# Alternative Anticoccidial Treatment of Broiler Chickens

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# Alternative Anticoccidial Treatment of Broiler Chickens

Alternatieve coccidiostatische behandeling van vleeskuikens

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*To*
*Ursula, Sami and Tariq*
*who taught me the nature of Love*

# Contents

# *Scope of the Thesis*

Coccidiosis is a major parasitic disease of poultry and is caused by the Apicomplexan protozoan of the genus *Eimeria*. Coccidiosis causes mortality, malabsorption, inefficient feed utilization, impaired growth rate in broilers and reduced egg production in layers (McDougald, 2003; Lillehoj *et al.*, 2004). In combination, these health and welfare problems inflict tremendous economic losses to the world poultry industry in excess of US$3 billion annually (Dalloul and Lillehoj, 2006). The invasion of the *Eimeria* sporozoites to the intestinal epithelium results in inflammation, leading to initiation of the immune response and resulting in massive infiltration of macrophages, granulocytes, and lymphocytes into the lamina propria. The macrophages modulate the severity of the infection and the lymphocytes, in particular the $CD^{+4}$ T cells, and act as inducer of an effective immune response (Jeurissen and Veldman, 2002). Both macrophages and lymphocytes are the sources of cytokine production in the intestine during *Eimeria* infection, thereby modulating the immune response (Lillehoj, 1994; Trout and Lillehoj, 1995; 1996; Breed *et al.*, 1997). Lowenthal et al. (1997) were able to decrease the negative effects of *E. acervulina* on body-weight gain of chickens by treating them with recombinant interferon-γ before the infection.

Conventional disease control strategies rely heavily on chemoprophylaxis and, to a certain extent, on live vaccines. However, drug-resistance in coccidial populations has been a constant threat to the continued success of prophylactic chemotherapy. Moreover, the increasing regulations and bans on the use of anticoccidial drugs coupled with the high costs of developing new drugs enhance the need for development of novel approaches and alternative control strategies for coccidiosis (Williams, 2006). This thesis describes two alternative anticoccidial treatments in broiler chickens: the use of a specific dietary supplement and a specific physical method.

The interaction between diet composition and coccidia is a classic area of interest. Prior to the availability of effective anticoccidial drugs, recommendations for coccidial control included the formulation of diets that were considered capable of reducing the severity of infection. Interest was directed towards the use of skim milk, butter, or whey as diet ingredients (Beach, 1925; Becker,

1937). Research that is more recent has focussed on the impact of coccidia infection on nutrient malabsorption which results in reduced weight gain. However, there are only few reports on alternative feedstuffs and natural products that might have a protective effect against infections with coccidia (Ruff and Allen, 1990, Allen *et al*., 1998; Allen and Fetterer, 2002).

MOS is a mannanoligosaccharide derived from the cell wall of the yeast *Saccharomyces cerevisiae*. There is suggestive evidence that ingestion of MOS may suppress pathogens of the intestinal mucosa in chickens and turkeys (Sonmez and Eren, 1999; Spring 1999a; 1999b; Spring *et al*., 2000; Iji *et al*., 2001; Delzenne, 2003). Attachment of pathogens to the epithelial cells of the gut is an essential step in the process of infection. Lectins (carbohydrates-binding proteins found on the exterior of the bacterial cells) are associated with fimbrial adhesion of bacteria. Lectins bind to gut epithelial cells by attaching to the oligosaccharide components of glycoconjugate receptors. Type-1 fimbrial adhesions, which are common in numerous species of *Escherichia coli* and *Salmonella,* are specific for mannan residues (Oyofo *et al*., 1989; Spring *et al*., 2000). Mannans aid in the resistance of pathogenic colonization by acting as a receptor analog for type-1 *fimbriae* and thereby decrease the number of available sites (Spring *et al*., 2000). Many studies reported that MOS improve gut health by increasing villi height, uniformity and integrity and modulate the gut and systemic immunity by acting as a non-pathogenic microbial antigen, giving an adjuvant-like effect (Ferket *et al.,* 2002; Loddi *et al.,* 2002). Fernandez et al. (2002) have reported an increase in the faecal *Bifidobacteria*, and reduction in susceptibility to *Salmonella enteritidis* colonization in young chickens fed a diet supplemented with mannanoligosaccharides or palm kernel meal.

Weak electromagnetic fields (EMF) may have beneficial effects on bones, joints, neurological disorders and wound healing (Bassett, 1993; Cane *et al*., 1993; Satter *et al*., 1999; Montesinos *et al*., 2000). The positive effect of EMF on wound healing may be mediated by controlling the proliferation of inflammatory lymphocytes. Various authors (Blank *et al*., 1992; Goodman *et al*., 1994; Mevissen *et al*., 1998; Simkó and Mattsson, 2004) have stated

that EMF causes stress at the cellular level, leading to the production of cytokines that enhance the immune response.

This thesis describes the effects of MOS and EMF in broiler chickens infected with *Eimeria* parasites. The question addressed was whether ingestion of MOS or exposure to EMF would counteract the coccidiosis-induced depression of growth performance and would reduce oocyst shedding and the severity of intestinal lesions. In other words, the question addressed was whether MOS or EMF could serve as an alternative to the anticoccidial drugs currently used. A literature review provides insight in the life cycle of coccidia and its relation with necrotic enteritis, the gut immune system, and its response during coccidiosis. The review also highlights the methods used in the control of coccidiosis, drug resistance, and alternatives to coccidial drugs (Chapter 1). In two different experiments, the effect of MOS was studied in broiler chickens infected with either a single dose of *E. tenella* (Chapter 2) or a mixture of *Eimeria* containing low doses of sporulated oocysts of *E. acervulina, E. maxima,* and *E. tenella* (Chapter 3). Possible effects of a weak electromagnetic field (EMF) on body weight, feed conversion ratio, feed intake, oocyst counts and severity of coccidial lesions were tested in broiler chickens infected with a mixture of *Eimeria* containing sporulated oocysts of *E. acervulina, E. maxima,* and *E. tenella* the infection being imposed by either crop gavage (Chapter 4) or by contaminated litter (Chapter 5). In Chapter 6 different methods of infection and their characteristics are described with emphasis on a nature-like-infection under controlled conditions. In the General Discussion section, an attempt is made to value MOS and EMF as alternative anticoccidial treatments of broiler chickens.

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*Chapter* **1**

# Coccidiosis in Poultry with Emphasis on Alternative Anticoccidial Treatments

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## Introduction

Coccidiosis is an infectious disease caused by protozoan parasite of the genus *Eimeria* (Tyzzer, 1932). It is a disease of almost universal importance in poultry production. The disease may strike any type of poultry in any type of facility. The parasite multiplies in the intestinal tract and causes tissue damage, resulting in diminished feed intake and nutrient absorption, reduced body-weight gain, dehydration, blood loss, and increased susceptibility to other diseases (Davies *et al.,* 1963; Turk, 1978; McDougald, 2003). The induced tissue damage and change in intestinal function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis (Helmboldt and Bryant, 1971, Maxey and Page, 1977). Caecal coccidiosis caused by *Eimeria tenella* may contribute to an increased severity of blackhead disease in chickens (McDougald and Hu, 2001). Coccidiosis remains one of the most expensive and common diseases of poultry production in spite of advances in chemotherapy, management, nutrition, and genetics. It costs chickens producers worldwide at least 3 billions $US annually (Dalloul and Lillehoj, 2006).

## Life cycle

Infection with coccidiosis follows the ingestion of viable oocysts, which are contaminants of food, dust, and water. After the oocysts are swallowed, they are subjected to the action of the digestive enzymes in the upper intestine and the grinding process in the gizzard, which lead to the liberation of sporozoites (excystation). Following the liberation, the sporozoites actively penetrate the epithelium of the intestine, and are then transported in macrophages through the lamina propria of the villi to reach the epithelium at the depth of the intestinal glands, where further developments occur (Jeurissen and Veldman, 2002; McDougald, 2003). Most *Eimeria* species have a characteristic site of invasion, and in chickens, these locations are used as diagnostic features. Following the penetration of the epithelial cells there is a period of growth during which the parasites becomes rounded, and is now termed trophozoites (Figure 1).

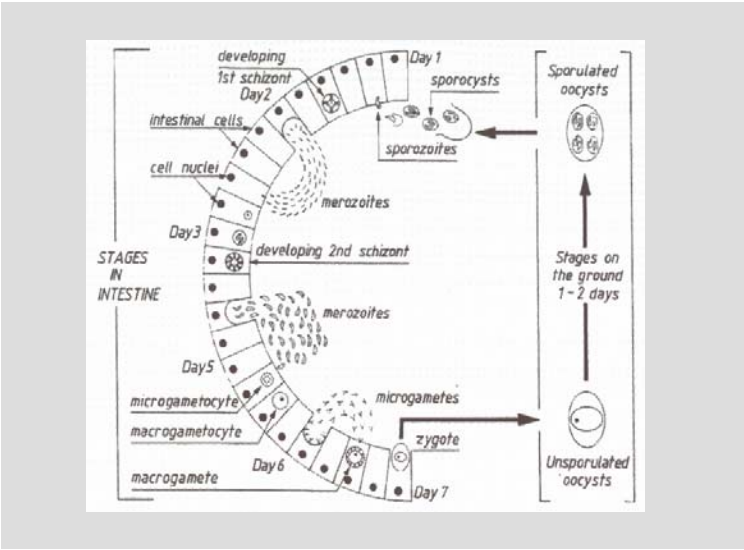


**Figure 1:** Overview of the various stages of *E. tenella*, which is typical for the genus *Eimeria*

At least 2 generations of asexual life cycles begin when the trophozoites have developed into schizonts and merozoites by a process called schizogony and merogony, respectively. This leads to the sexual phase, in which the small fusiform, motile microgametes seek out and unite with macrogametes to form a zygote. The resulting zygote will develop into an oocyst when a cyst wall develops around it. The oocyst will be extracted from the host tissues and is passed to the exterior with the faeces (Figure 2).

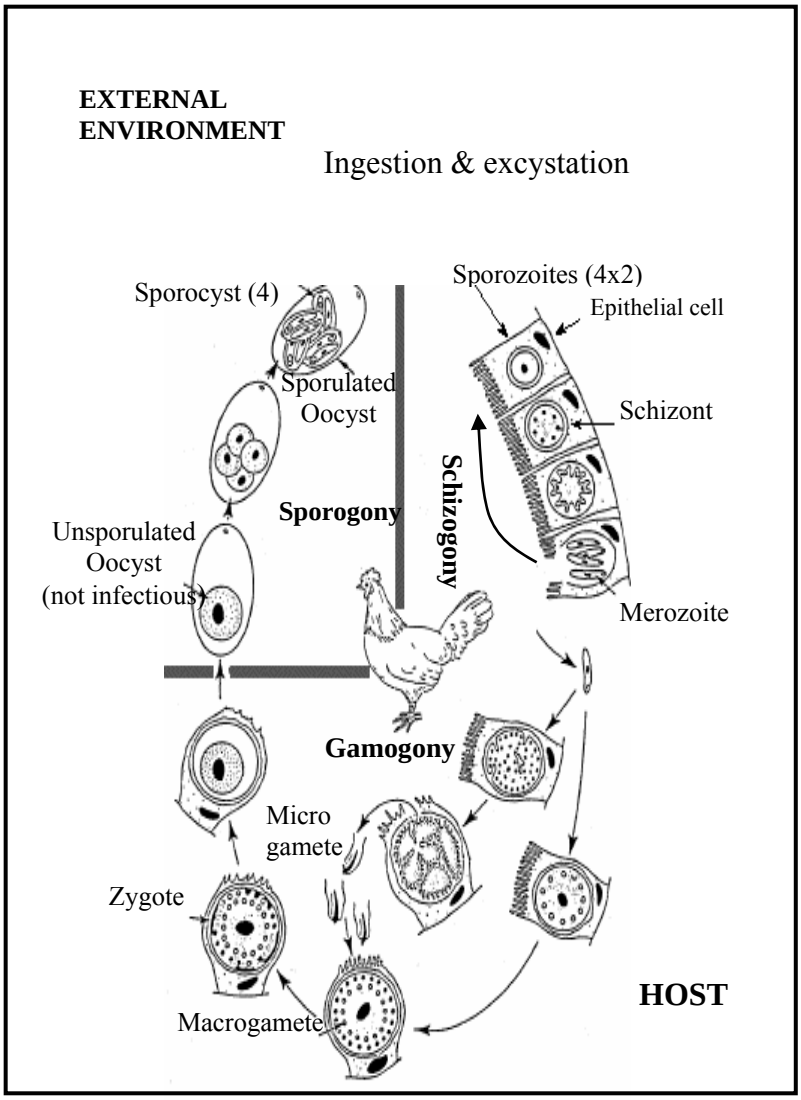


**Figure 2:** Stages of the life cycle take place in the external environment and in the host.

The period from the time of ingestion to the first appearance of the oocysts in the faeces is known as the ***prepatent period*** and the duration of this is a characteristic of the species, and it is used in species identification. The prepatent period varies from 93 hours (*E. acervulina*) to up to 22 days (*E. arloingi*). In some species (*E. tenella, E. necatrix*), the maximum tissue damage occurs when the

second generation of schizonts ruptures to release merozoites. Other species may have small schizonts, which cause little damage, but the gametocytes may elicit a strong reaction with cellular infiltration and thickened inflamed tissues (McDougald, 2003).

## Etiology

The coccidia of chickens have been the subject of intense study and there are more recorded details on their life cycle, physiology, pathology, and prophylactic and therapeutic control than on those of similar other parasites. There are many *Eimeria* species that can infect chickens, but there are seven species of *Eimeria* that parasitize chickens (*Gallus gallus*). These species are *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella* and they occur throughout the world wherever domesticated fowls are reared (Shirley and Long, 1990; Williams, 1998). Davies et al. (1963), Long et al. (1976), Vermeulen (1998) Allen and Fetterer (2002) and McDougald (2003) have summarized the criteria that are useful in the identification of species as follows:

1. Location of the lesion in the intestine (Figure 3).
2. Macroscopic appearance of the lesions.
3. Oocyst size, shape, and colour.
4. Size of schizonts and merozoites.
5. Minimum prepatent period in experimental infection.
6. Location of the parasite in the tissues (type of cell parasitized).
7. Immunogenicity against reference strain.
8. Stage of the life cycle that produces most tissue damage.
9. Molecular and biological approach: electrophoresis of metabolic enzymes (Shirley, 2000) and PCR (Tsuji *et al.,* 1997).

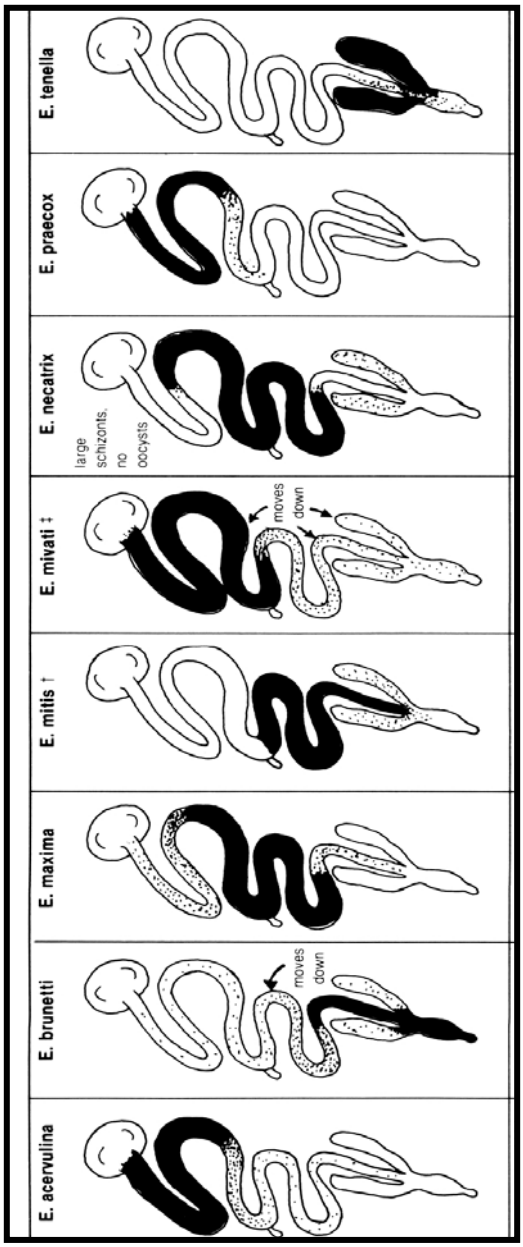


**Figure 3:** Location of the lesions caused by different *Eimeria* species in the gastrointestinal tract.

## Coccidiosis and Microflora

Schaedler (1973) stated that an "*ideal flora*" would allow optimum growth performance. Any alteration of the indigenous flora by diet, disease, or environment can be deleterious to the host. Extensive reviews (Jukes, 1955; March, 1979; Fuller, 1989; Ewing and Cole, 1994) on the role of microflora on animal performance support Schaedler's statement. Many studies have documented the important role of intestinal microflora in promoting the incidence and the severity of coccidiosis. It is confirmed that caecal coccidiosis does not occur in chickens fed a diet designed to depress putrefying bacteria, while it occurs in chickens fed a diet designed to promote proliferation of anaerobic putrefying bacteria in the intestine (Mann, 1977). It has been shown that clinical signs and mortality do not occur in bacteria-free chickens infected with surface-sterilized *E. tenella* oocysts, but chickens with two or more indigenous species of bacteria develop more severe lesions of coccidiosis and mortality than do their bacteria-free counterparts (Radhakrishnan, 1971; Johnson and Reid, 1972; Radhakrishnan and Bradley, 1972; Visco and Burns, 1972a&b). The indigenous bacteria aid in the development of large numbers of the endogenous stages of *E. tenella* and typical caecal coccidiosis in chickens (Bradley and Radhakrishnan, 1973). During the course of caecal coccidiosis, the growth of C*lostridium perfringens* and coliforms, especially *E. coli*, is stimulated and the growth of *Lactobacillus* spp. is suppressed (Johansson and Sarles, 1948; Radhakrishnan, 1971). Turk and LittleJohn (1987) studied the effects of *E. acervulina, E. necatrix, E. brunetti,* and *E. tenella* on the composition of the gut microflora. They reported that the number of the faecal anaerobes was increased on the 6$^{th}$ day of *E. acervulina* infection, on the 3$^{rd}$, 6$^{th}$, 7$^{th}$, and 14$^{th}$ days of *E. necatrix* infection, and on the 3$^{rd}$ and 6$^{th}$ days of *E. brunetti* infection. Turk and LittleJohn (1987) also reported an increase in faecal *Lactobacilli* in *E. necatrix*-infected birds during the period of 16 to 18 days post infection, and on the 8$^{th}$ day in *E. necatrix* infection. However, the faecal coliforms increased in all infections on the 6$^{th}$ day. The authors related the observed changes in the microflora population to the changes in the residual nutrients found in the gut, resulting from malabsorption of nutrients by the host due to the parasitic attack.

## Immunity development during coccidiosis

In coccidiosis infection, the chickens react in several ways. Following the ingestion of *Eimeria* oocysts, the non-specific portion of the immune system is antagonistic in the form of low pH, enzymes, and inflammatory reactions. This will limit the number of viable sporozoites that reach the site of infection. When the infection is established, the specific immunity system will become active in the form of specific antibodies and specific cellular immunity (Jeurissen and Veldman, 2002). Brandtzaeg et al. (1987) defined three general functions of the specific immune response GALT in the host defence against pathogenic infections, including coccidiosis:

1. Processing and presentation of antigens.
2. Production of intestinal antibodies.
3. Activation of cell-mediating immunity.

