment group to that the unchallenged controls in Treatment The $2^{-\Delta\Delta Ct}$ data analysis relies on the following formula:

$\Delta\Delta Ct$ = (Ct of target (IL-8, IL-12, IL-15 or IL-6) – Ct of GAPDH)$_{Treatment}$ – (Average Ct of target – average Ct of GAPDH) $_{Control}$.

Ct= Threshold cycle, relative measure of the concentration of target gene in the PCR reaction
Δ= Ct of target gene (treatment) - Ct of reference gene (control)
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase (reference gene)

CHAPTER IV

RESULTS AND DISCUSSION

A. First Study: Production and pathogenesis in broilers subjected to differently-timed *Eimeria* spp. challenge.

Table 16 shows the percent increase in weights of controls and groups of birds challenged at different ages of 14, 21, 28, and 35 days old, during the 6 days-incubation period following each challenge.

Table 16. Percentage of weight increase of controls and *Eimeria*-challenged birds after 6 days of challenge administered at different ages

Bird group[1]	Age at challenge (days)	Mean percentage of weight increase at 6 days period following each challenge		
		Controls	Challenged	SEM
1	14	96.3	88.2	
2	21	69.0	54.5	
3	28	41.2	31.7	
4	35	28.8	20.2	
Mean		58.8[a]	48.6[a]	10.0

[1]Each group started with 10 birds, except for group 4 that started with 8 birds
[a,b]Means in a row followed by the same superscripts are not significantly different (P>0.05)

All groups of birds challenged at different ages had a lower percentage of weight increase during the 6 days-incubation period compared to their respective control-unchallenged groups. This reduction in weight gain by the challenged group in comparison to controls was approximately of a mean equivalent to 10%. The mean % weight increase of all control groups didn't differ statistically from the challenged groups (P>0.05), in spite of the consistent trend of reduction in % weight gain by the challenged compared to control groups. However, this reduction of around 10 % in

weight gain by challenged birds is of great significance in the economy of intensive broiler production (Cahaner & Leenstra, 1992; Havenstein *et al*., 2003).

Table 17 shows the comparison of the mean feed conversion in control versus challenged groups. There was a consistent higher feed conversion in challenged groups compared to their respective controls, with a statistically higher mean conversion in challenged groups compared to controls ($P<0.0.5$). This inefficiency in feed conversion to weight by the challenged groups is most likely due to the pathogenesis of the *Eimeria* spp. challenge on the intestine, affecting negatively the feed digestion (Major *et al.*, 1978; Assis *et al.*, 2010). The feed conversion parameter is of paramount importance (Williams, 1999a; Havenstein *et al*., 2003) in this experimental design model that can statistically differentiate the performance between controls and challenged birds. In another words, each bird in the challenged groups has to eat an average of 3.1 Kg of feed to convert it to 1 Kg of live weight, while each bird in the controls has to eat only 1.7 Kg of feed to convert it to 1 Kg of live weight. This significant inefficiency in converting feed to live weight in challenged birds has a great negative impact in economic losses in intensive poultry production operations (Williams, 1999a). The respective hypothesis for Study B and Study C of the project was to determine if the *Echinacea*-based preparation (EBP) treatment (long term and intermittent respectively) can improve the feed conversion in *Eimeria*-challenged birds to be closer to the low values obtained by the control groups.

Table 17. Feed conversion of controls and *Eimeria*-challenged birds at 6 days-incubation period following challenges administered at different ages

Bird group[1]	Age at challenge (days)	Feed conversion during 6 days following challenge		
		Controls	Challenged	SEM
1	14	1.1	3.0	
2	21	1.7	3.3	
3	28	2.1	2.5	
4	35	2.1	3.7	
Mean		1.7[a]	3.1[b]	0.3

[1]Each group with 10 birds, except for group 4 that started with 8 birds
[a,b]Means in a row followed by different superscripts are significantly different ($P<0.05$)

Table 18 presents the data related to frequency of mortality in the control and challenged groups during the 6 days-incubation period following the challenge. The mortality in the groups challenged at 14 and 21 days of age was higher than their corresponding control groups. However, there was no mortality in groups of birds challenged at 28 and 35 days of age and their corresponding control groups, which resulted in insignificant difference in the mean frequency of mortality between all control and challenged groups ($P>0.05$). The challenge model seems to create mortality if given at 14 and 21 days of age but not in older birds (Brackett & Bliznick, 1952), In spite of the inefficiency in their feed conversion. The trend in getting higher mortality in birds challenged at 14 and 21 days of age compared to their corresponding controls will be useful in future evaluation of EBP ability to alleviate the mortality of birds challenged at younger ages.

Table 18. Frequency of mortality of controls and *Eimeria*-challenged birds at 6 days period following challenges administered at different ages

Bird group[1]	Age at challenge (days)	Frequency of mortality (%) in controls and *Eimeria*-challenged birds at 6 days period following challenges administered at different ages		
		Controls	Challenged	SEM
1	14	1 (10%)[a]	4 (40%)[a]	
2	21	1 (10%)[a]	2 (20%)[a]	
3	28	0 (0%)[a]	0 (0%)[a]	
4	35	0 (0%)[a]	0 (0%)[a]	
Mean		0.5 (5%)[a]	1.5 (15%)[a]	0.5

[1]Each group is started with 10 birds, except for group 4 starting with 8 birds
[a]Frequencies in a row followed by the same superscript are not significantly different (P>0.05)

Table 19 compares the gross lesions score in different parts of the intestine of controls and groups of chickens challenged at different ages, using Johnson & Reid (1970) method. The mean score of the lesions in different parts of the intestine of challenged birds was always higher in the groups of birds administered the challenge at earlier age of 14 and 21 days old compared to birds challenged at older ages of 28 and 35 days. This data correlates with the mortality data shown in Table 18, in which birds challenged at older ages of 28 and 35 days had no mortality, which could be related to milder mean score of lesions shown in Table 19 (Long *et al*., 1980; Ramsburg *et al.,* 2003; Chapman *et al.,* 2005). However these milder lesions in groups challenged at older ages of 28 and 35 days seem to raise the inefficiency of the feed conversion of these birds (Table 17). In addition, most of the examined intestinal parts had higher significant lesion scores in birds challenged at 14, 21, and 28 days of age compared to the same intestinal parts of their corresponding control groups (P<0.05). This significant difference in mean lesion score of the different parts of the intestine disappeared between the birds of the group challenged at 35 days of age compared to birds of its corresponding control (P>0.05).

The results related to mean lesion score of the different parts of the intestine in challenged versus controls is indispensable for future evaluation of EBP ability to reduce the significance in differences of mean score of gross lesions in different intestinal parts of the challenged compared to control groups.

Table 19. Mean score of lesions in different parts of the intestine in controls and *Eimeria*-challenged birds at the end of the 6 days-incubation period following challenges administered at different ages

Bird group[1]	Age at challenge (days)	Intestine part	Mean score of lesions at 6 days period following each challenge		
			Controls	Challenged	SEM
1	14	Duodenum	0.0^{a}	1.5^{b}	0.25
		Jejunum	0.0^{a}	1.1^{b}	0.18
		Ileum	0.0^{a}	1.6^{b}	0.25
		Cecum	0.0^{a}	1.1^{b}	0.21
2	21	Duodenum	0.2^{a}	2.0^{b}	0.27
		Jejunum	0.0^{a}	1.7^{b}	0.25
		Ileum	0.1^{a}	1.6^{b}	0.26
		Cecum	0.2^{a}	1.6^{b}	0.25
3	28	Duodenum	0.6^{a}	1.4^{a}	0.25
		Jejunum	0.3^{a}	1.6^{b}	0.25
		Ileum	0.0^{a}	0.6^{b}	0.15
		Cecum	0.0^{a}	0.9^{b}	0.22
4	35	Duodenum	0.25^{a}	0.5^{a}	0.18
		Jejunum	0.0^{a}	0.38^{a}	0.14
		Ileum	0.0^{a}	0.38^{a}	0.14
		Cecum	0.0^{a}	0.13^{a}	0.06

[1] Each group started with 10 birds, except for group 4 that started with 8 birds
[a,b]Mean score of lesions followed by different superscripts are significantly different ($P<0.05$). Lesion scores range between 0-3.

Table 20 demonstrates the data related to mean oocyst counts in different intestinal parts of controls versus groups of birds challenged at different ages. The control birds kept free from infection until the end of the experiment at the age of 41 days. This fact proved the efficiency of management followed in strict isolation units used for rearing the experimental birds. The mean oocyst counts was always significantly higher in all examined parts of the challenged groups compared to their corresponding controls ($p<0.0.5$), except in the group challenged at 35 days of age, in

which only the jejunum organ maintained a significantly higher mean oocyst counts compared to its respective organ of the corresponding control birds.

This data is important also for future evaluation of the EBP ability to reduce the significant differences in oocyst counts in different parts of the intestine of challenged birds compared to their controls (Ogbe *et al*., 2009).

Table 20. Mean oocysts count per gram of intestinal part[1] in controls and *Eimeria*-challenged birds at end of 6 days-incubation period following challenges administered at different ages

Bird group[2]	Age at challenge (days)	Intestine part	Mean[3] oocyst counts per gram of intestinal organ at end of 6 days period following challenges administered at different ages		
			Controls	Challenged	SEM
1	14	Duodenum	0^{a}	42254^{b}	9744
		Jejunum	0^{a}	67839^{b}	16177
		Ileum	0^{a}	112843^{b}	35342
		Cecum	0^{a}	96666^{b}	26708
2	21	Duodenum	0^{a}	33715^{b}	8584
		Jejunum	0^{a}	36636^{b}	9343
		Ileum	0^{a}	41896^{b}	10173
		Cecum	0^{a}	48041^{b}	14776
3	28	Duodenum	0^{a}	6578^{b}	1438
		Jejunum	0^{a}	12321^{b}	3075
		Ileum	0^{a}	19029^{b}	4566
		Cecum	0^{a}	7309^{b}	1978
4	35	Duodenum	0^{a}	15344^{a}	6956
		Jejunum	0^{a}	6506^{b}	1569
		Ileum	0^{a}	2912^{a}	921
		Cecum	0^{a}	2367^{a}	756

[1]A length of about 1 cm was cut from the middle of each intestinal organ, and a pool of the cut same organ from 3 birds in the same treatment was formed. Each pooled organ was weighed to calculate oocyst counts/gram of the pools organ.
[2]Each group started with 10 birds, except for group 4 that started with 8 birds.
[3]Mean oocysts count/intestinal organ is calculated from three pooled samples, in which each is a pool of three same organ collected from three birds.
[a,b]Oocysts count in a row followed by different superscripts are significantly different ($P<0.05$).

In conclusion, the experimental design followed in the first part of this research resulted in significant statistical differences in most measured parameters between controls and challenged birds, including the feed conversion, lesion scores and oocyst

counts in different parts of the intestine. In addition, and in spite of the insignificant differences in % weight gain and mortality frequency between controls and challenged groups, still there is a trend of reduction in weight gain and in getting higher mortality in challenged birds compared to controls, a fact that affects significantly the economy of broiler intensive husbandry. These significant differences in identified parameters, and the trend in differences of the other parameters, will allow for future evaluation of the efficacy of new developed immounomodulators or new coccidiostats aiming to counter the huge negative economic impact of coccidiosis in broiler industry around the world.

B. Second Study: Production and pathogenesis in long term-supplementations of *Echinacea*-based preparation (EBP) to broilers subjected to differently timed *Eimeria* spp. challenge.

Table 21 compares statistically the mean percent of weight increase in the 6 days-period following each of the four challenges administered to the 4 groups of broilers at the respective ages of 14, 21, 28, and 35 days. Negative control (NC) groups were not treated with *Echinacea*-based preparation (EBP) nor challenged, Positive control (NC) groups were not treated with EBP and challenged at different ages, C14 groups were treated with EBP for 1-14 days period and challenged at different ages, and C21 groups were treated with EBP for 1-21 days period and challenged at different ages.

Table 21. Mean percent of weight increase of Negative control (NC) groups, and *Eimeria*-challenged birds (PC, C14 and C21 groups), observed after 6 days of challenge that was administered at different ages

Age at challenge (days)	Percent weight increase of the 4 groups[1] in the 6 days period following each challenge				
	NC	PC	C14	C21	SEM[2]
14	88.9	22.9	88.5	86.7	
21	43.3	23.3	13.5	4.9	
28	53.5	20.4	18.9	18.2	
35	27.7	17.8	16.6	13.16	
Mean % weight increase	53.3[a]	21.1[a]	34.4[a]	30.7[a]	7.2

[1] Treatments in each group started with 10 birds
Negative controls (NC): deprived of EBP and *Eimeria* spp. challenge,
Positive controls (PC): deprived of EBP but *Eimeria* spp. challenged,
C14 and C21 were administered in drinking water the EBP for 1-14 and 1- 21 days period respectively and *Eimeria* spp. challenged.
[2]SEM= Standard Error of Means in the same row.
[a]Means of % weight increase in a row followed by the same superscript are not significantly different (P>0.05).