The role of the specific antibodies in immunity against coccidial infection is limited, but they are present in the circulation and mucosal secretions. The circulating IgY and the biliary IgA that are specific for coccidial parasites have been detected one week after the infection and reach peak values within 8-14 days and persist for two months (Lillehoj and Ruff, 1987). Lillehoj (1988) reported that bursectomised chickens could show full protection against coccidiosis in the absence of antibodies, illustrating that the role of antibodies is minor in the process of immunity against coccidiosis. However, *in vitro* studies showed that immune sera increased the phagocytosis of sporozoites and merozoites (Onaga and Ishii, 1980; Bekhti and Pery, 1989). It is possible that antibodies reduce the invasion of some, but not all *Eimeria* species, or enhance the intraluminal destruction of the sporozoites if they come into close contact with local antibodies before they enter the host cells (Lillehoj and Trout 1996). On the other hand, T cells have been reported to play an important role in the immune responses to coccidiosis (Rose and Hesketh, 1982; Rose *et al.,* 1988; Isobe and Lillehoj, 1993). Trout and Lillehoj (1995) studied the role of $CD4^+$ and the cytokines produced in coccidiosis infection, and found that depletion of $CD4^+$ cells have no effect on *E. acervulina* infection, but results in a significant increase in oocyst production following *E. tenella* primary infection. The authors suggested that this difference could

be related to the changes that occur during these infections or that the immune mechanisms may vary from one gut location to another. In contrast, depletion of $CD8^+$ results in a substantial increase in oocyst production following a challenge with *E. acervulina* infection in chickens. The direct role of the $CD8^+$ T cells in resistance to coccidiosis has not been proven yet. However, increased numbers of these cells were seen, and in direct contact with parasite-infected epithelial cells, in a tissue section of the gut following secondary infection, suggesting that infected epithelial cells may be the target of the cytotoxic T cells (Trout and Lillehoj, 1995; Lillehoj and Bacon, 1991).

## Control of coccidia

### *Prophylactic control with anticoccidial drugs*

More than 50 years anticoccidial feed additives have been used to prevent or treat coccidiosis in poultry. Anticoccidials can be classified as follows (Jeffers, 1997; Chapman, 1997; Allen and Fetterer, 2002):

**Chemicals:** These compounds are produced by chemical synthesis and have a specific mode of action against parasite metabolism, such as amprolium, nicarbazin and diclazuril.

**Polyether ionophores:** They are produced by fermentation of Streptomyces or Actinomadura and they are the most commonly used agents, such as salinomycin, monensin, lasalocid and narasin. They act through a general mechanism of altering ion transport and disrupting osmotic balance in the parasite.

*Mode of action of anticoccidial drugs*

Anticoccidials often have more than one biochemical effect, but each class of chemical compound is unique in its type of action exerted on the parasite and its development stage. Diverse modes of action have been described and this can be divided into several broad categories as follows (McDougald, 1982; 2003; Chapman, 1997):

**Drugs that affect cofactor synthesis:** Several drugs affect biochemical pathways that are dependent upon an important

cofactor. For instance, amprolium competitively inhibits the uptake of thiamine by the parasite.

**Drugs that affect mitochondrial function:** These drugs inhibit energy metabolism in the cytochrome system of the *Eimeria*. For instance, quinolones and clopidol inhibit electron transport in the parasite mitochondrion, but by different pathways.

**Drugs that affect membrane function:** Ionophores in common have the ability to form lipophylic complexes with alkaline metal cations ($Na^+$, $K^+$, and $Ca^{++}$) and transport these cations through the cell membrane and then affect a range of processes that depend upon ion transport, such as influx of sodium ions thus, causing severe osmotic damage. These drugs act against the extracellular stages of the life cycle of the *Eimeria*.

*Anticoccidial drugs resistance*

As early as 1963, the World Health Organization (WHO) defined resistance as "*ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevents their multiplication*". Such resistance may be relative (increasing doses of the drug being tolerated by the host) or complete (maximum doses being tolerated by the host) (Chapman, 1982). Anticoccidial drugs added to the feed are a good preventive measure and are well adapted to large-scale use, but prolonged use of these drugs leads inevitably to the emergence of *Eimeria* strains that are resistant to all anticoccidial drugs, including ionophores (Ruff and Danforth, 1966; Chapman, 1994; 1997; 1998; Allen and Fetterer, 2002). Resistance can develop quickly, as in the case of quinolones and clopidol, or it may take several years for the coccidia to become tolerant, as in the case of polyether ionophores (Chapman, 1997; McDougald, 2003).

*Origin of Resistance*

There are three important factors contributing to drug resistance in commercial poultry production (Jeffers, 1989; Chapman, 1997; Jeurissen and Veldman, 2002):

1. The intense and the continuous use of anticoccidial drugs in the poultry industry providing the basis for changing gene frequency through genetic selection.
2. Coccidia presence in the poultry facilities is ubiquitous and the large reproductive potential forms a large reservoir of genetic variation, which leads to the development of drug resistance.
3. The life cycle of *Eimeria* is complex and involves a period of asexual and sexual stages. The nuclei of the asexual stage of *Eimeria* contain haploid complement chromosomes while most drugs are active against this haploid stage, resulting in the removal of the most sensitive ones, enabling the more resistant ones to increase and thus rapidly becoming the dominant phenotype that spreads through the parasite population.

***Poultry House Management***

The high standard of flock hygiene, sanitation and poultry farm management helps in achieving optimal benefit from the anticoccidial drugs in preventing coccidiosis (Chapman, 1997). However, the sanitary practice alone is inadequate for complete elimination of coccidial oocysts. This is supported by the following: 1) there have been too many failures in sanitary programs; 2) oocysts are extremely resistant to common disinfectants; 3) house sterilization is never complete; and 4) an oocyst-sterile environment for floor-maintained birds could prevent early establishment of immunity and thus allow late outbreaks (McDougald, 2003).

## Alternatives for anticoccidial drugs

The extensive use of the anticoccidial drugs for prevention and control of coccidiosis in poultry has been a major factor in the success of the industry. This beneficial use of anticoccidial drugs is associated with a widespread drug resistance of coccidia in the United States, South America and Europe (Jeffers, 1974a&b; Litjens, 1986; McDougald *et al*., 1986; 1987; McDougald, 2003). The first line of defence against development of resistance is the use of shuttle programs (two or more drugs employed within a single flock) and frequent rotation of drugs (rotation of different compounds

between flocks) (Chapman, 1997; McDougald, 2003). Because of the pressure by the consumers to avoid chemotherapeutics, the high development costs and low profits, the pharmaceutical industry is reluctant to develop new anticoccidial products (Chapman, 1997). Thus, alternatives have been sought and are still being sought.

***Probiotics***

As early as 1908, it was proposed that the consumption of live microorganisms (mainly lactic acid bacteria) could improve intestinal heath and well-being of the host (Metchnikoff, 1908). Fuller (1989) has defined probiotics as "*a live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance*". Probiotic preparations may consist of a single strain *Lactobacilli* or *Streptococci* or may contain any number up to eight strains (Fuller, 1989; Timmerman *et al.,* 2004).

The use of probiotics aims to fasten the development of a stable and beneficial intestinal microflora, which will lead to improvement of intestinal health and modulate the immune system, enhancing host resistance to enteric pathogens (Jin *et al.,* 1996; 1998; 2000; Abdulrahim *et al.,* 1999; Zulkifli *et al.,* 2000). Tortuero (1973) demonstrated the antagonism between Lactobacilli and enterobacteria and showed that lactobacilli reduced the severity of clinical signs in *E. tenella* infection. Dalloul et al. (2003) reported that a *Lactobacillus* containing diet fed to broilers infected with *E. acervulina* resulted in an immunoregulatory effect on the local immune system and improved the broilers' resistance to *E. acervulina* infection. Furthermore, it has been reported that *lactobacillus* species inhibit the invasion of *E. tenella in vitro* (Tierney *et al*., 2004). Recently, Lee et al. (2007) reported that *Pediococcus acidilactici* effectively enhanced the resistance of birds and partially protected against the negative growth effects associated with coccidiosis.

***Prebiotics***

A prebiotic has been defined by Gibson and Roberfroid (1995) as "*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon that can improve the host health*". Prebiotics have the advantage, when compared with probiotics, that

they are targeting the bacteria already present and thus those that are adapted to the gastrointestinal tract environment.

Many studies have proved that the non-digestible polysaccharides inulin, oligofructose, and oligomannose, enhance the growth of the beneficial bacteria (*Bifidobacteria and Lactobacillus)* and reduce that of the pathogenic bacteria (*E.coli* and *Salmonella*) and also stimulate the immune system of the host (Hidaka *et al.,* 1986; Wang and Gibson, 1993; Gibson and Roberfroid, 1995; Gibson *et al.,* 1995; Gibson, 1999; Gibson and Fuller, 2000; Cummings and MacFarlane, 2002). Mannanoligosaccharides (MOS), derived from the cell wall of the yeast, can be considered as prebiotics. MOS is non-digestible and is utilized by lactic acid producing bacteria (Delzenne, 2003). MOS also competes with mannose-specific binding of type-1 *fimbriae* of pathogenic, gram-negative bacteria such as *E.coli* and *Salmonella*, resulting in a reduction of their colonization (Ofek *et al.,* 1977; Spring *et al.,* 2000). Fernandez et al. (2002) have reported an increase in faecal *Bifidobacteria* and a reduction in susceptibility to *Salmonella enteritidis* colonization in young chickens fed a diet supplemented with MOS. Addition of MOS to the diet of broilers reduced the severity of the infection due to either *E. tenella* alone (Elmusharaf *et al*., 2006) or a mixture of *E. acervulina, E. maxima* and *E. tenella* (Sun *et al*., 2005; Elmusharaf *et al*., 2007).

***Vaccines***

Vaccines are one of the most valuable public health tools that have been developed by man (Payette and Davis, 2001). The development of resistance of coccidia to anticoccidial drugs (Chapman, 1997, Williams, 2002), the concern about drug residues in poultry products (McEvoy, 2001; Young and Craig, 2001), the pressure imposed by consumers to avoid chemotherapeutics and the recent announcement by the EU to ban several anticoccidial drugs used in broilers (Farrant, 2001), have led to interest in the vaccination of poultry against coccidiosis. In addition to the vaccines currently available, many others are under development (Chapman *et al.,* 2002). Jeurissen and Veldman (2002) have listed factors making coccidiosis as a disease that can be controlled by vaccines. These factors are:

1. Immunity to avian coccidiosis is strongly species-specific.
2. Coccidiosis infection induces a quick and strong protective immunity.
3. Lack of antigenic variation in *Eimeria* species.

However, as described above, *Eimeria* exhibit a complex life cycle comprising stages both inside and outside of the host. During the in-host stage, there are both intracellular and extracellular stages and both asexual and sexual reproduction. This complexity provides the immune system with only three moments to inhibit *Eimeria* development. The first is when the sporozoites search for a site of penetration and actually bind with the epithelium. The second is when the sporozoites are in the villus epithelium, inside and between intraepithelial leucocytes. The third moment of possible attack by the immune system is during the passage of the lamina propria into the crypt epithelium (Jeurissen and Veldman, 2002).

There are four major brand of vaccines commercially available, and they are based on the use of wild type (Coccivac® D/B and Immucox®) and attenuated (Paracox® and livacox®) *Eimeria* species (Williams, 1998; 2002; Chapman *et al*., 2002). The non-attenuated vaccines contain a mixture of oocysts of wild-type-strain *Eimeria* that will not produce pathogenic effect, but induce immunity.

The methods of administration of vaccines have been reviewed by Williams (2002). In the past, vaccines were applied via drinking water or feed when the chickens are about one-week of age, but recently the method of vaccination is a single dose at day one with Coccivac D® (Chapman and Cherry, 1997a), Immuncox® (Chapman and Cherry, 1997b) and Coccivac B® (Chapman *et al*., 2002). Administration of vaccines with a single dose at day-one of age is important in initiating immunity as early as possible in broilers as they are reared only to about 6 weeks of age. However, some studies indicated that vaccination on day one could not evoke a strong immunity since the immune system in young chicks is immature (Rose, 1987). In contrast, other studies have shown that chicks infected at day one of age indeed are capable of building an effective immunity (Lillehoj, 1988; Bafundo and Jeffers, 1990; Stiff and Bafundo, 1993; Chapman and Cherry, 1997a&b). Moreover,

many scientists have reported that even embryos have a functional immune system (Fredericksen *et al*., 1989; Doelling *et al*., 2001). There are various methods of administration of coccidial vaccines, including intra-ocularly (Coccivac®), by hatchery spray (Coccivac® and Nobils®), by edible-gel (Immucox®), or by spraying on feed (Coccivac®, and Paracox®) (Chapman, 2000; Chapman *et al*., 2002; Williams, 2002).

As the immunological protection against *Eimeria* is strongly species specific (Rose, 1973; 1978), a number of species has been incorporated in vaccines, varying from two species (*E. acervulina* and *E. tenella* as in LivacoxD®) up to eight species (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. mivati, E. necatrix, E. praecx,* and *E. tenella* as in CoccivacD®) (Shirley and Millard, 1986; Williams, 1998). However, Williams (1998), and Chapman (2000) recommended the inclusion of *E. acervulina, E. maxima,* and *E. tenella* and the exclusion of *E. brunetti,* and *E. necatrix* in vaccines as the latter two species rarely infect younger chickens. Following vaccination, immunity is initially stimulated by the vaccine oocysts and is subsequently boosted and maintained by multiple re-infections initiated by the viable oocysts in the litter either originating from the vaccine or from local wild-type strains (Chapman, 1997; Chapman *et al*., 2002; Williams, 2002). This synchrony of infection development is called "trickle" infection and has been shown to be crucial in stimulating solid protective immunity (Joyner and Norton, 1973; 1976; Nakai *et al*., 1992; Chapman and Cherry, 1997a&b).

***Enzymes***

The use of exogenous enzymes in food processing started as early as 1900 and the majority of the enzymes have been derived from fermentation by microorganisms (Clarkson *et al*., 2001). When broilers fed diet rich in wheat, barley, oat, or rye, the presence of non-starch polysaccharides (arabinoxylans and β-glucans) can give rise to high viscosity in the small intestine thereby decreasing the contact of endogenous digestive enzymes and its substrates. This results in a decrease in absorption and broilers' performance, and increase in the size of the GIT, pancreas, and the liver (Yu *et al*., 1997; Choct and Bedford, 1999; Bedford, 2000; Choct and Sinlae, 2000; Silva *et al*., 2002). Wang et al. (2005) reported an improvement in broilers' performance, a reduction in the size of digestive organs

and the GIT size, and an increase in the total volatile fatty acids in the caecum, when a wheat-based diet was supplemented with the 200mg exogenous enzymes xylanase or β-glucanase per kg feed. Addition of exogenous xylanase has been found to improve the performance and to reduce ileal digesta viscosity in *Eimeria* infected birds (Cumming, 1992; 1994; Morgan and Bedford, 1995). It was concluded that intestinal viscosity and the size of the gizzard might affect the severity of the *Eimeria* infection. However, others did not observe effects of increased intestinal digesta viscosity on the severity of the *Eimeria* infection, when a large increase in viscosity was being induced by the inclusion of carboxymethyl cellulose in the feed (van der Klis *et al*., 1993; Banfield *et al*., 1999; 2002; Waldenstedt *et al.,* 2000a). It was concluded that the effects of enzyme addition on coccidial infections could be more related to factors, other than viscosity itself.

***Electromagnetic Fields (EMF)***

Electromagnetic fields (EMF) have been in use as therapeutic modalities for at least 40 years. It is well known that selected electromagnetic fields (EMF) can have beneficial effects on bones, joints, and neurological disorders, as well as wound healing (Bassett, 1993; Cane *et al.,* 1993; Satter *et al.,* 1999; Montesinos *et al.,* 2000). Anti-inflammatory aspects of EMF exposure have been reported to be due to the activation of $A_{2A}$ adenosine receptors in human neutrophils (Vallbona and Richard, 1999). Generally, inflammation is characterized by massive infiltration of T lymphocytes, neutrophils and macrophages into the damaged tissue (Gessi *et al*., 2000). In earlier studies, it has been reported that EMF mediate positive effects on wound healing, controlling the proliferation of inflammatory lymphocytes, and therefore demonstrating beneficial effects on inflammatory disease (Jasti, e*t al*., 2001). Several authors (Blank *et al.,* 1992; Goodman *et al*.,1994; Mevissen *et al.,* 1998; Simkó and Mattsson, 2004) have discussed the effects initiated by various EMF signals and stated that EMF causes stress at the cellular level and that this leads to production of cytokines and consequently a biological response, including an immune response. Recently, Elmusharaf et al. (chapters 4&5) have reported that exposure of broiler chickens to EMF antagonized the effects of coccidial infection in birds infected with a mixture of

sporulated oocysts containing *E. acervulina, E. maxima,* and *E. tenella*. It was found that the severity of the intestinal lesions mediated by *E. acervulina* and *E. maxima* were reduced in the EMF-treated birds.

***Dietary modulation of coccidia***

The study of the interactions between diet composition and coccidia is not a new area of interest. Before the availability of effective anticoccidial drugs, recommendations for coccidial control included the formulation of diets that were considered capable of reducing the severity of infection such as diets containing skim milk, buttermilk, or whey (Beach and Corl, 1925; Becker, 1937). But due to the development of the efficient, low-cost anticoccidial drugs caused lesser interest in dietary modulation. However, with the appearance of resistance to coccidiostats, the consumers' concern, and the expected regulations to ban the coccidiostats in the future, the possible role of nutrition has recently attracted interest (Allen *et al*., 1998; Gabriel *et al*., 2006).

*Vitamins and minerals*

Several vitamins influence the immune status and the resistance of the host against *Eimeria* infections. Many studies reported that vitamin A deficiency depresses T-lymphocyte response to mitogens (Friedman and Sklan, 1989a; Sklan *et al*., 1994) and reduces specific antibody production to protein antigens (Friedman and Sklan, 1989b). Recently, Dalloul et al. (2002) reported that vitamin A deficiency in chickens caused alteration in the IEL subpopulation, reduced the local cell-mediated immunity, and lowered the ability of birds to resist *E. acervulina* infection. Vitamin E and selenium generally improve resistance to coccidiosis, improve weight gain (Colnago *et al.,* 1984a; El-Boushy, 1988), and reduce mortality due to *E. tenella* infection (Colnago *et al*., 1984b). Vitamin C is known to possess immunity-enhancing effects in chickens and positive effect on birds' performance during coccidial challenge has been observed (Attia *et al*., 1979), but vitamin C had no effect on the lesion scores due to *E. tenella* or *E. acervulina* infection (Webber and Frigg, 1991). Moreover, Waldenstedt et al. (2000b) found that feeding a diet with extra vitamins A, C, $D_3$, K, and selenium had no beneficial effects on the performance of chickens with subclinical

infection caused by *E. maxima,* and *E. tenella*. Moreover, the authors reported that performance in the birds supplemented with vitamins was even poorer than in birds fed the control diet. These findings are in contrast with previous work of Colnago et al. (1984b) who fed 0.025 or 0.50 mg Se/kg of diet, noted a reduced mortality, an increase in body weight, and improved immunity against *E. tenella.* The review of Rivera et al. (2003) highlights the protective role of trace minerals specially zinc, on the immune response during parasitic infections. Rapp et al (2004) reported that dietary supplementation with a zinc- amino acid complex reduced gut lesion scores of broilers infected with *E. maxima*.

*Products rich in n-3 fatty acid*

The n-3 fatty acids are polyunsaturated fatty acids, the major fatty acids being eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found abundantly in fish oil, and alpha-linolenic acid (ALA), being a major component of flaxseed oil. As early as 1938, Murphy et al. reported that fish-liver oil exerts favourable control on the course of coccidiosis. Recently, Allen et al. (1996a&b; 1997a) performed a series of experiments using fish oil, flaxseed oil and flaxseed in diets fed to male chickens from day 1 of age through 3 weeks of age and challenged with *E. tenella* at 2 weeks of age. The authors reported a significant reduction in caecal lesion scores and in the histological examination, a significant reduction in the degree of parasitaization and retarded development of the *E. tenella* parasite was observed. The suggested mode of action is that the n-3 fatty acids infiltrate the tissues of the parasite, which in turn become more susceptible to oxidative attack by phagocytic cells. Additionally, n-3 fatty acids have been shown to enhance the immune response in birds infected with *E. tenella.* However, little if any response was seen in the birds' performance, which is of most importance in poultry production. The n-3 fatty acids were proven ineffective against moderate or severe infection with *E. maxima*, and did not counteract reduced body-weight gain and lesion scores. The reason for the differences in response between these two *Eimeria* species to dietary n-3 fatty acids is not yet known (Allen *et al*., 1997a).

*Betaine*

Betaine supplementation has been shown to have positive effects on the water balance of broiler chicks stressed by high ambient temperature or coccidiosis (Augustine and Danforth, 1999; Teeter, 1999), and to protect the cells from osmotic stress, allowing them to continue regular metabolic activities under conditions that would normally inactivate the cell (Ko *et al*., 1994). Augustine et al. (1997) reported that betaine, in combination with the ionophore salinomycin, had a significant positive effect on the performance of chickens infected with *E. acervulina, E. maxima,* and *E. tenella*, the effect being greater than that mediated by betaine or salinomycin alone. Moreover, the combination resulted in a slight decrease in development and invasion of the epithelium by *E. acervulina*, while there was an increase in the invasion of *E. tenella*. However, the diet supplemented with betaine alone decreased the invasion of *E. acervulina* and *E. tenella* as indicated by the number of sporozoites present in the intestinal epithelium after the challenge. Klasing et al. (2002) later clarified this effect when they found that chickens fed betaine had more lymphocytes in the epithelium and in the lamina propria during *E. acervulina* infection than those fed the diet without betaine. This effect of betaine could result in more effective clearance of sporozoites that explain the decreased numbers of sporozoites in the epithelium as observed by Augustine et al. (1997). In contrast, Waldenstedt et al. (1999) found that betaine as a single feed supplement significantly improved chickens' body weight and tended to reduce the feed conversion ratio during coccidiosis infection. When betaine was used in the combination with the ionophore narasine, betaine showed no effects on birds' performance when *E. tenella* was the major pathogenic species. Recently, Kettunen et al. (2001) reported that betaine supplementation of the diet improved the crypt-villus ratio in chickens infected with *E. maxima* and stabilized the intestinal epithelium structure. The exact action of betaine is not fully understood. However, Augustine et al. (1997) suggested that betaine might increase performance in chickens infected by coccidiosis by inhibition of coccidial invasion and indirectly by supporting intestinal structure and function that could enhance the ability of the infected chickens to withstand coccidial infection.

*Whole wheat*

The use of whole grains in broiler feeds is common practice in Europe (Forbes and Covasa, 1995). Chickens possess the ability to efficiently process and digest whole grains, primarily due to a significant increase in gizzard size, needed to grind the whole grains before passing down to the small intestine (Rose *et al.,* 1986). Many studies indicated that offering broilers a whole cereal grains and balanced pellets greatly reduced the severity of infection with *Eimeria* as based on the reduction in output of oocysts (Cumming, 1987; 1992; 1994). However, Waldenstedt (1998) and Banfield (1999; 2002) investigated the effects of whole wheat inclusion in broiler feeds with or without access to grit, and they observed no significant differences in faecal oocyst yields, lesion scores, or performance in birds infected with *E. tenella* or *E. maxima*. They concluded that the reduction in output of oocysts as caused by inclusion of whole cereals in the diet, and observed in the previous experiments, was not due to the increase in the viscosity of the digesta or the crushing of oocysts by an active gizzard and that whole wheat addition to the diet of broiler chickens provides no control of coccidiosis.

*Herbs and natural additive*

A number of natural herbs have been tested as anticoccidial dietary additives. Artemisinin isolated from *Artemisia annua*, is a naturally occurring endoperoxide with antimalarial properties. It has been found effective in reducing oocyst output from both *E. acervulina* and *E. tenella* infections when fed at levels of 8.5 and 17 ppm in starter diets (Allen *et al.,* 1997b). The mode of action is thought to involve oxidative stress. Extracts from 15 Asian herbs were tested for anticoccidial activity against *E. tenella* and the test criteria were survival rate, bloody diarrhoea symptoms, lesion scores, oocyst output, and technical performance. Of the species tested, extracts from *Sophora flavescens Aiton* was the most effective in reducing lesion scores, maintaining body weight gain, and reducing oocyst production. (Youn and Noh, 2001). Practical applications of these findings, such as the use of the products in starter rations or combinations of them with current anticoccidials or vaccines, appear possible and need to be investigated (Allen and Fetterer, 2002).

## Summary

Coccidiosis is an ubiquitous disease of almost universal importance in poultry production. The disease may strike any type of poultry in any type of facility and causes large economic losses. The immune responses of the body against coccidiosis are complex because *Eimeria* species exhibit a complex life cycle, which includes stages inside and outside the birds and the inside-stage, comprises extracellular and intracellular stages. The major component in coccidiosis control in the poultry industry since the 1940s has been the use of anticoccidial compounds. These compounds, when used in carefully designed prophylactic treatment programs are efficient in disease control. However, the inevitable development of drug resistance to chemical types of anticoccidials by avian coccidia, the increased pressure from consumers and governments to phase out the use of chemical anticoccidials in the diet of food animals has resulted in the need of a re-examination of another type of coccidial control. Among others, mannanoligosaccharide (MOS) has shown promising in suppressing pathogens of the intestinal mucosa of chickens and turkeys, but the studies need to be confirmed. Electromagnetic fields (EMF), n-3 polyunsaturated fatty acids and natural herbs all have potential anticoccidial activities, but more research is needed to define the condition of practical application and to unravel their modes of action.

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*Chapter* **2**

# Effect of a Mannanoligosaccharide Preparation on Eimeria tenella Infection in Broiler Chickens

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**Summary**

The hypothesis tested was that the feeding of mannanoligosaccharides (MOS) will suppress the signs of a coccidiosis infection in broilers. Two separate experiments were performed in which part of the broilers used were infected with *Eimeria tenella*. In each experiment there were three treatment groups: a negative control group fed the basal diet and two infected groups fed the basal diet without or with a commercial MOS preparation. The infection of the broiler chickens was successful based on the caecal lesions, oocyst shedding and schizonts in the lamina propria of the caecum but did not affect growth performance of the birds. In the infected birds fed the MOS preparation, the number of schizonts was reduced without a decrease in the severity of caecal lesions and without impact on growth performance. It is suggested that the MOS preparation had enhanced the immunity of the infected birds and thereby had decreased the number of schizonts. It is concluded that this study presents evidence for a protective effect of MOS against coccidiosis infection in broilers.

*Keywords*: Mannanoligosaccharides, basal diet, caecal lesions

## Introduction

Coccidiosis is a common infectious disease in poultry, causing major economic losses. The protozoan parasite of the genus *Eimeria* multiplies in the intestinal tract of poultry and produces tissue damage, resulting in reduced growth and increased susceptibility to pathogens (McDougald, 2003) such as *Clostridium perfringens,* leading to necrotic enteritis (Helmboldt and Bryant, 1971; Maxey and Page, 1977; Shane *et al*., 1985).

In bacteria-free chickens infected with surface-sterilized *Eimeria tenella* oocysts, clinical signs do not develop unlike in chickens with two or more indigenous species of bacteria (Johnson and Reid, 1972; Radhakrishnan, 1971; Visco and Burns, 1972a; 1972b). Apparently, indigenous bacteria are required for the occurrence of typical caecal coccidiosis in chickens. In the course of development of caecal coccidiosis, the growth of C*lostridium perfringens* and coliforms, especially *Escherichia coli*, is stimulated whereas the growth of *Lactobacillus* spp. is suppressed (Johansson and Sarles, 1948; Rahhakrishnan, 1971). *Lactobacillus* spp have been shown to inhibit the invasion of *E. tenella* in *vitro* (Tierney *et al*., 2004).

It is expected that in the near future the coccidiostatics currently used in animal feeds will be banned. Thus, there is a need for alternative agents to control coccidiosis in poultry. Perhaps, mannanoligosaccharide (MOS) preparations can be useful. These carbohydrate preparations are derived from the cell wall of the yeast *Saccharomyces cerevisiae* and have been reported to suppress pathogens in the intestinal mucosa of chickens and turkeys (Sonmez and Eren, 1999; Spring 1999a; 1999b; Iji *et al*., 2000; Spring *et al*., 2000). MOS may improve gut health as indicated by increased villi height, uniformity and integrity (Loddi *et al*., 2002) and they modulate gut and systemic immunity (Ferket *et al.,* 2002). Possibly even more important in relation to coccidiosis, dietary MOS supplementation increased the levels of *Bifidobacterium* spp. and *Lactobacillus spp* in the intestinal tract and depressed the number of *Enterobacteriaceae* (Fernandez *et al*., 2002). *Lactobacillus* spp. are known to compete with *Clostridium* spp. (Shane, 1985).

In the light of the above-mentioned, an increase in *Lactobacillus* spp. and a decrease in *Clostridium* spp might reduce caecal coccidiosis in broiler chickens. Consequently, it could be hypothesized that the feeding of MOS will suppress coccidiosis infection in broilers. In the present experiments, the hypothesis was put to the test by measuring the severity of *E. tenella* infection in broiler chickens fed a diet containing a commercial MOS preparation.

## Materials and Methods

*Animals and Diets*

Two separate experiments were performed. One-day old male broiler chicks (Ross 308) were purchased from a local hatchery. On arrival, they were wing-banded and randomly allocated to either the basal or the supplemented diet. Two groups received the basal diet and one group received the supplemented diet. The birds were housed in wire-floored, suspended cages that were placed in one room. Ambient temperature was gradually decreased from 32 $^{o}$C on day 1 to 25 $^{o}$C on day 21. Throughout the experiments, there was continuous lighting. The basal diet did not contain growth promoters or coccidiostatics; the composition was as follows (g/kg diet): wheat (+ xylanase), 250; corn, 321; soyabean meal (46.7% crude protein), 225; peas, 50; sunflower meal (32% crude protein), 40; potato protein, 15; fish meal (72% crude protein), 25; soyabean oil, 40; premix (Research Diet Services, Wijk bij Duurstede, The Netherlands), 5; limestone, 16; monocalcium phosphate, 7; phytase (Natuphos 5000G), 0.1; salt, 1.7; sodium bicarbonate, 1.7; L-lysine HCl, 0.8; D,L-methionine, 1.7.

The experimental diet was prepared by supplementation of MOS (Bio-Mos™, Alltech) at a level of 1 g/kg diet at the expense of an identical amount of corn starch. The diets were in pelleted form and fed *ad libitum* from arrival of the birds (day 1) to day 21 in experiment 1 and from days 1 to 14 in experiment 2. Tap water was freely available throughout the experiment. Body weights, feed, and water intakes were recorded at day of infection and 6 days post infection (PI).

*Experiment 1*

75-one-day-old broiler chickens were allocated randomly to three treatment groups of 25 birds each. Each group was then randomly divided into 5 replicates of 5 birds each. Each replicate was housed in a separate cage. On day 12 of the experiment, one group receiving the basal diet and the group receiving the supplemented diet were inoculated with 3500 sporulated oocysts of the *E. tenella* (Houghton) laboratory strain. The oocysts were obtained from the Animal Health Service Ltd. Poultry Health Centre (Deventer, The Netherlands). The oocysts were administered with 1 ml of tap water via an oral gavage directly into the crop. The negative control group fed the basal diet was given water only through gavage. Care was taken to prevent cross contamination throughout the experiment. At the end of the experiment (day 21), the birds were euthanized by cervical dislocation.

*Experiment 2*

In this experiment, the dose of *Eimeria* was raised and administered on day 8. 45-one-day-old chickens were randomly allocated to three groups of 15 chickens each, the groups consisting of 5 replicates of 3 birds each. Each replicate was housed in a separate cage. On day 8, one group receiving the basal diet and the group fed the supplemented diet were inoculated with 5000 sporulated oocysts of *E. tenella.* The oocysts were administered as described above. The negative control group was given water through gavage. On day 14, all birds were killed by cervical dislocation.

*Analyses and measurements*

The numbers of oocysts in faeces were determined in samples collected from each cage during the period of 3 to 6 days post infection in experiment 2. The modified Mc Master counting chamber techniques of Hodgson (1970) was used. A 10% (w/v) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of water) was prepared. After shaking thoroughly, 1 ml of the suspension was mixed with 9 ml of a salt solution (131 g of NaCl mixed into 1 L of water). Then, the suspension was put into the McMaster chamber using a micropipette and the number of oocysts

was counted and expressed per gram of faeces (Peek and Landman, 2003).

In both experiments, the severity of coccidial caecal lesions was scored while the investigator was blinded to treatment modality. The 0-4 scoring system described by (Johnson and Reid, 1970) was used. In experiment 1, tissue sections from caecum (about 4 μm thick) were routinely processed, paraffin embedded and stained with haematoxylin-eosin (HE). Microscopical lesions in the tissues were examined under the microscope (x 400). The presence of *E. tenella* schizonts in the lamina propia of the whole section of the caecum was scored on a 0- 2 scale (0 = no schizonts, 1 = 1 to 3 schizonts, 2 = mass of schizonts).

Fat digestibility was measured using the faeces collected for days 15 to 18 in experiment 1 and for days 8 to 11 in experiment 2. The acid hydrolysis method was applied to extract crude fat from the feed and excreta. Briefly, a 2-g dry sample was weighed in a flask and 2 ml of ethanol was added and the sample was shaken until fully wettest. Subsequently, 10 ml of 8 mol/l HCl was added and after mixing the flasks were placed in a water bath at 80 °C for 40 min. Then, 10 ml of ethanol was added and after cooling, 25 ml of diethyl ether was added and the sample was shaken for about 1 min. Then, 25 ml of petroleum ether was added and the sample was shaken for another 1 min. The supernatant consisting of ether-fat mixture was poured into a flask, and the precipitate was extracted further with 15 ml of both ethers. The supernatants were combined and the solvent was evaporated at 50 °C. The flask containing the residue was dried in a desiccator over night under vacuum. The residue was weighed and considered to be crude fat.

*Statistical analysis*

All data for each variable were subjected to univariate analysis of variance using SPSS (SPSS Inc, Chicago, USA). The oocyst values were logarithmically transformed [$\log_{10}(X+1)$] to create a normal distribution before being analyzed, and lesion scores were transformed using multinomial transformation. When significant treatment effects were disclosed, differences between the three treatments were evaluated by the post hoc multiple comparison least significant difference (LSD) test. Lesion scores

were compared using the non-parametric Mann-Whitney U test. Chi-square analysis was used to compare treatment group values for the semi-quantitative scores of schizonts found in the lamina propria of the caecum. The level of statistical significance was pre-set at $P < 0.05$.

## Results

In both experiments, body-weight gain during the interval of 6 days post infection showed no significant differences between treatments (Table 1). In the infected birds, the MOS preparation had produced a 7.7% increase in group-mean weight gain in experiment 1 and a 1.8% increase in experiment 2.

**Table 1:** Body weight gain (g/day/bird) of the broilers during 6 days post infection.

| Days post infection | Control | | MOS | Pooled | *P*-value |
|---|---|---|---|---|---|
| | Negative | Infected | Infected | SEM | |
| Experiment 1 | | | | | |
| 12 - 18 | 54 | 52 | 56 | 1.49 | 0.14 |
| Experiment 2 | | | | | |
| 8 - 14 | 41 | 38 | 38 | 2.69 | 0.70 |

Post infection feed intake in experiment 1 showed no significant difference between the three treatment groups. In experiment 2, the infected control group and birds given MOS displayed a significantly lower feed intake than the negative control group (Table 2). Group mean feed intake in the infected birds was increased by 5.8% after feeding MOS in experiment 1, but was not increased in experiment 2.

**Table 2:** Feed intake (g/day/bird) of the broilers during 6 days post infection.

| Days post infection | Control | | MOS | Pooled SEM | *P*-value |
|---|---|---|---|---|---|
| | Negative | Infected | Infected | | |
| Experiment 1 | | | | | |
| 12 - 18 | 71 | 68 | 72 | 2.12 | 0.14 |
| Experiment 2 | | | | | |
| 8 - 14 | 45[a] | 42[b] | 41[b] | 0.77 | <0.05 |

[a,b] Mean values within the same row with different superscript letter are significantly different ($P$<0.05).

Post infection feed conversion was not significantly affected by the treatments (Table 3). In experiment 2 the group-mean feed conversion ratio in the infected chickens was decreased by 3% after feeding the mannanoligosaccharide preparation.

**Table 3:** Feed conversion ratio (feed/gain) of the broilers during 6 days post Infection.

| Days post infection | Control | | MOS | Pooled SEM | *P*-value |
|---|---|---|---|---|---|
| | Negative | Infected | Infected | | |
| Experiment 1 | | | | | |
| 12 - 18 | 1.33 | 1.32 | 1.28 | 0.03 | 0.53 |
| Experiment 2 | | | | | |
| 8 - 14 | 1.30 | 1.30 | 1.24 | 0.04 | 0.64 |

Counting of oocysts in faeces was done in experiment 2 only. Table 4 illustrates that there was no significant difference between the infected control group and the infected group given MOS. No oocysts were detected in faeces obtained from the negative control group.

**Table 4:** Faecal oocyst counts [log (X+1)] in experiment 2.

| Days post infection | Control | | MOS | Pooled SEM | *P*-value |
|---|---|---|---|---|---|
| | Negative | Infected | Infected | | |
| Experiment 2 | | | | | |
| 11 – 14 | 0.00[a] | 4.80[b] | 4.71[b] | 0.23 | <0.05 |

[a,b] Mean values within the same row with different superscripts letter are significantly different ($P<0.05$).

In both experiments, the negative control birds showed no caecal lesions (Table 5). The mean lesion score and the frequency distribution of lesions scores did not differ significantly between the infected birds fed the diets without or with MOS (Tables 5 and 6).

**Table 5:** Lesion scores for the infected birds.

| Experiment | n | Infected Controls | Infected MOS | *P*-value |
|---|---|---|---|---|
| Experiment 1 | 25 | 1.10 ± 0.21 | 1.00 ± 0.17 | 0.83 |
| Experiment 2 | 15 | 1.70 ±0 .27 | 1.67 ± 0.23 | 0.91 |

Means ± SE

**Table 6:** Frequencies of lesion scores in infected birds and negative controls.

| Treatment | Lesion Scores | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Experiment 1[1] | | | | | |
| Negative Control | 25 | - | - | - | - |
| Infected Control | 8 | 10 | 4 | 3 | - |
| Infected, MOS | 9 | 10 | 4 | 2 | - |
| Experiment 2[2] | | | | | |
| Negative Control | 15 | - | - | - | - |
| Infected Control | - | 7 | 2 | 6 | - |
| Infected, MOS | 1 | 6 | 5 | 3 | - |

[1]n=25, [2] n=15

The scores for the numbers of *E. tenella* schizonts in the lamina propria of the caecum, as determined in experiment 1, are shown in Table 7. In the infected birds given MOS 13 out of 24 had

one or three schizonts whereas only one bird showed a mass of schizonts in the lamina propria. In the infected control group there were 12 out of 25 birds revealing a mass of schizonts and 6 animals displaying one or three schizonts in the lamina propria of the caecum. No schizonts were found in the negative control group. There was a significant difference in schizonts between the infected groups fed the diets without or with MOS.

**Table 7:** Frequency distribution of the number of schizonts in the lamina propria of the caecum in experiment 1

| Treatment | n | Schizonts Score (%) | | |
|---|---|---|---|---|
| | | 0 | 1 | 2 |
| Negative Control | 25 | 100 | 0 | 0 |
| Infected Control | 25 | 28 | 24 | 48 |
| Infected, MOS | 24 | 42 | 54 | 04 |

In both experiments there were no significant differences in fat digestibility between the treatment groups (Table 8). In experiment 1, MOS produced a higher group mean fat digestibility in the infected birds, but this was not seen in experiment 2.