In the *Eimeria* spp.-challenged treatments (PC, C14 and C21), the lowest mean % weight increase was obtained in the challenged treatment birds that were deprived of EBP (mean of 21.1%), compared to that obtained in C14 and C21 treatments, that were

treated respectively with EBP at 1-14 and 1-21 days periods of age (34.4 % and 30.7% respectively). However, the mean % weight increase was better in the Negative control birds (NC) that were deprived of EBP and not challenged with *Eimeria* spp. (53.3%).

It is worth nothing that a great improvement in weight gain of *Eimeria*-challenged and EBP-treated birds (C14 and C21) was noted in comparison to *Eimeria*-challenged and EBP-deprived birds (Positive control, PC), when *Eimeria* was inoculated at 14 days of age. This resulted in almost equal % weight gain in birds of C14 and C21 equivalent to 88.5 % and 86.7 % respectively, compared to the corresponding birds of the treatment PC that had a percent weight gain of only 22.9 %. The difference between the treatments C14 or C21 and PC was about 65 % which is of great economic significance to poultry industry (Cahaner & Leenstra, 1992; Havenstein *et al*., 2003).

It is worth nothing that the Negative control treatment (NC), had a % weight gain of 88.9%, equivalent to that obtained by the C14 and C21 birds for the same incubation period of *Eimeria* spp., following the challenge that occurred at 14 days of age. Unfortunately, the % weight gain in the EBP- treated birds (C14 and C21), that were challenged at 21, 28, and 35 days of age, didn't improve over that of the corresponding birds of PC that were deprived of EBP.

The C14 and C21 treatment birds challenged at 21 d of age and treated with EBP showed lower % weight increase than the corresponding PC treatment, deprived of EBP. The decrease in % weight increase of C14 and C21 compared to corresponding PC can justify that EBP treatment has an adverse effect and increased the pathogenesis of the disease (Botsoglou *et al*., 2004; Huntley *et al*., 2005). The long-term treatment of EBP in C21, resulted in marked adverse effect on % weight increase when was

observed at 28 days of age (*Eimeria*-challenge at 21days). The EPB treatments in C14 and C21 birds resulted in observed marked diarrhea that may be related to this adverse effect (Huntley *et al*., 2005).

Table 22 shows the mean feed conversion of the 4 treatments of broilers (NC, PC, C14, and C21) in the 6 days period following each of the four challenges administered at different ages.

Table 22.The mean feed conversion of Negative control (NC) groups, and *Eimeria*-challenged birds (PC, C14 and C21 groups), observed after 6 days of challenge that was administered at different ages

Age at challenge (days)	Feed conversion of the 4 groups[1] in the 6 days period following each challenge				
	NC	PC	C14	C21	SEM[2]
14	1.79	3.51	2.38	2.55	
21	1.39	3.21	1.96	7.24	
28	1.45	3.58	3.14	3.23	
35	1.52	1.725	1.80	1.92	
Mean	1.54[a]	3.01[a]	2.32[a]	3.735[a]	0.36

[1] Treatments in each group started with 10 birds
Negative controls (NC): deprived of EBP and *Eimeria* spp. challenge,
Positive controls (PC): deprived of EBP but *Eimeria* spp. challenged,
C14 and C21 were administered in drinking water the EBP for 1-14 and 1-21 days respectively and *Eimeria* spp. challenged.
[2]SEM= Standard Error of Means in the same row
[a]Means of feed conversion in a row followed by the same superscript are not significantly different (P>0.05).

Again we are seeing an improvement in feed conversion of EBP-treated birds challenged at 14 days of age in C14 and C21, with obtained respective feed conversion ratios of 2.38 and 2.55, compared to a conversion of 3.51 obtained in the corresponding treatment PC birds that were similarly challenged, but deprived of the EBP. The mean feed conversion among the challenged birds (PC, C14, and C21) was the best in C14 birds that were treated for 14 days with EBP (P<0.05), and this improvement is of economic importance in intensive poultry production operations (Williams, 1999a). It is worth nothing that the birds in C14 treatment (EBP- treated and *Eimeria*-challenged)

had in three out of the 4 periods, following the 4 different timed challenges, an improvement in feed conversion over birds of PC treatment that were deprived of EBP but challenged similarly. In addition, the feed conversion in the Negative control (NC) unchallenged birds was always better than the *Eimeria*-challenged birds in the PC, C14 and C21 treatments ($P<0.05$).

Table 23 shows the mean percent mortality of the 4 treatments of broilers (NC, PC, C14, and C21) in the 6 days period following each of the four challenges administered at different ages.

Table 23. Mean percent mortality of Negative control (NC) groups, and *Eimeria*-challenged birds (PC, C14 and C21 groups), observed after 6 days of challenge that was administered at different ages

Age at challenge (days)	Percent mortality in the 4 groups[1] during the 6 days period following challenges administered at different ages				
	NC	PC	C14	C21	SEM[2]
14	0%[a]	20%[a]	0%[a]	0%[a]	
21	0%[a]	20%[a]	0%[a]	0%[a]	
28	0%[a]	10%[a]	10%[a]	0%[a]	
35	0%[a]	0%[a]	0%[a]	10%[a]	
Mean % Mortality	0%[a]	12.5%[b]	2.5%[ab]	2.5%[ab]	1.8

[1] Treatments in each group started with 10 birds
Negative controls (NC): deprived of EBP and *Eimeria* spp. challenge,
Positive controls (PC): deprived of EBP but *Eimeria* spp. challenged,
C14 and C21 were administered in drinking water the EBP for 1-14 and 1-21 days respectively and *Eimeria* spp. challenged.
[2]SEM= Standard Error of Means in the same row
[a,b]Means % mortality in a row followed by different superscripts are significantly different ($P<0.05$).

The mean % mortality of 12.5 % in PC treatment (*Eimeria*-challenged birds and deprived of EBP) was significantly higher than that of Negative control (NC) treatment (0.0%). However the EBP treatment in C14 and C21 dropped the mortality to 2.5 % in both treatments, that was insignificantly different from that obtained in PC treatment ($P>0.05$). This reveals the impact of EBP administered either at 1-14 or 1-21

days of age on the survival of birds challenged with a cocktail of non-attenuated *Eimeria* spp.

Table 24 demonstrates the data related to the mean score of lesions in different intestinal organs of the 4 treatments (NC, PC, C14, and C21) at the end of the 6 days period following the 4 challenges at different ages.

Table 24. Mean score of lesions in different parts of the intestine in Negative control groups (NC), and *Eimeria*-challenged birds (PC, C14, and C21 groups), observed after 6 days of challenge that was administered at different ages

Age at challenge (days)	Intestine part	Mean score of lesions in the 4 groups[1] at 6 days period following each challenge				
		NC	PC	C14	C21	SEM[2]
14	Duodenum	0.2^{a}	1.1^{b}	1.3^{b}	1.6^{b}	0.14
	Jejunum	0.3^{a}	2.0^{b}	1.5^{b}	1.5^{b}	0.14
	Ileum	0.0^{a}	1.7^{b}	1.7^{b}	1.5^{b}	0.15
	Cecum	0.0^{a}	2.4^{b}	1.5^{b}	1.2^{ab}	0.21
21	Duodenum	1.2^{a}	2.4^{b}	1.8^{ab}	1.6^{ab}	0.16
	Jejunum	0.9^{a}	1.7^{a}	1.3^{a}	1.4^{a}	0.13
	Ileum	0.9^{a}	1.0^{a}	1.4^{a}	1.6^{a}	0.11
	Cecum	0.0^{a}	2.2^{b}	2.4^{b}	2.4^{b}	0.195
28	Duodenum	1.7^{a}	1.7^{a}	1.7^{a}	2.0^{a}	0.13
	Jejunum	0.5^{a}	1.6^{b}	1.7^{b}	1.9^{b}	0.13
	Ileum	0.6^{a}	2.1^{b}	1.1^{a}	2.2^{b}	0.14
	Cecum	0.3^{a}	2.5^{b}	2.5^{b}	2.3^{b}	0.18
35	Duodenum	1.3^{a}	2.1^{ab}	2.1^{ab}	2.5^{b}	0.13
	Jejunum	0.5^{a}	1.9^{b}	2.2^{b}	1.9^{b}	0.15
	Ileum	0.1^{a}	1.5^{b}	1.1^{b}	1.5^{b}	0.15
	Cecum	0.0^{a}	1.5^{b}	1.3^{b}	2.1^{b}	0.19

[1] Treatments in each group started with 10 birds
Negative controls (NC): deprived of EBP and *Eimeria* spp. challenge,
Positive controls (PC): deprived of EBP but *Eimeria* spp. challenged,
C14 and C21 were administered in drinking water the EBP for 1-14 and 1-21 days respectively and *Eimeria* spp. challenged.
[2]SEM= Standard Error of Means in the same row
[a,b]Mean score of lesions followed by different superscripts are significantly different ($P<0.05$). Lesion scores range between 0-3.

The lesion scores given in Table 24 were judged in the range of 0-3 using Johnson and Reid, (1970), and the mean score of the lesion in specific intestinal organ was compared among the 4 treatments (NC, PC, C14, and C21). The mean score of

lesions in the Duodenum, Jejunum, Ileum and Cecum of birds challenged at 14 days of age was significantly similar in *Eimeria*-challenged birds (PC, C14, and C21) that were EBP treated versus EBP deprived birds (PC treatment) ($P>0.05$). In addition, these mean scores in the 3 intestinal organs of all birds challenged at 14 days of age were significantly higher than those of the NC treatment birds ($P<0.05$). A peculiar result was obtained in the cecal lesions, showing similar low mean scores in birds that were EBP treated and challenged at 14 days of age (C14 and C21), equivalent respectively to 1.5 and 1.2 that were apparently lower than the cecal mean score lesions in birds of PC treatment (EBP deprived and *Eimeria*-challenged) (mean score of 2.4). This improvement in reduction of cecal mean lesion score of C14 and C21 that were EBP treated was also observed during our experimental observations, in which C14 and C21 treatment's litter was not tinged with blood which is an indication of decreased *Eimeria* pathogenicity (Conway & Mckenzie, 2007). This could be most likely responsible for the birds improvement in % weight increase (Table 21), feed conversion (Table 22), and mortality (Table 23). However, birds that were challenged at 21, 28 and 35 days of age respectively and treated with EBP (C14 and C21) did not show this improvement in cecal-mean score lesions compared to the corresponding *Eimeria*-challenged and EBP deprived birds of the PC birds. Moreover, in the majority of the data related to mean score lesions in Duodenum, Jejunum, Ileum and Cecum of birds challenged at 21, 28, and 35 days of age, there wasn't a consistent pattern of reduction in the mean score lesions in the EBP-treated birds in treatments C14 and C21 compared to the corresponding *Eimeria*-challenged birds that were deprived o the EBP (PC treatment), which could have reflected on statistical similarity in mean % weight increase, feed conversion, and mortality affecting negatively the positive data obtained in EBP treated

birds challenged at 14 days that showed improvements over *Eimeria*-challenged and EBP deprived birds.

The mean oocyst counts per gram of each of the 4 intestinal organs in the birds of the 4 treatments (NC, PC, C14 & C21) at the end of the 6 days-period following the challenges administered at different ages is shown in Table 25.

Table 25. Mean oocyst counts per gram of intestinal organ[1] in Negative control (NC) groups[2], and *Eimeria*-challenged birds (PC, C14 and C21 groups[2]), observed after 6 days of challenge that was administered at different ages

Age at challenge (days)	Intestine part	Mean[3]oocyst counts per gram of intestinal organ at end of 6 days incubation period following challenges administered at different ages				
		NC	PC	C14	C21	SEM[4]
14	Duodenum	0^{a}	8811^{a}	3124^{a}	5110^{a}	2399
	Jejunum	0^{a}	30467^{b}	8374^{a}	10938^{a}	3647
	Ileum	0^{a}	20401^{b}	7038^{ab}	14696^{ab}	2847
	Cecum	0^{a}	56865^{b}	17445^{ab}	27975^{ab}	7565
21	Duodenum	0^{a}	3161^{a}	1479^{a}	0^{a}	841
	Jejunum	0^{a}	9100^{b}	8098^{b}	10417^{b}	1384
	Ileum	0^{a}	11219^{b}	9478^{ab}	9428^{ab}	1622
	Cecum	0^{a}	12053^{ab}	20837^{bc}	37940^{c}	4519
28	Duodenum	0^{a}	6619^{a}	3842^{a}	4338^{a}	1220
	Jejunum	0^{a}	7495^{a}	9013^{a}	4658^{a}	1499
	Ileum	0^{a}	19880^{b}	8455^{ab}	8558^{ab}	2449
	Cecum	0^{a}	23868^{a}	2866^{a}	12159^{a}	3915
35	Duodenum	0^{a}	8444^{b}	2822^{ab}	3163^{ab}	1208
	Jejunum	0^{a}	8252^{a}	4597^{a}	11345^{a}	1705
	Ileum	0^{a}	3979^{a}	6340^{a}	10427^{a}	1547
	Cecum	0^{a}	5633^{a}	4690^{a}	11272^{a}	1651

[1]A length of about 1 cm was cut from the middle of each intestinal organ, and a pool of the cut same organ from 3 birds in the same treatment was formed. Each pooled organ was weighed to calculate oocyst counts/gram of the pools organ.
[2]Treatments in each group started with 10 birds
Negative controls (NC): deprived of EBP and *Eimeria* spp. challenge,
Positive controls (PC): deprived of EBP but *Eimeria* spp. challenged,
C14 and C21 were administered in drinking water the EBP for 1-14 and 1-21 days respectively and *Eimeria* spp. challenged.
[3]Mean oocyst counts/intestinal organ is calculated from three pooled samples, in which each is a pool of three individual organs collected from three respective birds.
[4]SEM= Standard Error of Means in the same row
[a,b,c] Oocyst counts in a row followed by different superscripts are significantly different ($P<0.05$).