**Table 8:** Apparent fat digestibility (% of intake) in the three treatment groups.

| Days post infection | Control | | MOS | Pooled SEM | *P*-value |
|---|---|---|---|---|---|
| | Negative | Infected | Infected | | |
| Experiment 1 | | | | | |
| 12-18 | 80.8 | 80.1 | 85.1 | 2.11 | 0.23 |
| Experiment 2 | | | | | |
| 8-14 | 81.2 | 81.7 | 81.4 | 0.38 | 0.69 |

## Discussion

In both experiments, there was a successful infection with *Eimeria tenella* as indicated by the caecal lesions, the shedding of oocysts and the presence of schizonts in the lamina propria. The birds had been inoculated with either 3500 or 5000 sporulated oocysts. McDougald (2003) reported that inoculation with 1000 to

3000 sporulated oocysts would be sufficient to cause bloody droppings, and other sign of infection. Conway et al. (1993) were only able to get a significant reduction in body weight at a dose of 10000 sporulated *E. tenella* oocysts. In the present experiments, no significant effect of infection on weight gain was seen. The lack of susceptibility of growth performance to the infection with *E. tenella* must relate to the birds, the pathogenicity and the dose of the *E. tenella* strain used, and/or the experimental conditions. It generally accepted that weight gain is the variable most sensitive to coccidiosis and anticoccidial efficacy of treatments (Barwick *et al.*, 1970; Johnson and Reid, 1970; Long 1970).

In experiment 1, the ingestion of MOS markedly increased group mean water consumption without reaching statistical significance. Unfortunately, water intake was not determined in experiment 2. It is not known why MOS would increase water consumption. The effect of MOS on water intake cannot be explained by the induction of diarrhoea, as this was not observed. Ferket (2002) has reported that birds fed a MOS preparation retained normal body temperature after exposure to a pro-inflammatory antigen. If such an effect of MOS also occurs after inflammatory stress imposed by invasion of the epithelium surface of the caecum by sporozoites of *E. tenella* an increase in water consumption would not be anticipated.

In experiment 1, the inoculation was done when the birds were aged 12 days and in experiment 2 the age was 8 days while the dose of sporulated oocysts was increased. Lesion scoring was performed at 9 days post infection in experiment 1 and at 6 days post infection in experiment 2. It would be expected that the infection would be more severe in experiment 2 than in experiment 1 (Rose, 1976; Johnson and Reid, 1970). Indeed, the lesions scores were higher in experiment 2 than in experiment 1. There was no effect of MOS on the caecal lesions. However, in experiment 1 feeding of the MOS preparation had caused a significant decrease in the number of schizonts found in the lamina propria of caecum of the infected birds. Only one out of 24 birds fed the diet supplemented with MOS had a mass of schizonts whereas large clusters of schizonts were seen in 12 out of 25 infected control chickens. The protective effect of MOS might be related to an

improvement of intestinal function (Loddi *et al.,* 2002) or immunity modulation (Ferket *et al.,* 2002). Jeurissen et al. (1996) found in immune chickens that significantly fewer sporozoites reached the crypt epithelium and that the formation of schizonts was inhibited. Sporozoites that had failed to reach the crypt epithelium within 48 hours after infection were detected within macrophages or were surrounded by them, pointing at control of the intensity of a primary infection.

A reduction in schizonts as seen in the infected birds fed MOS should be associated with lower caecal lesion scores mediated by the *E. tenella* infection (McDougald, 2003). It is not known why feeding of MOS reduced the number of schizonts without affecting the caecal lesion scores. The most pathogenic stage is the second generation of schizonts, which matures after 4 days of the production of clusters of large schizonts, which may contain hundreds of merozoites. The schizonts develop deep in the lamina propria so that the mucosa is damaged seriously when the schizonts mature and the merozoites are released. Clearly, further investigations will have to be done to check the reproducibility of the effect of the mannanoligosaccharide on the number of schizonts.

In both experiments, the apparent fat digestibility was not influenced by the infection with *E. tenella*. In contrast, Adams et al. (1996) showed that fat digestion was reduced from 86 to 21% in chickens infected with *E. acervulina*. The discrepancy between the two studies probably relates to the different species of *Eimeria* and the type of lesions that they induce. *E. tenella* induces lesions in the caecum whereas *E. acervulina* induces lesions in the small intestine. Fat digestion depends on the integrity of the small intestine rather than on that of the caecum.

In conclusion, these experiments describe a successful infection of broiler chickens with *E. tenella* as based on caecal lesions, oocyst shedding, and schizonts in the lamina propria of the caecum. The infection did not affect growth performance of the birds. In infected birds fed the MOS preparation, the number of schizonts was reduced without a decrease in the severity of caecal lesions. Perhaps MOS had enhanced the immunity of the infected birds thereby decreased the number of schizonts. The hypothesis tested in this study was that the feeding of MOS would suppress coccidiosis.

It could be concluded that evidence was found for a protective effect of the MOS preparation used, but this effect was not associated with a decrease in caecal lesions and improved growth performance.

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*Chapter* 3

# The Effect of an in-feed Mannanoligosaccharide Preparation (MOS) on a Coccidiosis Infection in Broilers

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**Abstract**

In the current study with broiler chickens, the objective was to assess whether a mannanoligosaccharide (MOS) preparation would have anticoccidial activity. One-day-old birds were given a single, mild coccidiosis infection with a mixture of three *Eimeria* species *Eimeria acervulina, Eimeria maxima* and *Eimeria tenella.* Infected and non-infected birds were fed a diet without or with 10g/kg MOS. Growth performance, feed intake and feed conversion were not different ($P>0.05$) among the treatment groups. The feeding of the MOS preparation reduced oocyst excretion and diminished the severity of coccidiosis lesions induced by *E. acervulina* ($P<0.05$) but did not affect the lesions mediated by *E. maxima* and *E. tenella* ($P=0.69$ and $P=0.84$ respectively). Further research is necessary to explain the observed effects in terms of mechanisms and to assess their practical relevance.

*Keywords*: Broilers, Coccidia, Mannanoligosaccharide.

## Introduction

The protozoan parasite of the genus *Eimeria* may cause coccidiosis in poultry, an infectious disease causing major economic losses through increased mortality and reduced growth (McDougald, 2003). In order to control coccidiosis in poultry, coccidial vaccination, in drinking water and in-feed supplementation of anticoccidial products are commonly used. However, it is expected that within five years from now (2012) the anticoccidial products currently used will be banned (van den Ban *et al*., 2005). Thus, there is an urgent need for alternative agents to control coccidiosis in poultry. In this context, mannanoligosaccharide (MOS) preparations, which are derived from the cell wall of the yeast *Saccharomyces cerevisiae*, can be useful in broiler chickens infected with *E. tenella*, the feeding of a MOS preparation was shown to reduce the number of the asexual stage schizonts in the lamina propria of the caecum (Elmusharaf *et al*., 2006). We interpreted the outcome of that study as evidence for a protective effect of MOS against coccidiosis infection and enhanced immunity in broilers. Jeurissen et al. (1996) found in immune chickens that significantly fewer sporozoites reached the crypt epithelium and that the formation of schizonts was inhibited.

In a previous study (Elmusharaf *et al*., 2006) the chickens infected with only one species of *Eimeria*. In the present study, the possible anticoccidial activity of MOS was further investigated in broiler chickens infected mildly with a mixture of three *Eimeria* species, *E. acervulina, E. maxima,* and *E. tenella*. Infected and non-infected birds were fed a diet without or with MOS. Growth performance, intestinal lesions, and oocyst excretion were measured.

## Materials and Methods

*Animals and Diets*

The experiment was of a factorial design, with/without MOS, and with/without *Eimeria* mixture. Two hundred and fifty-six-day 1-day-old male broiler chickens (Ross 308) were purchased from a local hatchery. On the day of arrival (Day 1), the birds were wing-banded, weighed, and randomly allocated to four treatment groups of 64 birds each. Each group was further divided into 8

replicates of 8 birds each. All replicates were housed in 32 separate wire-suspended cages equipped with plastic sides and bottoms covered with clean wood shavings. Continuous lighting was provided. The temperature in the cages was 32°C on arrival of the chickens and from day 8 of the experiment the temperature was decreased gradually by 2°C every day until it reached 20°C by day 14.

Two groups received the control diet and the other two groups were fed the basal diet supplemented with MOS. The diets did not contain growth promoters or anticoccidial products Table1 The experimental diet was prepared by adding a MOS preparation (Bio-Mos™, Alltech) at a level of 10g/kg diet at the expense of an identical amount of cornstarch. The diets were in pelleted form (2.5mm in diameter). Throughout the experiment, the birds had free access to their diet and tap water.

Body weights were measured on Days 1, 7, 14, and 19. Amounts of feed provided and leftovers were weighed per cage. Feed intakes were determined per week per cage and expressed as g/bird/day. Feed conversion ratio is calculated as feed intake per cage divided by weight gain of birds in the cage.

**Table 1:** Ingredients and composition of the basal diet.

| Ingredients | g/kg |
|---|---|
| Wheat (+ xylanase) | 250 |
| Corn | 321 |
| Peas | 50 |
| Soybean meal, (46.7%CP) | 225 |
| Sunflower meal (32% CP) | 40 |
| Fish meal (72%CP) | 25 |
| Potato protein | 15 |
| Soyabean oil | 40 |
| Premix[1] | 5 |
| Salt | 1.7 |
| Limestone | 13.5 |
| Sodium chloride | 5 |
| Calcium carbonate | 1.7 |
| Monocalcium phosphate | 4 |
| L-lysine HCl | 0.8 |
| D,L-methionine | 1.7 |
| L-Threonine | 0.5 |
| Natuphos 5000G (phytase) | 0.1 |
| Total | 1000 |
| Calculated chemical composition | |
| $AME_N$ (MJ/kg) | 11.7 |
| Crude protein (g/Kg) | 215.6 |

[1] The 10g premix consist of 24.0mg vitamin A (500000 IU/g), 6.0mg vitamin $D_3$ (100000 IU/g), 60.0mg vitamin E (500 IU/g), 6.6mg vitamin $K_3$ (purity, 22.7%), 100.0mg vitamin $B_{12}$ (purity, 0.1%), 2000.0mg biotin (purity, 0.01%), 1100.0mg choline chloride (purity, 50%), 1.1mg folic acid (purity, 90%), 65.2mg nicotinic acid (purity, 100%), 16.3mg d-pantothenate (purity, 92%), 4.5mg vitamin $B_6$ (purity, 100%), 12.5mg riboflavin (purity, 80%) 2.5mg vitamin $B_1$ (purity, 100%), 32.00mg $CuSO_4 \cdot 5H_2O$, 333.20mg $FeSO_4 \cdot H_2O$, 166.80mg MnO, 1.00mg $Na_2SeO_3 \cdot 5\ H_2O$, 220.00mg $ZnSO_4 \cdot H_2O$, 4.80mg $CoSO_4 \cdot 7H_2O$, 0.56mg KI, 100.00mg ethoxyquin, and 5742.94mg corn meal as carrier.

*Coccidiosis infection*

On Day 1, one group fed the control diet and one group fed the experimental diet were challenged with a mixture of *Eimeria* containing 900 sporulated oocysts of *E. acervulina* (Weybridge strain), 570 sporulated oocysts of *E. maxima* (Weybridge strain) and 170 sporulated oocysts of *E. tenella* (Houghton strain). The oocysts were obtained from the Animal Health Service Ltd., Deventer, The Netherlands. The oocysts were laboratory strains and the dose and the species used simulated commercially available live vaccines (Williams, 2002).

The sporulated oocysts were administered with 1 ml of tap water directly into the crop via a scaled 1-ml syringe. The non-infected groups were given 1 ml of oocyst-free water into the crop. In order to avoid cross contamination, the cages were equipped with plastic sides, and the non-infected groups were always taken care of first. On Day 14, one bird per cage per treatment was randomly selected and killed by cervical dislocation and used for lesion scoring. On Day 19, all remaining birds were killed by cervical dislocation, dissected and the different coccidial lesions were scored.

*Oocyst counting*

Fresh excreta samples were collected from the four corners and the middle of each cage on Days 5, 7, 9, 12, 14, 16, and 19 of the experiment for oocyst counting. Excreta collection was done in the evening and the samples were stored overnight in a refrigerator. The oocysts of each cage (8 samples/treatment) were counted the next day and the numbers expressed per g of excreta.

For oocyst counting, a modified McMaster counting chamber technique of Hodgson (1970) was used. A 10% (w/v) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of water) was prepared. After shaking thoroughly, 1 ml of the suspension was mixed with 9 ml of a salt solution (311 g of NaCl mixed into 1 L of water). Then, the suspension was put into the McMaster chamber using a micropipette and the number of oocysts was counted (Peek and Landman, 2003).

*Lesion scoring*

On days 14 and 19 of the experiment 8 and 24 birds per treatment, respectively, were randomly selected and coccidial intestinal lesion scored. The 0-4 lesion scoring system of Johnson and Reid (1970) was used. The areas scored were the upper, middle and the caecal regions of the intestine, which are the natural predilection sites for *E. acervulina, E. maxima,* and *E. tenella,* respectively. 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) is recorded for each chickens. The severity of coccidial lesions was scored while the investigator was blinded to treatment modality

*Statistical analysis*

All data for each variable were subjected to univariate analysis of variance using SPSS (SPSS Inc, Chicago, USA). The oocyst values were logarithmically transformed [$\log_{10}$ (X+1)] to create a normal distribution before being analyzed, and lesion scores were transformed using multinomial transformation. When significant treatment effects were disclosed, differences between the four treatments were evaluated by the post hoc multiple comparison least significant difference (LSD) test. Lesion scores were compared using the non-parametric Mann-Whitney test. The level of statistical significance was pre-set at $p < 0.05$.

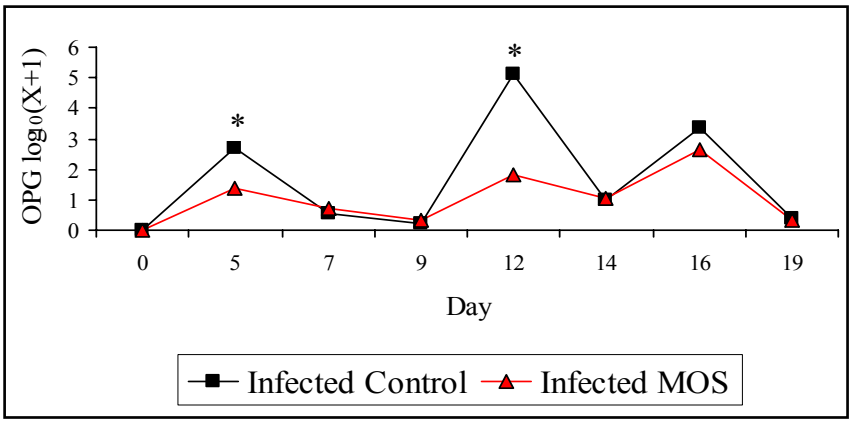
## Results

Chicks performance in the various intervals of the experiment showed no significant differences between all groups ($P > 0.05$) (Table 2).

Oocysts were not detected in the excreta obtained from non-infected groups. The pattern of oocyst shedding shows peaking on Days 5, 12 and 16. The two earlier peaks were most pronounced in the infected controls. The feeding of MOS significantly lowered ($P < 0.05$) the number of oocysts per g of faeces on days 5 and 12 of the experiment (Figure 1).

**Table 2:** Body weights gain (g) and feed conversion ratio (feed/gain) of broilers

| Variable | Control | | MOS | | Pooled SEM | *P*-value |
|---|---|---|---|---|---|---|
| | Uninfected | Infected | Uninfected | Infected | | |
| Body weight gain (g) | | | | | | |
| Days 1-7 | 86 | 97 | 85 | 93 | 4.9 | 0.293 |
| Days 7-14 | 241 | 247 | 253 | 229 | 9.2 | 0.319 |
| Days 14-19 | 264 | 264 | 265 | 262 | 11.9 | 0.999 |
| Feed conversion (feed/gain) | | | | | | |
| Days 1-7 | 1.26 | 1.20 | 1.22 | 1.22 | 0.04 | 0.73 |
| Days 7-14 | 1.48 | 1.46 | 1.37 | 1.48 | 0.06 | 0.48 |
| Days 14-19 | 1.54 | 1.45 | 1.44 | 1.43 | 0.09 | 0.81 |




*Statistically different $P<0.05$.
*N= 8 Samples/Treatment*

**Figure 1:** Faecal oocyst excretion (number of oocysts per g of faeces) by infected birds fed diets without or with a MOS preparation.

In the non-infected birds, no lesions were found for either Day 14 or 19. Lesion scores for day 14 of the experiment showed no significant difference (P>0.05) between the infected birds fed either the diet without or with MOS (data not presented). For Day 19 there were 24 dissected birds per treatment and the results showed a significant reduction (P<0.05) in *E. acervulina* lesions in the infected group fed the diet with MOS (Table 3). The lesions induced by *E. maxima* and *E. tenella* showed no difference (P>0.05) between the two infected groups.

**Table 3:** Mean lesion scores and their frequencies for Day 19 in infected birds fed diets without or with a MOS preparation

| Variable | | Infected Control | | | | | Infected MOS | | | | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean lesion scores | | | | | | | | | | | |
| *E. acervulina* | | 0.46[a] ± 0.1 | | | | | 0.13[b] ± 0.09 | | | | <0.05 |
| *E. maxima* | | 0.71 ± 0.14 | | | | | 0.63 ± 0.13 | | | | 0.690 |
| *E. tenella* | | 0.42 ± 0.13 | | | | | 0.33 ± 0.1 | | | | 0.841 |
| Frequencies of lesion scores[1] | | | | | | | | | | | |
| | ***Score*** | **0** | **1** | **2** | **3** | **4** | **0** | **1** | **2** | **3** | **4** |
| *E. acervulina* | | 13 | 11 | - | - | - | 22 | 1 | 1 | - | - |
| *E. maxima* | | 10 | 11 | 3 | - | - | 11 | 11 | 2 | - | - |
| *E. tenella* | | 16 | 6 | 2 | - | - | 16 | 8 | - | - | - |

[a,b] Mean ±SE values within the same row with different superscript letters are significantly different (*P*<0.05).
[1]number of bird dissected 24/treatment

## Discussion

In this experiment, the infection of the broiler chickens with coccidiosis was successful based on the intestinal lesions and oocyst shedding. No effect of the infection on chicks' performance was seen. The lack of effect of infection on growth performance may relate to the mildness of the infection. Under conditions of more severe infection with *Eimeria*, weight gain is generally reduced (Johnson and Reid, 1970; Conway *et al*., 1993; McDougald, 2003; Chapman *et al*., 2004).

The pattern of faecal oocyst excretion as found in this experiment was fairly predictable (Figure 1). This was because the initial infection dose was low and more or less equal to the dose of a non-attenuated vaccine. Williams (2002) reported that in vaccinated birds the oocyst stage of *Eimeria* that follow excystation initiate the vaccinal infection, hence stimulating immunity, and during subsequent recycling of infection maintains this immunity. The phenomenon establishes 'trickle' infections that have been shown to be very effective in stimulating protective immunity (Joyner and Norton, 1973a; 1976b). On Days 5 and 12, the infected birds fed the diet with MOS showed significant lower peak excretions of oocysts compared to the infected control (Figure 1). The MOS-induced reduction in oocysts production indicates that it might have anticoccidial activities. Thus, it can be concluded that the MOS preparation has the potential to lower the severity and the pressure of the infection and at the same time maintain the oocysts production, which is crucial for the re-infection and the maintenance of the immunity stimulated by the initial infection. We have shown earlier that the feeding of a MOS preparation reduced the number of the asexual stage schizonts in the lamina propria of the caecum broiler chickens infected with *E. tenella* (Elmusharaf *et al.,* 2006). The protective effect of MOS may be due to the increase in the villi length and improved integrity and uniformity of the gut as observed in chickens fed a diet supplemented with MOS (Loddi *et al*., 2002). Moreover, Ferket *et al.* (2002) observed modulation of the systemic immunity in birds fed a diet fortified with MOS. Williams (1995) has reported that there is reciprocity between the immune status of chickens and their excretion of oocysts.