There was a consistent improvement in reduction of the oocyst counts in the Duodenum, Jejunum, Ileum and Cecum of EBP-treated birds that were challenged at 14 days (C14 and C21 treatments) compared to counts obtained in PC treatment birds that were deprived of EBP and *Eimeria*-challenged at the same age. The reduced counts of oocysts in these 4 intestinal organs were insignificantly different from those obtained in birds of the corresponding NC treatment ($P>0.05$), due to large values of the SEM (Standard Error of Means). However, the mean oocyst counts in the Jejunum, Ileum and Cecum of PC treatment birds that were deprived of EBP and *Eimeria*-challenged at 14 days were consistently-significantly higher ($P<0.05$) than the means obtained by the corresponding NC treatment.

This pattern of improvement in the reduction of oocyst counts in the four intestinal organs wasn't seen in the birds that were challenged at 21, 28 and 35 days of age, which could explain their weak production performance presented in Tables 21, 22 and Table 23 (De Pablos *et al.*, 2010).

In conclusion, the improvement in the production of EBP-treated birds in treatments C14 and C21 was limited to the data obtained when the birds were challenged with *Eimeria* spp. at 14 days of age, which was associated with an apparent reduction in score of cecal lesions, and in the reduction in mean oocyst counts of the four intestinal organs that were insignificantly different from the oocyst counts data obtained by the corresponding challenged treatment that was deprived of EBP but challenged with *Eimeria* (PC).

It is recommended that the following Study C should have an intermittent supplementation of EBP in drinking water at the ages of 1-3, 8-10, 15-17 and 22-24

days, since the long-term administration of this preparation for 21 days proved to have negative effect on performance (Böhmer, 2009; Zahid, 2009).

C. Third Study: **Protection, production, and immuno-modulation in broilers with intermittent administration of *Echinacea*-based preparation (EBP) against challenges with *Eimeria* spp. alone and *Eimeria* spp. plus *Clostridium perfringens***

The percent mortality in the birds of the 5 treatments at the ages of 14, 28, and 34 d and their averages are given in Table 26. There was no significant difference in the mortalities, which is most likely due to the short challenge-incubation time of 6 days, allowing for only around one life cycle of the *Eimeria* spp (Allen *et al*., 1973). Future investigations could extend the incubation period beyond an earlier challenge time, to allow for two or more *Eimeria* spp. life cycles to occur, thus creating higher cytopathic effects in the intestine, with probably higher mortalities in non-protected birds.

Table 26. Mortality in differently treated broilers and up to market age of 34 d.

Treatment[1]#	% mortality at different ages (days)			Average % mortality
	14	28	34	
1	0.0	5.0	5.3	3.4[a]
2	0.0	5.0	0.0	1.7[a]
3	0.0	5.0	0.0	1.7[a]
4	0.0	5.0	0.0	1.7[a]
5	0.0	0.0	0.0	0.0[a]
SEM[2]				0.64

[1]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *Cl. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *Cl. perfringens.*
SEM[2]: Standard Error of Average % mortalities in the same column.
[a]Averages of % mortality in the last column, followed by the same alphabet superscript are not significantly different (P>0.05).

The averages of the weight of birds in the 5 treatments at different ages are shown in Table 27 The birds in Treatment 1 (EBP treated and deprived of challenge) resulted in consistent higher average weights at 14, 28, and 34 d. of age compared to the

weights of all 4 challenged-treatments. Among the 4 challenged treatments, birds in Treatment 4 and 5 that were deprived of EBP had the lowest weights at the market age of 34 d (1596 and 1608.5, respectively), compared to that of their counter –similarly challenged and EBP treated-birds of Treatment 2 (1609g) and Treatment 3 (1660g) (Hein, 1968a; Waletzky, 1970; Kipper *et al*., 2013).

Table 27. Average weight of differently treated broilers at different ages

Treatments[1]#	Average weight at different days of age		
	14	28	34
1	304.0[b]	1249.2[a]	1776.7[a]
2	294.0[a,b]	1149.4[a]	1609.2[a]
3	282.8[a,b]	1200.0[a]	1660.6[a]
4	254.3[a]	1190.3[a]	1596.7[a]
5	276.8[a,b]	1193.5[a]	1608.5[a]
SEM[2]	55.43	182.0	25.81

[1]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *Cl. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *C. perfringens.*
SEM[2]: Standard Error of Averages in the same column
[a-b]Averages of recorded weights in each column that are followed by different alphabet superscripts are significantly different ($P<0.05$).

The Feed Conversion ratios of the birds in the 5 treatments at different ages are shown in Table 28. An improvement in reduction of the average feed conversion ratio down to 1.64 was observed in birds of Treatment 3 that were EBP treated and challenged with *Eimeria* spp. and *Cl. Perfringens* compared to the 1.70 ratio obtained by the similarly challenged but EBP -deprived birds of Treatment 5 (Hein, 1976). EBP caused improvements in feed conversion in the co-infected broilers which indicates that feed conversion improvement at 34 d responded to EBP under more stressed conditions (Botsoglou *et al.,* 2004; Bozkurt *et al.,* 2012). In this study the

Eimeria infection was not severe to cause significant mortality or body weight loss at 34 d.

Table 28. Feed conversion ratios in differently treated broilers at different ages

Treatment[1]#	feed conversion at different ages (days)			Average feed conversion
	14	28	34	
1	1.58	1.90	1.65	1.71[a]
2	1.59	2.14	1.82	1.85[a]
3	1.59	1.86	1.46	1.64[a]
4	1.77	1.60	1.78	1.72[a]
5	1.65	1.81	1.63	1.70[a]
SEM[2]				0.044

[1]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *Cl. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *Cl. perfringens.*
SEM[2]: Standard Error of Averages of feed conversion in the same column
[a]Averages of feed conversion ratio in the last column, followed by the same alphabet superscript are not significantly different (P>0.05).

The impact of EBP treatment on protection against the challenge, as deduced from the average oocyst counts and the average lesion scores in each of the 4 intestinal organs (Johnson & Reid, 1970; Conway & Mckenzie, 2007), is shown in Tables 29 and 30, respectively. The average oocyst counts and the average lesion scores in most of the 4 intestinal organs were consistently lower in the EBP -treated and challenged birds compared to their counter EBP deprived birds that were similarly challenged.