Intestinal lesion scores on Day 19 of the experiment showed that *E. acervulina* lesions in the infected birds fed MOS were significantly reduced ($P<0.05$), whereas the severity of lesions produced by the two other species of *Eimeria* were reduced but not significantly. The absence of the lesions due to *E. maxima* and *E. tenella* on day 14 is probably due to the very low initial dose and the low oocyst productive potential of both species compared to *E. acervulina* (Brackett and Bliznick, 1952). The lesions seen on Days 14 and 19 should be considered as secondary and third lesions (Williams and Andrews, 2001). Thus, it appears that the MOS preparation had anticoccidial activity which counteracted the secondary and third (due to the recycling of the infection after the initial infective dose) attack by *E. acervulina* and not the secondary and third attack by *E. maxima* and *E. tenella*.

In conclusion, the results of this experiment show that supplementation of the diet with MOS preparation reduced oocyst excretion on day 5 and 12 of the experiment and diminished the severity of *E. acervulina* lesions but not those due to *E. maxima* and *E. tenella* infection.

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*Chapter* **4**

# Antagonistic Effect of Electromagnetic Field Exposure on Coccidiosis Infection in Broiler Chickens

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**Summary**

The hypothesis tested was that exposure of broiler chickens to EMF (electromagnetic field) may reduce the signs of coccidiosis infection in broiler chickens based on recent insights in immunology. The experiment had a 2x2 factorial design. There was an uninfected and infected group not receiving further treatment. Other uninfected and infected groups were subjected to EMF treatment. In the cages of the EMF-treated birds, a field strength of 5 µTesla rms was created for a period of 30 min per day. Infected birds were given a single dose of a mixture of *Eimeria* species ($1.76x10^4$ sporulated oocysts of *Eimeria acervulina*, $1.25x10^4$ sporulated oocysts of *Eimeria maxima,* and $7.5x10^3$ sporulated oocysts of *Eimeria tenella*) through gavage into the crop. Infection with the *Eimeria* mixture induced intestinal lesions, shedding of oocysts and reduction in growth performance. Exposure of broiler chickens to EMF antagonized the effects of infection. In the EMF-treated birds, the infection caused no effect on weight gain and feed intake whereas the severity of intestinal lesions mediated by *Eimeria acervulina* and *Eimeria maxima* was less than in the infected controls. It is suggested that EMF has anticoccidial activities and its application could serve as an alternative to the anticoccidial drugs currently used in poultry production.

*Keywords:* Coccidia, Electromagnetic Fields, Broilers.

## Introduction

Coccidiosis is a common infectious disease in poultry that is caused by the protozoan parasite of the genus *Eimeria*. The signs of coccidiosis are lesions in the intestine and associated impaired growth performance. In addition, coccidiosis increases the susceptibility to pathogens (McDougald, 2003). The invasion of the *Eimeria* sporozoites into the intestinal epithelium initially results in inflammatory reactions, leading to an effective immune response (Jeurissen and Veldman, 2002). It could be hypothesized that inhibition of the inflammatory reactions and stimulation of the immune response would diminish the signs of coccidiosis infection in poultry. Exposure to electromagnetic field (EMF) may have an anti-inflammatory effect (Vallbona and Richard, 1999; Jasti e*t al.,* 2001) and it has been reported that EMF signals stimulate the production of cytokines, mediating an enhanced immune response (Blank *et al.,* 1992; Goodman *et al.,* 1994; Mevissen *et al.,* 1998; Simkó and Mattsson, 2004).

In the light of the above-mentioned, we hypothesized that exposure of broiler chickens to EMF may reduce the signs of coccidiosis infection in broiler chickens. In the present experiment, our hypothesis was tested. Preliminary results have been reported in abstract form (Cuppen *et al.,* 2006).

## Materials and Methods

*Birds and housing*

288-one-day-old female broilers (Ross 308) were purchased from a local hatchery. On the day of arrival (day 1), they were wing-banded and randomly housed in wire-floor, suspended cages. Each cage was 60x50x38 cm and was provided with thick foil and wood shavings as litter. Continuous lighting was provided throughout the experiment. The temperature in the cages at arrival was 32 °C and then gradually decreased to 20 °C at the end experiment.

*Diets*

Starter and grower diets were used. The diets did not contain growth promoters or anticoccidial drugs. The starter diet

was offered until day 13, followed by the grower diet. The ingredient composition of the diets was as follows (g/kg diet as fed; starter/grower): wheat (250/500), soybean meal (49 % crude protein) (345.5/253.7), corn (275.00/122.90), animal fat (40.00/50.00) Rape seed meal, extracted (20.00/3.00), soybean oil (18.70/1.39), corn gluten 60 (1.0522/0.00), premix (0.50/0.50), salt (0.2119/1.902), sodium bicarbonate (2.367/2.295), monocalcium phosphate (10.995/4.276), limestone (14.674/10.469), 99% DL-methionine (2.695/0.2146), 98.5% L-lysine HCl (1.723/2.418), 98% L-threonine (0.638/0.855), phytase (Natuphos5000G) (0.10/0.10). Throughout the experiment, the birds had free access to feed and tap water.

*Experimental design*

The experiment had a 2x2 factorial design. On day 13, the broilers were weighed and divided over the 4 experimental groups so that the weight distributions of the groups were similar. There was an uninfected and infected group not receiving further treatment. Other uninfected and infected groups were subjected to EMF treatment, which was started on day 1. The infection with a mixture of *Eimeria* species was done on day 15. The two groups not receiving EMF treatment consisted of 10 replicates each (2x10) and the other two groups had 8 replicates each (2x8). Each replicate was a cage with 8 birds initially. The cages of the uninfected and infected groups had evenly distributed locations.

Under each cage of the EMF treated birds, there was a magnetic coil, consisting of 50 loops of electricity wire with 1.5 $mm^2$ cross section. The coils were connected to an experimental exposure system of Immunent BV. The Netherlands, containing a signal generator controlled by a Cygnal C8051F126 microprocessor from Silabs, USA which regulate the period of time and the EMF per cage. The coil was placed directly under and along the boarders of the cage. No attempt was made to achieve uniformity of the magnetic field in cage as previous experiments (see Cuppen *et al.*, 2000b) has shown equal response from 0.3 to 50 µTesla. In the cages, the field strength varied between 5 and 10 µTesla as shown by measurements at 7 different points. Coil resistance was 0.9 Ohm and the current was less than 60 mA rms. Therefore, heat delivery was less than 50 mW for 30 minutes per cage so that no detectable

temperature increase was caused by the exposure. In addition, no detectable sound was produced by the coils at these very low exposure settings. The birds were observed multiple times during treatment and no behavioural changes were seen. The EMF treatment period lasted for 30 minutes and was given to each cage consecutively and once per 24 hours. The field strength within the cages was set to 5 μTesla rms, verified by a FW Bell 5180 Tesla meter with a MOS51-3204 Low field probe (www.fwbell.com). In order to avoid any effect of EMF exposure on the other groups, the EMF-free and EMF-positive, groups were housed in adjacent rooms within the facility. The distance between the EMF groups and the control groups was 3 meters and the thickness of the wall between the two rooms was 20 cm.

On day 15 of the experiment the birds in 18 cages (144 birds) were individually challenged with the mixture of *Eimeria* containing $1.76x10^4$ sporulated oocysts of *E. acervulina* (Weybridge strain), $1.25x10^4$ sporulated oocysts of *E. maxima* (Weybridge strain) and $7.5x10^3$ sporulated oocysts of *E. tenella* (Houghton strain). The oocysts were laboratory strains and were obtained from the Animal Health Service Ltd., Poultry Health Centre (Deventer, The Netherlands). The sporulated oocysts were administered with 1 ml of tap water via a scaled 1-ml syringe directly into the crop. Likewise, the negative groups were given 1 ml of water only. In order to avoid cross contamination between uninfected and infected groups, the sides of all cages were partially equipped with plastic. Birds of the uninfected groups were always fed and weighed prior to the infected groups. On day 21 of the experiment two randomly selected birds per cage (16 or 20/experimental group) were euthanized by cervical dislocation, dissected and the coccidial lesions were scored.

*Performance measurements*

Birds were weighed individually on days 15 and 21. Feed intake was measured per cage on a weekly basis. Average feed intake per broiler within a cage was calculated and corrected for dropouts, if any. Mortality was registered on a daily basis.

*Infection measurements*

The number of oocysts per gram faeces (OPG) was determined for excreta collected on day 6 post infection (PI). Oocyst shedding was assessed on one sample of homogenized fresh excreta collected from each cage. The modified McMaster counting chamber technique of Hodgson (1970) was used. A 10% (w/v) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of water) was prepared. After shaking thoroughly, 1 ml of the suspension was mixed with 9 ml of a salt solution (311 g of NaCl mixed into 1 L of water). Then, the suspension was put into the McMaster chamber using a micropipette and the number of oocysts was counted and expressed per gram of faeces (Peek and Landman, 2003).

The severity of coccidial lesions was scored on day 6 PI while the investigator was blinded to treatment modality. The 0-4 scoring system described by (Johnson and Reid, (1970) was used.

*Statistical analysis*

The oocyst values were logarithmically transformed [$\log_{10}(X+1)$] to create a normal distribution and lesion scores were transformed using multinomial transformation. Lesion scores for the various experimental groups were compared using the non-parametric Mann-Whitney U test. Performance and oocyst data were subjected to the LSD test. The statistical program SPSS (SPSS Inc, Chicago, USA) was used. The level of statistical significance was preset at $P< 0.05$.

## Results

*Growth performance*

Post-infection mortality was one or two animals per treatment group. Final body weight of the uninfected broilers exposed to EMF was significantly lower than that of uninfected controls, but body weights of the two infected groups were similar (Table 1).

**Table 1:** Post infection growth performance of the broilers in the four experimental groups

| Day in experiment | Control[1] | | EMF[2] | | Pooled SEM |
|---|---|---|---|---|---|
| | Uninfected | Infected | Uninfected | Infected | |
| Body weight (g) 21 (6 days PI) | 888[a] | 812[b] | 828.[b] | 804[b] | 14.63 |
| Weight gain (g/day) 15-21 (1-6 days PI) | 59[a] | 45[c] | 53[b] | 52[b] | 10.88 |
| Feed intake (g/day) 15-21 (1-6 days PI) | 106[a] | 93[b] | 95[b] | 93[b] | 2.01 |
| Feed conversion ratio (FCR) 15-21 (1-6 days PI) | 1.67[a] | 1.94[b] | 1.65[a] | 1.68[a] | 0.13 |
| Water intake (ml/day) 16-21 (2-6 days PI) | 162[ab] | 159[b] | 151[bc] | 147[c] | 2.75 |

[a,b,c,] Mean values within the same row with different superscript letter are significantly different ($P$<0.05)
[1]n= 80 birds per experimental group; [2]n= 64 birds per experimental group.

The post-infection daily growth rate of the infected EMF group was significantly higher ($P$ <0.01) than that of the infected control group. The uninfected birds exposed to EMF showed a significantly lower feed intake and lower weight gain than the uninfected control birds. Post-infection feed intake of the infected EMF birds was similar to that of the infected controls. Feed conversion in control birds was significantly raised by the infection, but there was no change in the EMF-treated birds (Table1). The infection with coccidiosis had no effect on water intakes as measured during days 2-6 post infection (Table 1).

*Coccidiosis infection*

The OPG values were not different for the infected control and EMF birds (Table 2). In excreta of the uninfected birds, no oocysts were detected.

T**able 2:** Number of oocysts per gram faeces (OPG), expressed as $\log_{10}(X+1)$ for the four experimental groups.

| Day in experiment | Control | | EMF | | Pooled SEM |
|---|---|---|---|---|---|
| | Uninfected | Infected | Uninfected | Infected | |
| 21 (day 6 PI) | 0[a] | 5.698[b] | 0[a] | 5.964[b] | 0.20 |

[a,b,] Mean values with different superscript letter are significantly different ($P$ <0.05).
n= 10 replicates for the control group and 8 replicates for the EMF group.

Lesions caused by *E. acervulina* and *E. maxima* were significantly lower in the infected group exposed to EMF than in the infected controls (Table 3). Lesion scores related to the infection with *E. tenella* showed no influence of EMF treatment. No coccidial lesions were seen in the two uninfected groups.

**Table 3:** Lesion scores and frequencies for the three *Eimeria* spp. in the infected birds on day 21 in the experiment (day 6 PI).

| Treatment | *n* | *E. acervulina* | *E. maxima* | *E. tenella* |
|---|---|---|---|---|
| Mean Lesion Scores | | | | |
| Infected control | 20 | 1.95[a] ± 0.15 [1] | 2.30[a] ± 0.15 | 2.65 ± 0.13 |
| Infected EMF | 16 | 1.19[b] ± 0.10 | 1.50[b] ± 0.13 | 2.00 ± 0.20 |
| *P*-value | | <0.05 | <0.05 | 0.21 |

| Lesion Scores | | *E. acervulina* | | | | | *E. maxima* | | | | | *E. tenella* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Scores*** | **0** | **1** | **2** | **3** | **4** | **0** | **1** | **2** | **3** | **4** | **0** | **1** | **2** | **3** | **4** |
| Treatment | *n* | | | | | | | | | | | | | | | |
| Infected control | 20 | - | 5 | 11 | 4 | - | - | 2 | 10 | 8 | - | - | 1 | 5 | 14 | - |
| Infected EMF | 16 | - | 13 | 3 | - | - | - | 8 | 8 | - | - | - | 5 | 6 | 5 | - |

*n*= number of bird dissected

[1a,b] Mean ± SE values within the same column with different superscript letter are significantly different (P<0.05).

## Discussion

It is clear that the infection with one single dose of the *Eimeria* mixture was successful in the control birds. The infection had induced intestinal lesions, shedding of oocysts and reduction in growth performance. These effects of the infection have been described earlier (Conway *et al.*, 1993; Adams *et al.*, 1996; McDougald, 2003). The novel observation is that exposure of broiler chickens to EMF antagonized the effects of infection with the three *Eimeria* species. In the EMF-treated birds, the infection produced no effect on feed intake, weight gain and feed conversion ratio. Furthermore, the severity of intestinal lesions mediated by *Eimeria acervulina* and *Eimeria maxima* were significantly less than in the infected controls. The EMF-mediated reduction of the severity of lesions in the duodenum and the mid gut most likely was associated with less loss of digestive capacity, which in turn was reflected by the observed unchanged growth performance. In the infected control birds, on the other hand, the more severe lesions due to *E. acervulina* and *E. maxima* were mirrored by a decrease in weight gain and increase in feed conversion ratio. It is well known that the invasion of the epithelial wall by *E. acervulina* and *E. maxima* results in morphological changes of the brush border and decreased activities of digestive enzymes (Fernando and McCraw, 1973; Allen, 1987). It would appear that EMF treatment also has beneficial effects in laying hens. Keirs et al. (2005) reported that EMF exposure in commercial egg-layer flocks improved production, which may have important welfare and economic implications.

The lower intestinal lesion scores for *E. acervulina* and *E. maxima* in the infected birds exposed to EMF were not associated with less oocyst shedding and with lower lesion scores for *E. tenella*. The lack of effect of EMF on faecal oocyst numbers could relate to the fact that all oocysts were counted in excreta collected at only one time point, i.e. at 6 days PI. The counts probably mainly reflected the oocysts of *E. maxima* and *E. tenella* and few oocysts of *E. acervulina* because of the respective prepatent periods (McDougald and Reid, 1991). Alternatively, the lack of effect of EMF on oocyst counts could be explained by the phenomenon that sub-optimal levels of anticoccidial treatments cause the infected animals to produce more oocysts than their unmedicated counterparts

(Brackett and Bliznick, 1949; Barwick and Casorso, 1970; Williams, 1973; Reid, 1975). The sub-optimal level of anticoccidial treatment is thought to reduce the burden of the coccidial infection so that the proportion of surviving sporulated oocysts has extra room in the intestinal epithelium and becomes more reproductive. Factors that might contribute to the differences in response of *E. tenella* and *E. acervulina* and *E. maxima* to EMF include site of parasite invasion, host immune reaction at the infection site and parasite metabolism (Allen *et al.,* 1997). Furthermore, *E. acervulina* and *E. maxima* are more immunogenic than *E. tenella* (Rose and Long, 1962).

The molecular basis underlying the observed antagonistic activity of EMF on coccidiosis infection in broiler chickens is unknown, but it could relate to one or more of the various biological effects that have been described. EMF exposure may have an anti-inflammatory effect (Vallbona and Richard, 1999; Cronstein *et al.,* 1999; Montesinos *et al.,* 2000) and beneficial effects on the nervous system have been recognized (Kaszuba *et al.,* 2005). The efficacy of EMF in increasing blood flow and wound healing has been documented (Cameron, 1961; Goldin *et al.,* 1981; Gessi *et al.,* 2000). Possibly, EMF treatment of the broiler chickens had resulted in increased peripheral blood flow and massive infiltration of macrophages into the damaged tissue, leading to the observed reduction of coccidial lesions. Jeurissen et al. (1996) reported that in immune chickens, fewer sporozoites will reach the crypt epithelium and the formation of schizonts is inhibited. Sporozoites that had failed to reach the crypt epithelium within 48 hours after infection were detected within or were surrounded by macrophages, indicating that the presence of these cells can moderate the intensity of a primary infection.

Further work on the effect of EMF exposure on coccidiosis infection in broiler chickens is needed. The uninfected birds treated with EMF showed a lower feed intake and higher weight gain than did the uninfected control birds. Whether or not this observation was caused by a room effect is not known. The control birds and EMF-treated birds had to be housed in different rooms, albeit that they were adjacent. As mentioned above, the mechanism by which EMF exerts its anticoccidial effect is not known. However, it can be concluded that the exposure of the broilers by low EMF could be

useful in controlling coccidiosis. The potential of EMF signals on broilers during coccidiosis needs to be verified in controlled field trails. Perhaps, EMF exposure could serve as an alternative to the anticoccidial drugs currently used.

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*Chapter* 5

# Exposure of Broiler Chickens to a Weak Electromagnetic Field Reduces the Impact of a Natural-Like Eimeria Infection

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**Summary**

The possible anticoccidial activity of an electromagnetic field (EMF) was investigated in broiler chickens with a natural-like coccidiosis infection. There was an uninfected and infected group not receiving further treatment. Another uninfected and infected group were subjected to EMF treatment. The infection was induced by adding to the litter a mixture of *E. acervulina, E. maxima,* and *E. tenella*. EMF treatment lasted for 30 minutes per day; the field strength within the cages was set to 5 µTesla rms. Infection with *Eimeria* resulted in a transient, but significant reduction of growth performance in the control chickens. Exposure to EMF counteracted the effect of infection on growth performance. EMF treatment had no effect on oocyst shedding. In the infected birds exposed to EMF, the lesion scores related to the three *Eimeria* species were generally lower than in the infected controls. Due to cross-contamination, the uninfected birds also showed intestinal lesions, the severity being less than in the infected chickens. In the uninfected birds, EMF treatment also had significantly reduced the severity of the lesions. It is concluded that EMF exposure protects against coccidiosis in broiler chickens and that it could serve as an alternative to anticoccidial drugs.