Table 29. Average oocyst counts of *Eimeria* spp in each of the 4 intestinal organs of differently treated broilers at 34 days of age

Treatment[1]#	Average oocyst counts/gram of each of 4 intestinal organs			
	Duodenum	Jejunum	Ileum	Cecum
1	0.00[a]	0.00[a]	0.00[a]	0.00[a]
2	13571.65[a]	598812.59[a]	121587.26[a]	19538.88[a,b]

3	33562.41[a]	601029.65[a]	5338.74[a]	51061.28[a,b]
4	17652.93[a]	127633.68[a]	22489.90[a]	25851.11[a,b]
5	66432.20[a]	676054.33[a]	113325.52[a]	86476.61[b]
SEM[2]	9880	107695	22365	10289

[1]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *Cl. Perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *Cl. Perfringens.*
SEM[2]: Standard Error of Averages in the same column
[a-b]Averages of oocyst counts/g of each intestinal organ in a column that are followed by different alphabet superscripts are significantly different (P<0.05).

Table 30. Average lesion scores of each of the 4 intestinal organs of differently treated broilers at 34 days of age

Treatment[1]#	Average lesion scores of each of 4 intestinal organs			
	Duodenum	Jejunum	Ileum	Cecum
1	1.00[a]	0.56[a,b]	0.11[a]	0.00[a]
2	0.61[a]	0.50[a]	0.28[a,b]	0.06[a]
3	1.00[a]	1.44[c,d]	0.78[b,c]	0.61[b]
4	1.17[a]	1.23[b,c]	0.82[b,c]	0.71[b]
5	1.05[a]	1.95[d]	1.10[c]	0.85[b]
SEM[2]	0.067	0.097	0.078	0.070

[1]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *Cl. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *Cl. perfringens* (Fig. 39).
SEM[2]: Standard Error of Averages in the same column
[a-d]Averages of lesion score in each intestinal organ in a column that are followed by different alphabet superscripts are significantly different (P<0.05).

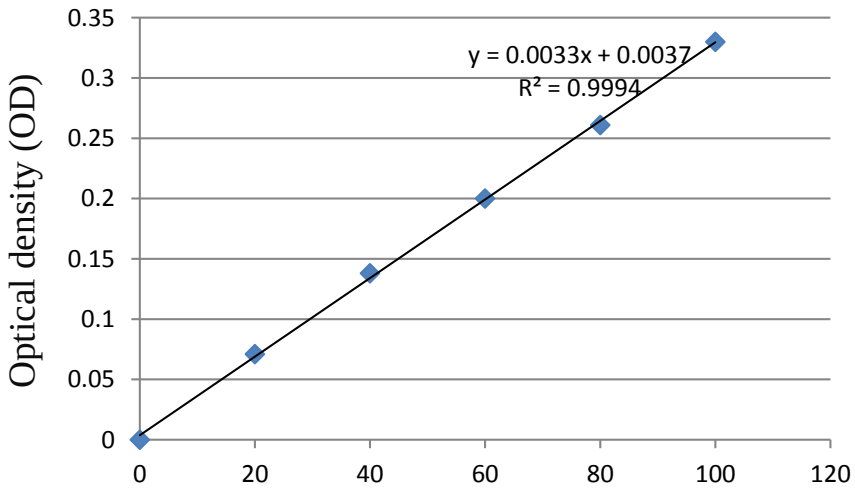
Fig. 39. Intestinal Jejunum, Lesion Score +3, treatment # 5: *Eimeria* spp. *+Cl. perfringens* infected and nontreated with EBP

Activated Macrophages and phagocytic leukocytes produce free radicals such as reactive oxygen species and nitric oxide (NO) that are toxic to bacteria and some parasites (Rosen *et al*., 1995; Adams *et al.,* 1990; Liew *et al.,* 1990; Gazzinelli *et al.,* 1993; Oswaldet *et al*., 1994; Petray *et al.,* 1994)**.** NO production is an effective mechanism in macrophages to kill intracellular protozoa and is involved in resistance and immunity to *Eimeria.* A significant increase in plasma nitrite and nitrate was seen in primary infections with *Eimeria acervulina, Eimeria tenella* and *Eimeria maxima* 6 days post infection. Sporulated oocysts of mixed infection of four species (*E. acervulina, E. maxima, E. necatrix, E. tenella*), when were inoculated to birds at 26 days of age, stimulated NO production and increased plasma nitrite and nitrate at 6, 10 and 14 days post infection progressively (Pirali Kheirabadi *et al.,* 2011) . NO could be toxic to coccidia as well as cells harboring them. Vascular iNOS (induced NO synthase) in the cecal blood vessels responsible for NO synthesis in vascular endothilum, promoted vasodilation in the infected ceca and enhanced hemorrhage. Inhibition of this mucosal iNOS did not affect the increase in plasma nitrite and nitrate levels and degree of parasite colonization in the ceca at 6 days post inoculation; however, its inhibition altered Gross lesions and hemorrhage (Ovington and Smith, 1992; Allen, 1994; Allen

and Teasdale, 1994b; Allen, 1997 a, b; MacMicking *et al.*, 1997). Nitrate (NO_2^-) and nitrate (NO_3^-) are stable forms of (NO) arising from the reaction of NO with peroxides (0_2^-) and oxyhemoglobin **(**Ignarro *et al.*, 1993). Increasing levels of the two ions in plasma is an indication of increased NO production within the tissue at time of infection (Stuehr and Marletta, 1985).

β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase responsible for 0_2^- production in the mucosa of the intestine, increased the potential for oxidative destruction and mucosal tissue sloughing in the *E.maxima* infected birds (Allen, 1997a). These free radicals are secreted by number of phagocytic cell types in response to stimulation by IFN-gamma (Oswald *et al.*, 1994; Petray *et al.*, 1994) and TNF-alpha **(**Adams *et al.*, 1990; Gazzinelli *et al.*, 1993**).** Higher levels of plasma $NO_2^- + NO_3^-$ in response to primary infection was reported with resistant breeds to coccidiosis (Allen and Lillehoj, 1998), but not in immune birds, suggesting a role of $NO_2^- + NO_3^-$ - in innate resistance rather than acquired immunity to coccidiosis.

The Standard curve (Fig. 40) is established relating the concentration of the serum Nitrite to the Optical Density at a wave length of 548 nm. The R square of the regression equation was 0.9994 with a P value < 0.05.



Concentration of the serum Nitrite (micromolar)

Fig. 40. The Standard curve relating the concentration of the serum Nitrite to the Optical Density at a wave length of 548 nm

The average serum Nitrite levels (NO_2^-), reflecting the phagocytes activities, in the broilers of the 5 treatments at challenge day (28 days of age) and 6 days post challenge (34 days of age) are shown in Table 31.

Table 31. The average serum Nitrite levels in broilers of the 5 treatments at the day of challenge (28 days of age) and 6 days post challenge (34 days of age)

Treatments[1]#	Average serum Nitrite level (micromolar) at 28 and 34 days of age		
	28 d	34d	SEM[3]
1	6.33[a,1]	0.61[a,1]	1.55
2	7.00[a,1]	5.91[a,1]	1.71
3	6.58[a,1]	1.64[a,2]	0.89
4	3.30[a,1]	4.10[a,1]	1.28
5	3.72[a,1]	1.97[a,1]	1.22
SEM[2]	0.84	0.83	

[1]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *Cl. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *Cl. perfringens.*
[a]Averages of serum Nitrite levels in a column that are followed by same alphabet superscript are insignificantly different ($P<0.05$).
[1,2]Averages in a row followed by different Arabic numerical superscript are significantly different
SEM[2]: Standard Error of Averages in the same column.
SEM[3]: Standard Error of Averages in the same row.

The intermittent EBP treatment in the first 4 weeks of the broiler's life in Treatments 1, 2, and 3 was able to result in almost double the average level of serum Nitrite at 28 days of age compared to the broilers that were deprived of the EBP in

Treatments 4 and 5. This higher trend in the level of serum Nitrite in EBP treated groups indicates a presence of inflammation and immuno-modulation of the phagocytes by the active ingredients of *Echinacea*. This suggests an increased activity of iNOS and NO production due to increased levels of interferon-gamma and TNF-alpha in EBP treated groups (Adams *et al.,* 1990; Gazzinelli *et al.,* 1993; Oswald *et al.,* 1994; Petray *et al.,* 1994). This serum Nitrite maintained its highest level at 6 days post challenge (34 d. of age) in broilers of Treatment 2 (5.91 micromolar) compared to the other treatments at the same age. Actually, this highest level of Nitrite in broilers of Treatment 2 at 6 days post challenge (Table 31) was associated with a consistent lowest average of oocysts and lesion scores in the 4 intestinal organs (Tables 29 and 30), which is contradicting to what was cited in literature (Brouet.& Ohshima,1995; Allen *et al.,* 1998). Significant reduction in the plasma $NO_2^-$and NO_3^- was found in *E. maxima* and curcumin treated birds, which explains the reduction in Gross Lesions due to this specie. Gross lesions due to *E.tenella* and *E.maxima* at day 6 were scored higher with increased plasma nitrite and nitrate due to increased vasodilation and mucosal sloughing (Allen, 1997a, b). Mucosal iNOS followed an increasing pattern as confirmed by qPCR mRNA levels in *E.acervulina* and *E. maxima* infections (Hong *et al*., 2006c). EBP may have potentiated systemic iNOS and protected the cells in the intestine from vascular iNOS and free radicals that might have caused increased blood and injury to the mucosa. The clear trend in immuno-modulation of the systemic phagocytes by EBP in Treatments 1, 2, and 3, did help in the greatest decline in multiplication of the *Eimeria* species and the greatest reduction of their corresponding intestinal lesions in birds challenged by *Eimeria* spp. alone.

In addition, the higher Nitrite levels at 28 d. of age in birds of Treatment 3 (EBP treated and challenged by both the *Eimeria* species and *Cl. perfringens*) compared to those of Treatment 5 (deprived of EBP and with a similar challenge to birds in Treatment 3) helped also in the consistent reduction of averages oocysts and lesion scores in birds of Treatment 3 compared to those in Treatment 5. However, the reduction in these averages in birds of Treatment 2 was of higher magnitude than that obtained by birds of Treatment 3, which is most likely due to the severity of the mixed infection created by the *Eimeria* spp. and *Cl. Perfringens* in birds of Treatment 3 that the immuno-modulation of phagocytes by EBP was not able to handle as effectively as in the case of challenge by *Eimeria* spp. alone. Histological examination of the intestinal tissue could have correlated between the Nitrite levels and the changes in the intestinal tissue at the ages of 28d. and 34d. of the birds.

The means of normalized amount of IL-8 chemokine transcript quantified in each of the 4 intestinal organs are shown on Table 32. It is worth noting that the normalization was done according to House Gene GAPDH and to transcripts of respective organs in control birds of Treatment 1; an example, if you read a mean of 'one' it indicates that the detected mean is one fold of the control mean, which indicates that the detected mean doesn't differ from the control group.

Table 32. Pro-inflammatory IL-8 cytokine in 4 intestinal organs of the differently treated broilers normalized[1] in relation to the controls of Treatment #1 at market age of 34d

Treatment[2]#	[1]Normalized mean amount of IL-8 in different intestinal organs relative to that of control birds in treatment #1			
	Duodenum	Jejunum	Ileum	Cecum
2	1.47^{a}	$2.28x10^{9\ a}$	0.44^{a}	0.38^{a}
3	0.19^{a}	$827.78x10^{9\ a}$	3.09^{a}	0.17^{a}
4	135.76^{b}	$36.57x10^{4\ a}$	0.00^{a}	1822.44^{a}
5	1285.01^{c}	255.11^{a}	45.49^{b}	1092.76^{a}

SEM[3]	123.39	$278.84x10^9$	5.74	314.64

[1]Normalized amount of IL-8 = $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct$ = (Ct of target (IL-8) – Ct of GAPDH)$_{Treatment}$ – (Average Ct of target (IL-8) – Average Ct of GAPDH) Control group 1.
[2]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *C. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *C. perfringens.*
SEM[3]: Standard Error of Means in the same column
[a-c]Averages of relative amount of IL-8 for each specified intestinal organ in a column, followed by different alphabet superscript are significantly different ($P<0.05$).

The means of normalized IL-8 in Table 32 show a great nearness of the mean fold to number 'One' in three out of the 4 examined organs namely, the duodenum, ileum, and cecum of birds in Treatment 2 and 3 that were treated with EBP, compared to higher folds in most data of respective intestinal organs of birds in Treatments 4 and 5 that were deprived of EBP.

IL-8 play a role in the initiation of the inflammation at the site of infection and its reduction is correlated to a decrease in the inflammatory response to *Eimeria* infections (Laurent *et al.,* 2001; Swaggerty *et al.,* 2004; Withanage *et al.,* 2004; Hong *et al*., 2006c). This IL-8 in EBP treated birds was significantly reduced in the duodenum compared to that detected in duodenum of EBP-deprived birds of Treatments 4 and 5 that had a respective similar challenge, which suggests a decrease in the inflammatory response between the mentioned treatments. The IL-8 was cited in literature as a potent stimulator of neutrophil activation (similar to hetrophils in chicken) and chemotaxis within the mucosa (Baggiolini *et al.*, 1989; Sturm *et al.,* 2005). Attracting phagocytic cells to the site of infection in the mucosa might increase mucosal NO production. Down regulation of IL-8 at the site of infection could have helped in alleviation of the injuries by decreasing NO production in the mucosa (Allen, 1997a, b). This fact reflect

the alleviation of challenge-immune injuries by EBP treatment in birds of Treatments 2 and 3, manifested in down regulation of transcription of IL-8 chemokine that is responsible to magnify the attraction of phagocytes and T-cells to the infection sites.