*Keywords*: Electromagnetic Fields, Coccidia, Broilers

## Introduction

Coccidiosis is a common infectious disease in poultry, causing major economic losses. The protozoan parasite of the genus *Eimeria* multiplies in the intestinal tract of poultry and produces tissue damage, resulting in reduced growth and increased susceptibility to pathogens (McDougald, 2003) such as *Clostridium perfringens,* leading to necrotic enteritis (Maxey and Page, 1977; Shane *et al*., 1985). The *Eimeria* species have a complex life cycle that occurs outside the host (exogenous stages) and inside the host (indigenous stages). During the indigenous stage, there are both intracellular and extracellular stages and both asexual and sexual reproduction. The complex life cycle of the *Eimeria* is associated with complex host immune responses to the parasite (Lillehoj and Trout, 1994; 1996). The invasion of the *Eimeria* sporozoites into the intestinal epithelium results in massive infiltration of macrophages, granulocytes, and lymphocytes into the lamina propria. The macrophages modulate the severity of the infection, and the lymphocytes, in particular $CD4^+$ T cells, act as inducer of an effective immune response (Jeurissen and Veldman, 2002). Both macrophages and lymphocytes are the source of cytokine production in the intestine during *Eimeria* infection and thereby modulate the immune response (Lillehoj, 1994; Trout and Lillehoj, 1995; Breed *et al*., 1997). It has been reported that an electromagnetic field (EMF) causes stress at the cellular level, leading to the production of cytokines followed by an enhanced immune response (Goodman *et al*., 1994; Mevissen, *et al*., 1998; Simkó and Mattsson, 2004). We hypothesized that exposure of broiler chickens to EMF may antagonize the effects of coccidiosis infection. Indeed, in broiler chickens infected with an *Eimeria* mixture containing *E. acervulina, E. maxima,* and *E. tenella,* the exposure to EMF was shown to counteract the decrease in weight gain and feed intake as well as the development of intestinal lesions (Cuppen *et al*., 2006).

In our previous study (Cuppen *et al*., 2006) the chickens had been infected with a mixture of *Eimeria* by a single dose administered through gavage into the crop. Infection through gavage with a single high dose of inoculum has no similarity with the field situation and might result in results with no practical relevance (Long, 1979). Standardized infection through the litter

mimics the field situation, but can be done under controlled conditions and allows experimental flexibility and a large number of experimental units within the research facility.

In the present study, the possible anticoccidial activity of EMF was further investigated. In broiler chickens, a natural progression of coccidial infection was simulated by adding to the litter a mixture of *E. acervulina, E. maxima,* and *E. tenella*. The effects of EMF exposure on growth performance, intestinal lesions, and oocyst excretion were measured. Preliminary data of the present study has been published in abstract form (Cuppen *et al.,* 2006).

## Materials and Methods

*Birds and housing*

272-one-day-old female broilers (Ross 308) were purchased from a local hatchery. On the day of arrival (day 1), they were wing-banded and randomly housed in wire-floor, suspended cages. Each cage was provided with thick foil and wood shavings as litter. Continuous lighting was provided throughout the experiment. The temperature in the cages on arrival of the birds was 32 °C and then gradually decreased to 20 °C at the end experiment.

*Diets*

Starter and grower diets were used. The diets did not contain growth promoters or coccidiostatics. The starter diet was offered until day 13, followed by the grower diet. The ingredient composition of the diets was as follows (g/kg diet as fed; starter/grower): wheat (250/500), soybean meal (49 % crude protein) (345.5/253.7), corn (27.50/12.29), animal fat (4.00/500), extracted rape seed meal (2.00/3.00), soybean oil (1.87/1.39), corn gluten (1.0522/0.000), premix (0.50/0.50), salt (0.2119/0.1902), sodium bicarbonate (0.2367/0.2295), monocalcium phosphate (1.0995/0.4276), limestone (1.4674/1.0469), DL-methionine (0.2695/0.2146), L-lysine HCl (0.1723/0.2418), L-threonine (0.0638/0.0855), phytase (0.0100/0.010). Throughout the experiment, the birds had free access to feed and tap water.

*Experimental design*

The experiment had a 2x2 factorial design with 8 replicates, each replicate consisting of 8 birds. On day 13, the broilers were weighed and divided over the 4 experimental groups so that the weight distributions of the groups were similar. There was an uninfected and infected group not receiving further treatment. Another uninfected and infected group was subjected to EMF treatment, which was started on day one. The infection with a mixture of *Eimeria* species was done on day 15 (see below). Under each cage of the EMF treated birds, there was a magnetic coil, consisting of 50 loops of 1.5 $mm^2$ electricity wire. The coils were connected via a relays bank to an amplifier and signal generator controlled by a microprocessor, which regulated the period of time and the EM field per cage. The EMF treatment period lasted for 30 minutes and was given to each cage consecutively and once per 24 hours. The field strength within the cages was set to 5 µTesla rms, verified by a FW Bell 5180 Tesla meter with a MOS51-3204 Low field probe. In order to avoid any effect of EMF exposure on the other groups, the EMF-free and EMF-infected groups were housed in adjacent rooms within the facility. The cages of the uninfected and infected groups had evenly distributed locations.

*Experimental infection procedure*

To test the anticoccidial activity of the EMF, a natural simulation of a coccidial infection within the broilers' test facility was developed. This was accomplished by placing both infected groups on day 15 in new litter seeded with sporulated coccidial oocysts. The infected chickens would pick up the oocysts from the seeded litter. Individual birds would ingest different numbers of oocysts at different times, which mimic a natural, progressive infection. The uninfected groups were placed in a clean litter. Sporulated oocysts of *E. acervulina* (Weybridge strain), *E. maxima* (Weybridge strain), and *E. tenella* (Houghton strain) laboratory strains were used in this study. The numbers of each species mixed into the wood shavings were equivalent to $1.76x10^4$ *E. acervulina,* $1.25x10^4$ *E. maxima* and $7.5x10^3$ *E. tenella* per bird. The infected litter was spread across the back half of the cage. The oocysts were obtained from the Animal Health Service Ltd., Poultry Health Centre (Deventer, the Netherlands).

*Performance measurements*

Birds were weighed individually on days 15, 21, 28 and 35. Feed intake was measured per cage on a weekly basis. Average feed intake per broiler within a cage was calculated and corrected for dropouts, if any. Mortality was registered on a daily basis.

*Infection measurements*

The number of oocysts per gram faeces (OPG) was determined for excreta collected on days 6, 13 and 21 post infection (PI). Oocyst shedding was assessed on one sample of homogenized fresh excreta collected from each cage. The modified McMaster counting chamber technique of Hodgson (1970) was used. A 10% (w/v) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of water) was prepared. After shaking thoroughly, 1 ml of the suspension was mixed with 9 ml of a salt solution (311 g of NaCl mixed into 1 L of water). Then, the suspension was put into the McMaster chamber using a micropipette and the number of oocysts was counted and expressed per gram of faeces (Peek and Landman, 2003).

The severity of coccidial lesions was scored on day 6, 13 and 21 PI while the investigator was blinded to treatment modality. The 0-4 scoring system described by (Johnson and Reid, 1970) was used.

*Statistical analysis*

The oocyst values were logarithmically transformed [$\log_{10}(X+1)$] to create a normal distribution and lesion scores were transformed using multinomial transformation. Lesion scores for the various experimental groups were compared using the non-parametric Mann-Whitney U test. Performance and oocyst data were subjected to the LSD test. The statistical program SPSS (SPSS Inc, Chicago, USA) was used. The level of statistical significance was preset at $P< 0.05$.

## Results

*Growth performance*

Post-infection growth performance data are presented in Table 1. The infection caused a significant growth depression

during the first two weeks in the control birds, but not in those exposed to EMF. During days 1-6 PI, the uninfected EMF-treated chickens displayed lower weight gain than did the uninfected controls, but this difference had disappeared during the subsequent periods. In the period of 7-13 days PI, the infected EMF-treated chickens grew significantly faster than did the infected controls. The infection produced a decrease in feed intake in the control chickens during days 1-6 and 7-13 PI, but feed intake was left unchanged in their counterparts exposed to EMF. In the control birds, the infection caused an increase in the feed conversion ratio during days 7-14 PI. In the EMF-treated chickens, the infection did not influence the feed conversion ratio.

**Table 1:** Post-infection growth performance for the four experimental groups.

| Variable and days PI | Control[1] | | EMF[2] | | Pooled SEM |
|---|---|---|---|---|---|
| | Uninfected | Infected | Uninfected | Infected | |
| Body weight (g) | | | | | |
| Day 6 | 738[a] | 658[b] | 690[b] | 684[b] | 15.75 |
| Day 13 | 1183[a] | 1021[b] | 1191[a] | 1216[a] | 25.04 |
| Day 21 | 1644[a] | 1539[b] | 1701[a] | 1686[a] | 37.31 |
| Weight gain (g/day) | | | | | |
| Days 1-6 | 50[a] | 45[b] | 45[b] | 46[ab] | 1.54 |
| Days 7-13 | 65[a] | 56[b] | 66[a] | 67[a] | 2.08 |
| Day 14-21 | 61 | 65 | 67 | 68 | 3.93 |
| Feed intake (g/day) | | | | | |
| Days 1-6 | 85 [a] | 75 [b] | 76 [b] | 76 [b] | 2.20 |
| Days 7-13 | 114[b] | 106[b] | 116[a] | 115[a] | 2.68 |
| Day 14-21 | 139 | 142 | 141 | 136 | 5.01 |
| Feed conversion ratio (FCR) | | | | | |
| Days 1-6 | 1.71 | 1.69 | 1.67 | 1.64 | 0.037 |
| Days 7-13 | 1.77[a] | 1.91[b] | 1.76[a] | 1.72[a] | 0.050 |
| Days 14-21 | 2.36 | 2.20 | 2.18 | 2.02 | 0.111 |

[a,b,c] Mean values within the same row with different superscript letter are significantly different ($P$<0.05).
Data are presented for 8 cages per experimental group.

*Coccidiosis infection*

The uninfected chickens were found to shed oocysts with the faeces, pointing at cross contamination (Table 2). On day 6 PI, the OPG values were significantly higher in the infected than the uninfected birds, irrespective of whether they had been exposed to EMF or not. Among the EMF-treated chickens the infected animals consistently showed higher OPG values than did their uninfected counterparts. Among the control chickens, the infected and uninfected animals had similar OPG values on days 13 and 21 PI

**Table 2:** Number of oocysts per gram faeces (OPG) expressed as $\log_{10}(X+1)$ for the four experimental groups

| Variable and days PI | Control[1] | | EMF[2] | | Pooled SEM |
|---|---|---|---|---|---|
| | Uninfected | Infected | Uninfected | Infected | |
| Day 6 | 1.719[a] | 4.033[b] | 2.168[a] | 3.988[b] | 0.606 |
| Day 13 | 3.259[ab] | 4.577[b] | 2.051[a] | 4.384[b] | 0.553 |
| Day 21 | 4.041[a] | 3.815[a] | 2.358[b] | 3.659[a] | 0.387 |
| Day 6 | 1.719[a] | 4.033[b] | 2.168[a] | 3.988[b] | 0.606 |

[a,b,] Mean values within the same row with different superscript letter are significantly different ($P$<0.05).
Data are presented for 8 cages per experimental group.

The uninfected birds showed intestinal lesions as would be expected based on oocyst shedding (Table 3). However, the severity of the lesions in the uninfected birds was consistently lower than in the infected chickens. Lesion scores due to *E. acervulina* and *E. maxima* on days 6 and 21 PI in the infected chickens were significantly lower in EMF-treated animals than in the controls. The lesions caused by *E. tenella* were less severe on days 13 and 21 PI for the infected EMF-treated chickens versus the infected controls. On days 13 and 21 PI, the uninfected chickens exposed to EMF had lower lesion scores than the uninfected controls, but the difference not always reached statistical significance.

**Table 3:** Intestinal lesion scores per *Eimeria* spp. for the four experimental groups

| Score per *Eimeria spp* | Control[1] | | EMF[2] | | *P*-value |
|---|---|---|---|---|---|
| | Uninfected | Infected | Uninfected | Infected | |
| Day 6 PI | | | | | |
| *E. acervulina* | $0.44^{a} \pm 0.16$ | $2.44^{b} \pm 0.13$ | $0.44^{a} \pm 0.13$ | $2.00^{c} \pm 0.13$ | <0.05 |
| *E. maxima* | $0.13^{a} \pm 0.09$ | $2.25^{b} \pm 0.17$ | $0.06^{a} \pm 0.06$ | $1.69^{c} \pm 0.12$ | <0.05 |
| *E. tenella* | $0.13^{a} \pm 0.09$ | $1.50^{b} \pm 0.13$ | $0.06^{a} \pm 0.06$ | $1.56^{b} \pm 0.18$ | <0.05 |
| Day 13 PI | | | | | |
| *E. acervulina* | $1.06^{a} \pm 0.19$ | $1.69^{cd} \pm 0.15$ | $0.33^{b} \pm 0.13$ | $1.44^{ac} \pm 0.16$ | <0.05 |
| *E. maxima* | $0.56^{a} \pm 0.16$ | $1.63^{bc} \pm 0.15$ | $0.00^{d} \pm 0.00$ | $1.13^{b} \pm 0.18$ | <0.05 |
| *E. tenella* | $0.19^{a} \pm 0.10$ | $1.31^{b} \pm 0.15$ | $0.07^{a} \pm 0.07$ | $0.94^{c} \pm 0.11$ | <0.05 |
| Day 21 PI | | | | | |
| *E. acervulina* | $1.38^{ab} \pm 0.27$ | $1.75^{b} \pm 0.17$ | $0.94^{a} \pm 0.19$ | $1.25^{a} \pm 0.13$ | <0.05 |
| *E. maxima* | $1.50^{ad} \pm 0.33$ | $2.13^{cd} \pm 0.15$ | $0.50^{b} \pm 0.22$ | $1.25^{a} \pm 0.18$ | <0.05 |
| *E. tenella* | $0.56^{a} \pm 0.16$ | $1.38^{b} \pm 0.15$ | $0.13^{c} \pm 0.09$ | $1.17^{b} \pm 0.11$ | <0.05 |

$^{a,b,c,d}$ Mean values ± SE within the same row with different superscript letter are significantly different ($P$<0.05).
Data are presented for 16 birds per experimental group per time point.

## Discussion

It was assumed that the use of litter contaminated with oocysts would simulate a natural coccidiosis infection. Oocyst shedding in the infected birds was found to peak on day 13 PI followed by a decline on day 21 PI. This pattern of oocyst shedding agrees with findings in commercial broiler operations (Hamet *et al.*, 1986; McDougald and Reid, 1991; McDougald, 2003). The presence of oocysts in the excreta of the uninfected chickens indicates that cross contamination had occurred. Although we attempted to prevent cross contamination, we feel that it does not affect the purpose of this study, i.e. studying the effect of EMF exposure on the severity of coccidiosis infection. The lesions in the uninfected chickens were less severe than in the infected ones. It could even be suggested that because of the cross contamination, the effect of EMF at two levels of infection can be evaluated.

Infection with *Eimeria* resulted in a significant reduction of growth performance in the control chickens, which was no longer apparent at the end of the experiment. An initial depression of technical performance followed by compensatory weight gain has been reported in chickens immunized against coccidiosis by hatchery spray (Mathis, 1999; Mathis and Long, 2001). Exposure to EMF counteracted the effect of infection on growth performance. In the EMF-treated chickens, the infection did not reduce body weight, weight gain and feed intake and did not raise the feed conversion ratio. The positive effect of EMF on the infected birds in this experiment may extend to laying hens. Keirs et al. (2005) reported that exposure of egg-layer flocks to EMF improved production, which may have important welfare and economic implications.

Contrary to growth performance, the number of oocysts in excreta was not significantly influenced by EMF exposure. This observation may not be surprising. Oocyst shedding only reflects the intensity of the infection at the time of sampling (Hodgson, 1970). Indeed, various workers have reported that oocyst production correlates only poorly with the performance of broiler chickens (Jeffers, 1978; Williams and Catchpole, 2000; Williams and Andrews, 2001). Moreover, Reid (1975) has criticized the use of oocyst shedding as the only index of the anticoccidial activity of drugs because medicated chickens may even produce more oocysts

than do unmedicated chickens. If any, in this study, the anticoccidial activity of EMF was associated with a tendency towards diminished excretion of oocysts.

Examination of the intestine on day 6 PI showed that in the infected birds exposed to EMF the lesion scores related to *E. acervulina* and *E. maxima* infection were significantly lower than in the infected controls, but the lesions due to the *E. tenella* were not affected by EMF. On days 13 and 21 PI, the group mean lesion scores for all three *Eimeria* species were lower in infected chickens exposed to EMF than in the infected controls. In the uninfected birds, there was no effect of EMF on intestinal lesions on day 6 PI, but on days 13 and 21 PI, EMF treatment had significantly reduced the severity of the lesions. Factors that might contribute to the differences in response of *E. tenella* and *E. acervulina* and *E. maxima* to EMF include site of parasite invasion, host immune reaction at the infection site and parasite infective stage (Allen *et al.,* 1997), Furthermore, *E. acervulina* and *E. maxima* are more immunogenic than *E. tenella* (Rose and Long, 1962). On day 13 PI, the lesion scores due to *E. acervulina*, *E. maxima* and *E. tenella* in both infected groups were on average lower than on day 6 PI. The opposite was seen in the uninfected chickens. The lesions on day 13 PI in the infected chickens are secondary lesions induced by re-infection as a consequence of the initial infection (Williams and Andrews, 2001; Williams, 2003). Oocysts that are excreted initially after a coccidiosis infection are less pathogenic than oocysts shed later because the latter have completed their life cycle in the host (Jeffers, 1975; Ryley *et al.,* 1976).

The molecular basis underlying the observed antagonistic activity of EMF on coccidiosis infection in broiler chickens is unknown, but it could relate to one or more of the various biological effects that have been described. EMF exposure may have an anti-inflammatory effect (Vallbona and Richard, 1999; Cronstein *et al.,* 1999; Montesinos *et al.,* 2000) and beneficial effects on the nervous system have been recognized (Kaszuba *et al.,* 2005). The efficacy of EMF in increasing blood flow and wound healing has been documented (Cameron, 1961; Goldin *et al.,* 1981; Gessi *et al.,* 2000). Possibly, EMF treatment of the broiler chickens had resulted in increased peripheral blood flow and massive

infiltration of macrophages into the damaged tissue, leading to the observed reduction of coccidial lesions. Jeurissen et al. (1996) reported that in immune chickens, fewer sporozoites will reach the crypt epithelium and the formation of schizonts is inhibited. Sporozoites that had failed to reach the crypt epithelium within 48 hours after infection were detected within or were surrounded by macrophages, indicating that the presence of these cells can moderate the intensity of a primary infection.

Further work on the effect of EMF exposure on coccidiosis infection in broiler chickens is needed. The uninfected birds treated with EMF showed a lower feed intake and weight gain than did the uninfected control birds. Whether or not this observation was caused by a room effect is not known. The control birds and EMF-treated birds had to be housed in different rooms, albeit that they were adjacent. As mentioned above, the mechanism by which EMF exerts its anticoccidial effect is not known. From a practical point of view, it would be relevant to find out whether optimizing the conditions of EMF could increase its anticoccidial activity. Perhaps, EMF exposure could serve as an alternative to the anticoccidial drugs currently used.

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*Chapter* **6**

# Efficacy and Characteristics of different Methods of Coccidiosis Infection in Broiler Chickens

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**Abstract**

In five different experiments with broiler chickens, different methods of infection with *Eimeria* species were used. In this paper, we compare and contrast the various methods, anticipating that the data may contribute to the selection of the most appropriate model of coccidiosis in broiler chickens. Such a model is important to speed up the screening of potential coccidiostatics. Administration through gavage into the crop of relatively low doses of either *Eimeria tenella* alone, or in combination with *Eimeria acervulina* and *Eimeria maxima*, did not influence body-weight gain and feed intake, but did induce intestinal lesions and faecal shedding of oocysts. The administration of an identically high number of sporulated oocysts in the form of a mixture of the three *Eimeria* species, either through a single dose by gavage or through the litter, produced similar lowering effects on body-weight gain or feed intake, similar degrees of severity of intestinal lesions and similar rates of faecal oocyst shedding. Depending on the variables considered of interest, the present data may indicate the most appropriate model. The model using infection with oocysts through the litter may optimally mimic the field situation in combination with controlled conditions and allowing experimental flexibility and a high number of experimental units within the research facility.