Chickens that were inoculated orally with *C. perfringens* following a primary challenge with *E.necatrix* inoculation, had significantly increased numbers of *C. perfringens* especially in the jejunum and ileum where the endogenous stages of *E.necatrix* takes place (Baba *et al.*, 1997). This fact could explain the high Il-8 in the Jejunum of treatments #2 and #3 (table 32). The IL-8 in the Jejunum was significantly increased in the documented research, boosting immune response caused by *Echinacea*-based preparation (EBP) as a result of the significant increase in number of *C. perfringens* and pathogencity of *Eimeria necatrix*. This boosted immune response injury was much less than the injury caused by *E.necatrix* alone or with *C. perfringens,* which explains the decrease in lesion scores of these treatments. Gross lesions due to host immune response and not the parasite reproduction was documented in literature, to evaluate flock performance to vaccination (Williams, 2003).

The amounts of IL-6, IL-12, and IL-15 in the four intestinal organs of all of the experimental birds were below the detectable limits of the applied Real-time PCR protocol namely, below 20.9ng/mcl of reverse transcribed DNA (Table 33).

Table 33. IL-6, IL-12, and IL-15 cytokines in 4 intestinal organs of the differently treated broilers normalized[1] in relation to the controls of Treatment #1 at market age of 34d

ND: Not Detectable
[1]Normalized amount of IL-8 = $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct$ = (Ct of target (IL-8) – Ct of GAPDH)$_{Treatment}$ – (Average Ct of target (IL-8) – Average Ct of GAPDH) $_{Control\ group\ 1}$.
[2]The five treatments are:
Treatment#1, EBP treated and deprived of challenge
Treatment#2, EBP treated and challenged with *Eimeria* spp. alone
Treatment#3, EBP treated and challenged with both *Eimeria* spp and *C. perfringens*
Treatment#4, deprived of EBP treatment and challenged with *Eimeria* spp alone
Treatment#5, deprived of EBP treatment and challenged with both *Eimeria* spp. and *C. perfringens*

In conclusion, the weights of challenged birds in two treatments that were administered the EBP were improved compared to that in counter birds of the two other treatments that were similarly challenged but deprived of the EBP. In addition, EBP improved the reduction of feed conversion of birds challenged with both the *Eimeria* spp. and *Cl. Perfringens* compared to similarly challenged birds that were deprived of EBP. Moreover, the EBP treatment resulted in a consistent reduction in oocyst and lesion scores of the 4 intestinal organs compared to that observed in similarly challenged birds that were deprived of EBP. This consistent reduction in oocysts and lesion scores in the EBP treated birds was associated with a higher level of serum Nitrite at the challenge day, associated with a significant reduction of chemokine IL-8 at the duodenal level, and a reduction trend at the ileal and cecal levels too, which could be responsible for the

Treatment[2]	Normalized mean amount of IL-6, IL-12, and IL-15 in different intestinal organs relative to that of control birds in treatment #1			
	Duodenum	Jejunum	Ileum	Cecum
2	ND	ND	ND	ND
3	ND	ND	ND	ND
4	ND	ND	ND	ND
5	ND	ND	ND	ND

alleviation of immune injuries (lower lesion scores) in birds of Treatments 2 and 3, thus improving their performance in production.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

The concluded results of Study A provide a novel base-line data of Kock's Postulate in a chicken model for future evaluation of new anti-coccidial drugs. The data is related to reproduction of *Eimeria* intestinal pathogenesis in a broiler Ross breed, marketed on a wide scale in the poultry industry. The Koch's postulate was achieved by challenges of chickens at different ages with a constant amount of $1.76x10^5$ sporulated oocysts of *Eimeria* of eight spp., equivalent to a 100 x dose recommended by Intervet's manufacturer for the non-attenuated Coccivac® vaccine.

In Study B the long-term application of *Echinacea*-based prepration (EBP) proved to have an adverse effect on the broiler performance. This adverse effect was due to the diarrhea that was obvious during our experimental observations in the EBP treated groups. At 21 d. of age, an improved performance in weight gain and in lower feed conversion of the EBP-treated and challenged groups was apparent compared to challenged and untrearted birds. On the other hand, in Study C, the non-challenged EBP-treated groups had an improved weight and lower feed conversion compared to the non-challenged untreated groups at 14 days of age. Therefore, further studies on growth promotion, by the intermittent application of EBP between 1-3d. and 8-10d. of bird's age, could be conducted in the future. However, the ceassation of EBP application after 14 days of age may have a negative effect on immuno-modulation and protection against *Eimeria* spp. Study C, proved also that the EBP has an immuno-modulation effect, an observation that is detected upon the measuring of the plasma nitrite and IL-8. This immune-modulating effect was associated with an improved performance, especially at 14 days of age.

Further studies could be done on other parameters such as IFN-gamma to evaluate the impact of EBP on cell mediated immunity. Actually, the IFN-gamma is also a parameter used for assessing the resistance in birds to *Eimeria* infection. In addition, testing titers of birds by ELISA against *Eimeria* in EBP-treated and nontreated groups could also give an indication of the Humoral Immuno-modulation. Previous documentation showed that an increase in titers against *Eimeria* spp. was associated with an increase in resistance to the disease. The addition of the carotenoid parameter in future investigations could enable us to have a better conclusion on the impact of EBP in diminishing the pathogenesis of *Eimeria* spp.

It is recommend in future studies to optimize the dosage of EBP in broilers. The 4.6ml of EBP/liter of drinking water was used in long-term and intermittent studies. Lower dosage might give the same immuno-modulating effect with more enhanced performance and a lower cost of the used material. A feasibility study can be conducted on whether the cost of EBP intermittent application at ≤ 4.6ml/liters in drinking water could lead to lowering the cost of controlling the 8 *Eimeria* spp. In addition, it is worth attempting to conduct an economical analysis on the growth promotion effect of EBP-intermittent administration in broiler chicken.

Finally, the data presented for EBP activity against *Eimeria* spp. alone or co-infected with *C. perfringens* in Study B & C is preliminary and needs further investigation involving different *Eimeria* spp. pathogenicity and field strains as a challenge, proper dosing of EBP, different intermittent adminstration days of EBP, and comparison of effectiveness and safety to the commercial coccidiostats.

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