*Keywords:* Infection Models, Coccidia, Broilers.

## Introduction

Coccidiosis is an infectious disease caused by the protozoan parasite of the genus *Eimeria* (Tyzzer, 1932). The disease is almost universal in poultry production and *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella* are considered as the pathogenic species (McDougald, 2003). Infection with coccidiosis follows the ingestion of viable oocysts, which are contaminants of food, dust, and water. After the oocysts are swallowed, the sporozoites are liberated and a life cycle in the host tissues begins, leading to the faecal excretion of newly formed oocysts. Coccidiosis remains one of the most expensive and common diseases in poultry production (Steven, 1998; Dalloul and Lillehoj, 2006).

Given the importance of coccidiosis in poultry production, consumer concern and the expected ban on the use of coccidiostatics (Van den Ban *et al*., 2005), there is great interest in the development of alternatives in the prevention and treatment of the disease. In order to study the efficacy of alternatives, a poultry model of coccidiosis is needed. In the course of our studies on alternative approaches to counteract coccidiosis in broiler chickens, we have used different models of infection (Chapters 2-5). In this paper, we compare and contrast the various methods that we have used. It is anticipated that the data presented here may contribute to the selection of the most appropriate infection model of coccidiosis in broiler chickens.

## Materials and Methods

*Animals, diet, and experimental designs*

One-day-old broiler chicks (Ross 308) were purchased from a local hatchery. On arrival (day 1) they were wing-banded, weighed and randomly allocated to the treatments. The birds were housed in wire-suspended cages equipped with plastic sides. The cages had either their wire floor as such (experiments 1 and 2) or covered with plastic and clean wood shavings as bedding (experiments 3, 4 and 5). Ambient temperature was gradually decreased from 32 $^{o}$C on day 1 to 20 $^{o}$C at the end of the experiments. During the entire experimental periods there was

continuous lighting. The basal diet used did not contain growth promoters or coccidiostatics. The composition was as follows (g/kg diet): wheat (+ xylanase), 250; corn, 321; soyabean meal (46.7% crude protein), 225; peas, 50; sunflower meal (32% crude protein), 40; potato protein, 15; fish meal (72% crude protein), 25; soyabean oil, 40; premix (Research Diet Services, Wijk bij Duurstede, The Netherlands), 5; limestone, 16; monocalcium phosphate, 7; phytase (Natuphos 5000G), 0.1; salt, 1.7; sodium bicarbonate, 1.7; L-lysine HCl, 0.8; D,L-methionine, 1.7. The diets were fed *ad libitum* as from arrival of the birds until the end of the experiments. Tap water was freely available.

Table 1 gives a brief overview of the five experiments that were carried out. Between the experiments, there are differences in the coccidiosis infection as to time point, *Eimeria* species and dose, and route of oocyst administration. Below the experimental designs are described in more detail.

**Table 1:** Overview of the five experiments

| Experiment | Day of infection | Route of infection | *Eimeria* species (dose of sporulated oocysts / bird) | Measurements |
|---|---|---|---|---|
| 1 | 12 | Gavage into crop | *E. tenella* (3500) | Growth performance lesion scores |
| 2 | 8 | Gavage into crop | *E. tenella* (5000) | Growth performance, lesion scores, OPG[1] |
| 3 | 1 | Gavage into crop | *E. acervulina* (900)<br>*E. maxima* (570)<br>*E. tenella* (170) | Growth performance, lesion scores, OPG |
| 4 | 15 | Gavage into crop | *E. acervulina* (17600)<br>*E. maxima* (12500)<br>*E. tenella* (7500) | Growth performance, lesion scores, OPG |
| 5 | 15 | Through litter | *E. acervulina* (17600)<br>*E. maxima* (12500)<br>*E. tenella* (7500) | Growth performance, lesion scores, OPG |

[1]OPG = number of oocysts per gram fresh faeces.

*Experiment 1*

The control and infected group consisted of 5 replicates of 5 birds each. Each replicate was housed in a separate cage and all birds were fed the basal diet. On day 12, one group was inoculated with 3500 sporulated oocysts of the *E. tenella* (Houghton strain) laboratory strain. The oocysts were obtained from the Animal Health Service Ltd., Poultry Health Centre (Deventer, The Netherlands). The oocysts were administered with 1 ml of tap water via an oral gavage directly into the crop. The control group was given water only through gavage. Care was taken to prevent cross contamination throughout the experiment. At the end of the experiment (day 21), the birds were euthanized by cervical dislocation.

*Experiment 2*

The control and infected group consisted of 5 replicates of 3 birds each. Each replicate was housed in a separate cage and all birds were fed the basal diet. On day 8, one group of birds was inoculated with 5000 sporulated oocysts of *E. tenella*. The oocysts were administered as described for experiment 1. The control group was given water through gavage. On day 14, all birds were killed by cervical dislocation.

*Experiment 3*

The control and infected group consisted of 8 replicates of 8 birds each. Each replicate was housed in a separate cage and all birds were fed the basal diet. On day 1, one group was challenged with a mixture of *Eimeria* containing 900 sporulated oocysts of *E. acervulina* (Weybridge strain), 570 sporulated oocysts of *E. maxima* (Weybridge strain), and 170 sporulated oocysts of *E. tenella* (Houghton strain). The oocysts were laboratory strains and the dose and strains simulated commercially available live vaccines (Williams, 2002). The sporulated oocysts were administered with 1 ml of tap water directly into the crop via a scaled 1-ml syringe. The non-infected birds were given 1 ml of oocyst-free water into the crop. On day 14, one bird per cage per treatment was killed by cervical dislocation and used for lesion scoring. On day 19, all remaining birds were killed by cervical dislocation.

*Experiment 4*

The control and the infected group consisted of 10 replicates, each replicate comprising 8 birds. Each replicate was housed in a separate cage and all birds were given the basal diet. On day 15, the chickens of one group were individually challenged with a mixture of *Eimeria* containing $1.76x10^4$ sporulated oocysts of *E. acervulina* (Weybridge strain), $1.25x10^4$ sporulated oocysts of *E. maxima* (Weybridge strain) and $7.5x10^3$ sporulated oocysts of *E. tenella* (Houghton strain). The sporulated oocysts were laboratory strains and administered with 1 ml of tap water via a scaled 1-ml syringe directly into the crop. The negative groups were also given 1 ml water via a scaled 1-ml syringe directly into the crop, but without oocysts. On days 21 and 28, two birds per treatment were killed by cervical dislocation and on day 35 the remaining birds were killed.

*Experiment 5*

The control and infected group consisted of 8 replicates of 8 birds each. Each replicate was housed in a separate cage and all birds were given the basal diet. On day 15, a simulation of a natural coccidial infection within the broilers' test facility was created. The infected replicates were placed in new litter seeded with sporulated oocysts. Individual birds would pick up the oocysts from the litter in different amounts and at different times, thus mimicking a natural, progressive infection. The uninfected replicates were placed in clean litter. Sporulated oocysts of *E. acervulina* (Weybridge strain), *E. maxima* (Weybridge strain), and *E. tenella* (Houghton strain) laboratory strains were used. A fixed amount of each species in a mixture ($1.76x10^4$ *E. acervulina*, $1.25x10^4$ *E. maxima* and $7.5x10^3$ *E. tenella* per bird) were mixed into wood shavings and spread evenly across the back half of the cage. On days 21 and 28, two birds per treatment were killed by cervical dislocation and on day 35 the remaining birds were killed.

*Sampling and measurements*

*Technical Performance*

In experiments 1 and 2, body weights were recorded on the day of infection and at 6 days post infection (PI). In experiments 3, 4 and 5, body weights were measured on the day of the infection, 6

days PI and then weekly until the end of each experiment. For the various time intervals, feed intakes were determined and expressed as g/bird/day. Feed conversion ratios (FCR) were calculated as feed intake per cage divided by weight gain of birds in the cage.

*Oocyst Counting*

The number of oocysts per gram of faeces (OPG) in experiment 2 was determined in faecal samples collected on days 5-7 PI. In experiment 3 OPG was determined for days 5,7,9,12,14 and 19 PI. In experiments 4 and 5, OPG was determined for days 6, 13 and 21 PI. A modified McMaster counting chamber technique of Hodgson (1970) was used. A 10% (w/v) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of water) was prepared. After shaking thoroughly, 1 ml of the suspension was mixed with 9 ml of a salt solution (311 g of NaCl mixed into 1 L of water). Then, the suspension was put into the McMaster chamber using a micropipette and the number of oocysts was counted (Peek and Landman, 2003).

*Lesion Scoring*

Lesion scoring in experiments 1 and 2 was performed on day 6 PI, whereas in experiments 3 and 4 it was done for days 14 and 19 PI and days 6 and 13 PI, respectively. In experiment 5, lesion scoring was performed for days 6, 13 and 21 PI. The severity of coccidial lesions was scored while the investigator was blinded to treatment modality. In all experiments, the 0-4 scoring system described by Johnson and Reid (1970) was used. The lesions caused by the three species of *Eimeria* were monitored separately when applicable.

*Statistical analysis*

To identify statistically significant treatment effects, the technical data for each variable were subjected to the Independent-Samples T Test procedure to compare the infected and uninfected means using SPSS (SPSS Inc, Chicago, USA). Prior to statistical testing, the oocyst values were logarithmically transformed [$\log_{10}(X+1)$] to create a normal distribution and lesion scores were transformed using multinomial transformation. The level of statistical significance was pre-set at $P < 0.05$.

## Results

*Growth performance*

Body weight gain in all 5 experiments is illustrated in Table 2. In experiments 1 and 2, there was no significant difference in weight gain between control and infected birds. Likewise, in experiment 3 the birds infected by gavage on day 1 showed unaffected weight gain. The birds infected by gavage on day 15 with the three *Eimeria* species (experiment 4) displayed a significant decrease in weight gain during the period of two weeks PI. In experiment 5, the infection with the three *Eimeria* species had induced a significant decrease in weight gain, which was no longer apparent in the period of 14-21 days PI.

In experiments 1, 2 and 3, feed intake and feed conversion ratios were similar for control and infected birds. In experiment 4, feed intake during days 1-6 PI was significantly lowered by the infection, but was unaffected thereafter. In experiment 5, a significantly lower feed intake in the infected birds also was recorded for days 1-6 days PI. The feed conversion ratio for the infected birds was significantly higher during days 1-6 and 7-14 PI in experiment 4, but in experiment 5 such an effect was not seen.

**Table 2:** Post-infection (PI) technical performance in the five experiments

| Days PI | Body Weight Gain (g/d) | | | Feed Intake (g/d/bird) | | | FCR | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Infected | *P*-value | Control | Infected | *P*-value | Control | Infected | *P*-value |
| Experiment 1 | | | | | | | | | |
| 1-6 | 54±1.2 | 52±1.5 | 0.53 | 71±1.2 | 68±2.8 | 0.70 | 1.33±0.02 | 1.32±0.03 | 0.72 |
| Experiment 2 | | | | | | | | | |
| 1-6 | 41±1.5 | 39±2.5 | 0.46 | 45±0.9 | 41±1.7 | 0.11 | 1.29±0.23 | 1.27±0.06 | 0.75 |
| Experiment 3 | | | | | | | | | |
| 1-7 | 12±0.9 | 14±0.4 | 0.16 | 15±1.2 | 17±0.6 | 0.38 | 1.26±0.05 | 1.20±0.02 | 0.29 |
| 8-14 | 34±1.1 | 35±0.9 | 0.57 | 51±2.9 | 51±1.5 | 0.91 | 1.48±0.09 | 1.46±0.06 | 0.85 |
| 15-19 | 53±2.5 | 53±1.8 | 0.98 | 79±3.5 | 76±5.0 | 0.65 | 1.54±0.13 | 1.45±0.08 | 0.56 |
| Experiment 4 | | | | | | | | | |
| 1-6 | 59$^{a}$±1.5 | 45$^{b}$±1.6 | <0.001 | 106±1.5 | 93±2.1 | 0.34 | 1.67$^{a}$±0.33 | 1.94$^{b}$±0.05 | <0.001 |
| 7-13 | 77$^{a}$± 1.4 | 69$^{b}$± 1.4 | 0.001 | 123$^{a}$±3.6 | 121$^{b}$± 3.2 | <0.001 | 1.60$^{a}$±0.04 | 1.76$^{b}$±0.05 | 0.02 |
| 14-21 | 86± 2.3 | 77± 5.6 | 0.17 | 149±7.5 | 158±5.4 | 0.36 | 2.00±0.14 | 1.84±0.06 | 0.30 |
| Experiment 5 | | | | | | | | | |
| 1-6 | 50$^{a}$±1.2 | 45$^{b}$±1.9 | 0.03 | 85$^{a}$±1.28 | 75$^{b}$±1.9 | <0.001 | 1.71±0.02 | 1.69±0.05 | 0.79 |
| 7-13 | 65$^{a}$±2.5 | 56$^{b}$±2.5 | 0.02 | 113±1.8 | 106±4.2 | 0.14 | 1.77±0.06 | 1.91±0.04 | 0.06 |
| 14-21 | 61±3.9 | 65±2.4 | 0.36 | 139±4.5 | 142±5.4 | 0.68 | 2.36±0.14 | 2.20±0.08 | 0.33 |

$^{a,b}$ Mean values for each variable within the same row bearing different superscript letters reflect a significant difference between control and infected animals ($P$<0.05).

*Lesion Scores*

In experiments, 1, 2 and 3 the control birds were found to be free of lesions throughout the experiment. However, in experiments 4 and 5 cross contamination had occurred (from day 13 PI and day 6 PI, respectively) which was indicated by lesion scores in the controls that were in general one unit lower than those seen in their infected counterparts. The lesion scores for the infected birds in all experiments are presented in Table 3.

**Table 3**: Lesion scores and their location for the infected birds in the five experiments

| Days PI | Location of Lesions | | *Eimeria* species | | |
|---|---|---|---|---|---|
| | | *n* | *E. acervulina* | *E. maxima* | *E. tenella* |
| Experiment 1 | | | | | |
| 6 | Caecum | 25 | - | - | 1.1 ± 0.21 |
| Experiment 2 | | | | | |
| 6 | Caecum | 15 | - | - | 1.70 ±0 .27 |
| Experiment 3 | | | | | |
| 14 | Duodenum, jejunum, caecum | 8 | 0.6 ± 0.18 | 0 | 0 |
| 19 | Duodenum, jejunum, caecum | 24 | 0.5± 0.1 | 0.7 ± 0.14 | 0.4 ± 0.13 |
| Experiment 4 | | | | | |
| 6 | Duodenum, jejunum, caecum | 20 | 2.0± 0.15 | 2.3± 0.15 | 2.7 ± 0.13 |
| 13 | Duodenum, jejunum, caecum | 20 | 1.7 ±0.15 | 1.6 ±0.15 | 1.1 ±0.18 |
| Experiment 5 | | | | | |
| 6 | Duodenum, jejunum, caecum | 16 | 2.4 ±0.13 | 2.3 ±0.17 | 1.5±0.13 |
| 13 | Duodenum, jejunum, caecum | 16 | 1.7±0.15 | 1.6 ±0.15 | 1.3 ±0.15 |
| 21 | Duodenum, jejunum, caecum | 16 | 1.8 ±0.17 | 2.1±0.15 | 1.4±0.15 |

Mean± SE; *n* = number of birds dissected

In experiments 1 and 2 the caecal lesions scores were similar. For experiments 3, 4 and 5, the mean scores for the lesions in duodenum, jejunum, and caecum are given. The small dose of oocysts used in experiment 3 resulted on day 14 PI in an average duodenal lesion score of 0.6 for *E. acervulina* and absence of lesions due to *E. maxima* and *E. tenella*. On day 19 PI, lesions scores below 1 were seen for all three species of *Eimeria*. After infection with identical amounts of the three *Eimeria* species in experiments 4 and 5, but administered through gavage or litter, respectively, similar lesion scores were observed in the infected birds. The scores all had values well above 1.

*Oocysts in faeces*

OPG was not determined in experiment 1. In experiment 2, the OPG was measured in a faecal sample pooled for days 5-7 PI. In experiments 3-5, oocyst shedding was followed during the time intervals indicated in Table 4.

**Table 4**: Number of oocysts per gram faeces (OPG) expressed as $\log_{10}(X+1)$ for the infected birds in the five experiments

| Days PI | *n* | OPG |
|---|---|---|
| Experiment 2 | | |
| 5-7 | 5 | 4.80±0.28 |
| Experiment 3 | | |
| 5 | 8 | 2.70±0.44 |
| 7 | 8 | 0.54±0.10 |
| 9 | 8 | 0.20±0.05 |
| 12 | 8 | 5.10±0.78 |
| 14 | 8 | 1.01±0.36 |
| 16 | 8 | 3.38±1.99 |
| 19 | 8 | 0.39±0.13 |
| Experiment 4 | | |
| 6 | 10[1] | 5.70±0.35 |
| 13 | 10 | 4.02±0.34 |
| 21 | 10 | 2.24±0.52 |
| Experiment 5 | | |
| 6 | 8 | 4.03±0.12 |
| 13 | 8 | 4.58±0.20 |
| 21 | 8 | 3.82±0.22 |

Mean± SE; *n* = number of replicates

The OPG pattern in experiment 3 showed alternating peaks and dips. In experiments, 4 and 5 there were high OPG values on days 6 and 13 PI, followed by a fall on day 21 PI.

## Discussion

In all five experiments, there was a successful infection with *Eimeria* species as indicated by the intestinal lesions and the shedding of the oocysts. In experiments, 1, 2 and 3 the infection had no influence on growth performance. The lack of effect of infection on growth performance may relate to the relatively low numbers of sporulated oocysts used. This observation corroborates earlier work (Conway *et al.,* 1993). The higher numbers of oocysts used in experiments 4 and 5 did indeed lower weight gain and feed intake PI. In experiment 4, feed conversion was clearly increased by the infection. The impact of higher infection doses on weight gain and feed intake has also been reported by others (Barwick *et al.,* 1970; Johnson and Reid, 1970; Long 1970; Conway *et al*., 1993; McDougald, 2003). In experiments 4 and 5 the reduction in the weight gain during days 1-6 PI was 24 and 13 %, respectively, and the decrease in feed intake was 12 % for both experiments. The similar effect of infection on feed intake in experiments 4 and 5 may indicate that the route of the infection had no additional impact. Coccidiosis is an infectious disease that may reduce body-weight gain in broilers as a result of the combination of reduced feed intake and reduced digestibility and absorption of macronutrients (Adams *et al*., 1996).

The absence of the intestinal lesions due to *E. maxima* and *E. tenella* on day 14 PI in experiment 3 may be explained by the low dose used and their low oocyst productive potential (Brackett and Bliznick, 1952). The lesion scores for *E. acervulina* and *E. maxima* in experiments 4 and 5 were similar. This again indicates that the administration of equal doses of *E. acervulina* and *E. maxima* through either gavage or litter had no differential effect. However, in experiment 5 the lesion scores due to *E. tenella* on day 6 PI were much lower than those seen in experiment 4 on the same day PI. This difference may relate to the slow progress of the infection in experiment 5 in which the oocysts were administered through the litter. The severity of the symptoms caused by *E. tenella* infection depends on the rate of the infection and the susceptibility of the

birds. When the chickens gradually pick up the oocysts, they may become resistant before clinical effects appear as opposed to administration of a massive dose by gavage (Davies *et al*., 1963; McDougald, 2003).

In addition to growth performance and lesion scores, oocyst shedding also is a good index of the success of infection with the *Eimeria.* In experiment 3 it was attempted to enhance immunity of the birds by the combination of an initial parasitic stage provided by an initial low coccidiosis infection dose and subsequent boosting the immunity by multiple re-infections with oocysts shed in the litter as does a non-attenuated vaccine (Chapman, 2000; Williams, 2002). Indeed, the pattern of faecal oocyst excretion was as expected, mainly because the initial infection dose was low and more or less equal to the dose of a non-attenuated vaccine. Williams (2002) reported that in vaccinated birds, the oocyst stage of *Eimeria* that follow excystation initiates the vaccinal infection, hence stimulating immunity, and that during subsequent recycling of infection this immunity is maintained. The phenomenon establishes 'trickle' infections that have been shown to be very effective in stimulating protective immunity (Joyner and Norton, 1973; 1976). Such infection model can be used to study the induction of the immunity by recycling of the infection in the presence of an agent with coccidiostatic activity. The prophylactic effects of such agents are obviously assisted by the chicken immune responses to coccidial challenges by oocyst accumulation in the litter (Jeffers, 1989; Philips, 1994; 1997; Chapman, 1999; Williams, 2002). Moreover, this model can be used to screen feed additives that have the potential to enhance the immunity of the host against *Eimeria* infection (Elmusharaf *et al*., 2007).

In experiments 4 and 5, oocyst shedding on day 21 PI was lower than on days 6 and 13 PI, the difference being greater in experiment 4. The high dose of the *Eimeria* mixture administered by gavage caused a high OPG value on day 6 PI followed by a steady reduction days 13 and 21 PI. This pattern may be explained by a crowding effect resulting from the boosting of the initial effect of the challenge dose by the secondary and the third infection produced by recycling of the oocysts shed in the litter (Tyzzer *et al.,* 1932; Brackett and Bliznick, 1952; Williams, 2001; 2002). The pattern of

oocyst shedding should be taken into account when interpreting the results of oocyst production in the testing of anticoccidial agents (Williams, 2001). The oocysts shedding pattern seen in experiment 5 resembles that in field situations (Hamet *et al.,* 1986; McDougald and Reid, 1991; McDougald, 2003). The infection through the litter on day 15 of the experiment resulted in a higher and lower oocyst shedding on days 13 and 21 PI respectively than that on day 6 PI. This observation is consistent with the outcome of an epidemiological survey showing that the oocysts in the litter or droppings of broiler chickens are usually most numerous at 4 weeks of age and generally decline as the birds become immune to further infection (Reyna *et al*., 1982, Williams *et al.,* 1996). In the field, outbreaks of coccidiosis arise in one of three situations, that is in young, susceptible stock placed on litter in which a lethal concentration of oocysts is already present, in young stock placed on litter under conditions suitable for the sporulation and survival of oocysts so that the rate of infection is greater than the rate at which resistance develops, and in adult stock which has been reared under conditions with minimal or no infection (Davies *et al*., 1963). The infection model based on infected litter may be more reliable than a model based on one large single dose to individual birds. Long (1979) reported that the use of a single massive level of inoculum in testing the efficacy of an anticoccidial agent may limit the ability to evaluate the expected challenge condition encountered in the field. A reduced sensitivity of *Eimeria* to some anticoccidials such as ionophores has been suspected for years (Chapman, 1982; Stephan *et al.,* 1997; Li *et al.,* 2004). The commercial success of ionophores indicates that the challenge conditions in the field are compatible with their different efficacy profiles (Jeffers, 1978; Chapman, 1984; McKenzie *et al*., 1989; Bafundo and Jeffers, 1990).

It generally accepted that technical performance in particular weight gain, is the most sensitive and informative measure of anticoccidial efficacy (Barwick *et, al.,* 1970; Johnson and Reid, 1970; Long, 1970) and high levels of oocysts inocula are generally needed to achieve measurable suppression of weight gain in the infected unmedicated birds (Conway *et al*., 1993; Chapman, 1998; McDougald, 2003). This would imply that the infection models used in experiments 1-3 are unsuitable. However, the infection model used in experiment 3 can be used to investigate the

response of broiler chickens to immunostimulators when the criterion of efficacy is based on oocyst shedding and lesion scores. An additional, later challenge through the litter could be done to test for the immunity development and to evaluate the anticoccidial under investigation using lesion scores, oocyst shedding, and technical performance as criterions. Infection through gavage with a single high dose of inoculum has no similarity with the field situation and it might result in results with no practical relevance. Infection through the litter mimics the field situation in combination with controlled conditions and allowing experimental flexibility and a large number of experimental units within the research facility.

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# *General Conclusions*

This thesis deals with two alternative anticoccidial treatments of broiler chickens: the ingestion of a preparation containing mannanoligosachharides (MOS) and exposure to a weak electromagnetic field (EMF). In this section the main conclusions from the thesis are listed per chapter and discussed briefly. In order to evaluate the potential of the two alternative treatments, the effect of a currently used coccidiostat is described first. Under experimental conditions identical to those described in Chapter 5 the effect of salinomycin (60 mg per kg diet) was studied. Salinomycin is one of the most commonly used anticoccidial ionophere agent and it is thought to act by disrupting cationic cross-membrane gradients (McDougald, 1982; 2003) by interfering with the ion transport system ($K^+$, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$). As a result, salinomycin arrests the development of coccidia early in their life cycle, before the host-cell-damaging stages are reached.

In the experiment, salinomycin was present in the diet as from day 1 and litter contaminated with a mixture of sporulated *Eimeria* oocysts containing $1.5x10^4$ *E. acervulina* (Weybridge strain) $1.5x10^4$ *E. maxima* (Weybridge strain) and $7.5x10^3$ *E. tenella* (Houghton strain) per bird was put in the cages on day 15. There were 8 birds in each cage with 6 replicates. The outcome of the experiment is given in Tables 1-3. Table 1 shows that salinomycin had no significant effect on the technical performance of the birds when compared with the birds of the infected control group placed on contaminated litter when they were 15 days old.

**Table 1:** Technical performance of broilers in the two treatment groups

| Treatment [n] Days in experiment | Infected Control | Salinomycin | *P*-value |
|---|---|---|---|
| Body weight (g) | | | |
| Day 1 | 50 ± 1.2[1] | 50 ± 0.5 | 0.77 |
| Day 7 | 179± 5.9 | 186 ± 5.9 | 0.29 |
| Day 15 | 460 ± 15.6 | 454 ± 6.1 | 0.74 |
| Day 21 | 915 ± 28.4 | 933 ± 7.5 | 0.55 |
| Day 28 | 1487 ± 36.3 | 1513 ± 20.9 | 0.55 |
| Body-weight gain (g) | | | |
| Day 1-7 | 129 ± 6.1 | 136 ± 2.3 | 0.33 |
| Day 7-15 | 281 ± 10.1 | 268 ± 4.9 | 0.28 |
| Day 15-21 | 455 ± 13.1 | 479± 3.3 | 0.12 |
| Day 21-28 | 576± 7.4 | 596 ± 15.8 | 0.28 |
| Day 1-28 | 1437 ± 36.6 | 1463 ± 20.7 | 0.55 |
| Feed Intake (g/d/bird) | | | |
| Day 1-7 | 24 ± 0.5 | 24 ± 0.2 | 0.74 |
| Day 7-15 | 55 ±1.6 | 54 ± 0.9 | 0.69 |
| Day 15-21 | 123 ± 3.2 | 125 ± 0.9 | 0.36 |
| Day 21-28 | 148 ± 9.3 | 170 ± 3.1 | 0.07 |
| Feed Conversion Ratio (FCR) (feed/gain) | | | |
| Day 1-7 | 1.21 ± 0.04 | 1.15 ± 0.02 | 0.12 |
| Day 7-15 | 1.47 ± 0.02 | 1.51 ± 0.02 | 0.13 |
| Day 15-21 | 1.47 ± 0.02 | 1.43 ± 0.01 | 0.09 |
| Day 21-28 | 1.675 ± 0.12 | 1.74 ± 0.03 | 0.17 |

[1] Means ± SE; [n]= 6 replicates per experimental group

Previous studies with broilers, reported that addition of 60 mg/kg salinomycin resulted in negative effects, on body weight and feed conversion ratio (Wheelhouse *et al.*, 1985; Chappel and

Babcock, 1997; Demirulus *et al*., 2006). However, others reported positive effects on technical performance of broilers when salinomycin was supplemented to the diet of broilers (Kiiskien and Anderson, 1987; Ferratto *et al*., 1988).

The OPG counts (oocysts per gram faeces) results are illustrated in Table 2. The salinomycin group showed significantly lower oocysts shedding when compared to the control group at 6 days post infection, but not at 13 days post infection. This is explained by the mode of action of salinomycin; the anticoccidial agent blocks the life cycle of *Eimeria* by arresting or killing the sporozoites or early trophozoites (McDougald, 2003).

**Table 2:** OPG counts (oocysts per gram faeces) expressed as $\log_{10}(X+1)$ in the two treatment groups.

| Treatment [n] Days Post Contamination | Infected Control | Salinomycin | *P*-value |
|---|---|---|---|
| Day 6 | $4.96^{a} \pm 0.121$[1] | $3.47^{b} \pm 0.239$ | 0.001 |
| Day 13 | $4.45 \pm 0.106$ | $3.97 \pm 0.249$ | 0.103 |

[a,b] Mean values within the same row with different superscript letter are significantly different ($P<0.05$); [n]= 6 replicate per experimental group.

Lesion scores were performed 6 days after placement of the birds in the contaminated litter and the results are presented in Table 3.

**Table 3:** Lesion scores day 6 and day 13 post contamination in the two treatment groups

| Treatment [n] Days Post Contamination | Infected Control | Salinomycin | *P*-value |
|---|---|---|---|
| Day 6 | | | |
| *E. acervulina* | 1.58[a] ± 0.193[1] | 1.08[b] ± 0.083 | 0.027 |
| *E. maxima* | 1.67 ± 0.188 | 1.42 ± 0.149 | 0.545 |
| *E. tenella* | 1.75 ± 0.179 | 1.42 ± 0.149 | 0.180 |
| Day 13 | | | |
| *E. acervulina* | 2.00 ± 0.246 | 1.50 ± 0.195 | 0.135 |
| *E. maxima* | 2.00[a] ± 0.123 | 1.33[b] ± 0.142 | 0.003 |
| *E. tenella* | 1.83[a] ± 0.167 | `1.33[b] ± 0.142 | 0.036 |

[a,b] Mean values within the same row with different superscript letter are significantly different ($P<0.05$); [n]= 24 birds per experimental group.

The data show that lesion scores due to *E. acervulina* were significantly lower in the salinomycin group when compared to those of the infected group, while the lesion scores due to *E. maxima* and *E. tenella* showed no significant difference between the salinomycin group and the infected group. This might be due to the short prepatent period of *E. acervulina* and/or the high potential productivity of this parasite (Brackett and Bliznick, 1949; Barwick and Casorso, 1970; Williams, 1973; Reid, 1975; McDougald and Reid, 1991). This is supported by the lesion scores of day 13 post contamination showing that the lesions scores for *E. maxima* and *E. tenella* were significantly higher in the infected control birds than in their counterparts given salinomycin, whereas there was no difference between the lesions due to the *E. acervulina* or oocyst shedding 13 days after contamination. However, many workers reported that lesion scores do not increase linearly with oocyst dose, and that low levels of inocula may produce fairly high lesion scores (McKenzie *et al*., 1989a,b,c; Conway *et al*., 1993). Moreover, there is no direct correlation between the number of oocysts excreted in the faeces and the severity of lesion scores (McKenzie *et al*., 1989a; b; c; Conway *et al*., 1993).

## Effect of MOS

*E. tenella* is the most common and most pathogenic parasite towards chickens and it is regularly associated with outbreaks of acute disease with a high rate of mortality. Literature data indicate that indigenous bacteria are required for the occurrence of typical caecal coccidiosis in chickens. Mannanoligosaccharides (MOS) have been reported to suppress pathogens and to enhance the growth of the beneficial bacteria in the intestinal mucosa of chickens and turkeys. In this thesis two studies are presented in which the hypothesis was tested that MOS might counteract a caecal coccidiosis infection in broiler chickens. When the broilers were fed diet supplemented with MOS, there was a significant decrease in the number of schizonts in the lamina propria, but this reduction was not associated with a decrease in the caecal lesions or improvement in the performance of the broilers fed the MOS-supplemented diet. It is generally accepted that weight gain is the most important variable to assess the anticoccidial efficacy of treatments (Barwick *et al*., 1970; Johnson and Reid, 1970; Long, 1970; Chapman, 1998).

It can be concluded that evidence was found for a protective effect of the MOS preparation against mild *E. tenella* infection, but MOS had no effect on the performance of the infected or non-infected broilers.

The question was addressed whether MOS would have anticoccidial activity in broilers mildly infected with a mixture of three *Eimeria* species: *E. acervulina, E. maxima,* and *E. tenella*. The dose and the species used simulated commercially available live vaccines and hence it would establish 'trickle' infections that have been shown to be very effective in stimulating protective immunity. From the oocysts excretion pattern seen in the infected broilers fed MOS, it appeared that the MOS preparation had the potential to lower the severity and the pressure of the infection and at the same time maintained oocysts production, which is crucial for the re-infection and the maintenance of the immunity stimulated by the initial infection. Moreover, *E. acervulina* lesions in the infected birds fed MOS were significantly reduced; the severity of lesions produced by the two other species of *Eimeria* was also reduced, but not significantly. In this study, the MOS preparation failed to show

any improvement of body weight and feed conversion. However, it is possible that under conditions other than those in the present studies, MOS could have positive effect on performance.

It is clear that both MOS and salinomycin failed to improve technical performance of the broilers, but both treatments reduced the parasitological impact of the *Eimeria* parasite, this effect being more pronounced for salinomycin than for MOS.

**Effect of EMF**

In the search of an alternative for the chemical coccidiostats commercially used, and with the help of literature data on the beneficial application of the EMF as to inflammatory reactions and the immune response, the hypothesis was tested that exposure of broiler chickens to EMF could reduce the signs of coccidiosis infection. In broiler chickens infected with a mixture of *Eimeria* species through gavage in the crop, EMF mediated a reduction in the severity of the lesions in the duodenum due to *E. acervulina* and in the mid gut due to *E. maxima*. EMF did not reduce the number of the oocysts shed with the faeces and this could be relate to the fact that oocysts were counted in excreta collected at only one time point, i.e. at 6 days post infection. The counts probably mainly reflected the oocysts of *E. maxima* and *E. tenella* and few oocysts of *E. acervulina* because of their prepatent periods. In conclusion, it appeared that exposure of broiler chickens to EMF antagonized the effects of infection with the three *Eimeria* species and in the EMF-treated birds, the infection produced no effect on feed intake, weight gain and feed conversion ratio. In the non-EMF treated birds, the infection reduced growth performance.

The main objective of the study presented in chapter 5 was to find out whether the EMF results described in the chapter 4 would be reproducible in a nature-like environment using the same dose, and species of *Eimeria*. The infection was induced by adding to the litter a mixture of three sporulated *Eimeria* species. Mimicking nature within the experimental facility was successful as indicated by the oocyst-shedding pattern and the reduction in body weight seen in the infected groups. Exposure to EMF counteracted the effect of infection. In the EMF-treated birds, the infection did not reduce body weight, weight gain and feed intake, and did not raise

the feed conversion ratio. However, the number of oocysts in excreta was not significantly influenced by EMF exposure. This observation is consistent with previous studies, reporting a poor correlation between oocyst production and the performance of broiler chickens. The group mean lesion scores for all three *Eimeria* species were lower in infected chickens exposed to EMF than in the infected controls. The effect of EMF generally was less pronounced on *E. tenella* than on *E. acervulina* and *E. maxima*. It is likely that the differences in response of the three *Eimeria* species to EMF is due to the site of parasite invasion, host immune reaction at the infection site and parasite infective stage. Based on our findings, it seems that the EMF technique has anticoccidial activity, but research is needed to investigate the mechanism of action. Perhaps, EMF exposure could serve as an alternative to the chemical anticoccidial drugs currently used.

When comparing the efficacy of salinomycin and EMF, it is clear that EMF was able to reduce the oocysts shedding, lesion scores and to improve technical performance of the broiler chickens whereas salinomycin failed to affect performance.

## Perspectives of MOS and EMF treatment

As described in chapter 1, there is a need of alternative anticoccidial treatments in broilers chickens. This thesis focuses on MOS and EMF as possible alternatives.

MOS are marketed with claims that they enhance growth performance and health. MOS would modulate the immune system, increase villi length, and improved the integrity and uniformity of the gut, leading to enhanced growth performance. The present studies show that MOS has anticoccidial potential and was able to decrease the number of the schizonts in the lamina propria and to decrease the oocysts shedding but it failed to stimulate growth performance of the chickens. In contrast, the studies with EMF showed that it has the potential to positively influence the parasitological and technical parameters. Based on the present work, further studies with EMF should focus on the molecular basis underling the observed antagonistic activity on coccidiosis infection in broiler chickens. Further studies on the efficacy and effects of MOS should be performed to identify the optimal conditions

required for its efficacy in broilers. Finally, in order to screen and test the efficacy of potential anticoccidials, an experimental model should be used that optimally mimics the field situation in combination with controlled conditions, high experimental flexibility and a large number of experimental units within the research facility.

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# Summary

The extensive use of chemically synthesized anticoccidial drugs for prevention and control of coccidiosis in poultry is a major factor in the success of the poultry industry. These drugs, when used in carefully designed prophylactic treatment programs are efficient in disease control. However, the development of drug resistance to all chemical types of anticoccidials and the increased pressure from consumers and governments to phase out the use of chemical anticoccidials in the diet of food animals has resulted in the need of alternatives to be sought.

The present thesis explored a mannanoligosaccharide (MOS) preparation and a low electromagnetic field (EMF) as possible alternative to anticoccidial drugs in broiler chickens. The MOS preparation is derived from the cell wall of the yeast *Saccharomyces cerevisiae*. It is claimed that under practical conditions MOS suppresses pathogens in the intestinal mucosa, improves gut health and uniformity, and modulates gut and systemic immunity. Low EMF is created by magnetic coil consisting of loops of electricity wire. The coils are connected via a relay bank to an amplifier and signal generator that is controlled by a microprocessor. It is proposed that EMF signals have an anti-inflammatory effect, stimulate the production of cytokines, enhance the immune response, increase peripheral blood flow, and promote the infiltration of macrophages into damaged tissues.

The aim of the studies was to evaluate the role of MOS and EMF as possible alternative treatment of coccidiosis infection in broilers. In the broiler chickens, the effect of MOS and EMF was determined on technical performance, fat digestibility, oocyst shedding, and intestinal lesions. Furthermore, the different methods used to establish an infection with *Eimeria* species are compared and contrasted, anticipating that the outcome would contribute to the selection of the most appropriate model of coccidiosis in broiler chickens, thus speeding up the screening of agents with potential anticoccidial activity.

The literature review in this thesis describes that coccidiosis as caused by the parasite of the genus *Eimeria* is a serious, ubiquitous, and complex disease in poultry. Coccidiosis is a disease of almost universal importance in poultry production and can cause large economical losses. The *Eimeria* parasites multiply in the intestinal tract and cause tissue damage, which results in depressed feed intake, diminished nutrient digestion and absorption, dehydration, blood loss, and increased susceptibility to other disease-evoking agents. For many years, the control of coccidiosis by chemotherapy or chemoprevention by administration of anticoccidial drugs via the feed has been common practice and found to be reliable. The success of anticoccidial products is negatively influenced by drug resistance, toxicity resulting from misuse and a limited spectrum of activity. Moreover, because of the consumers' concern and the expected ban of the chemical anticoccidials, the poultry industry would benefit from alternatives. Hence, research is carried out to find possible natural anticoccidial alternatives.

Two separate experiments were conducted to describe the effect of the commercial preparation of MOS (Bio Mos®) in broilers that were infected with either 5000 or 3500 sporulated oocysts of *E. tenella* when they were aged 8 or 12 days, respectively. A diet based on corn and soybean meal with or without MOS was used. The male broiler chickens were housed in suspended wire-bottomed cages. It was shown that in the infected birds fed the MOS preparation, the number of schizonts was reduced, but without improvement in growth performance, caecal lesions and fat digestibility. However, it cannot be excluded that under conditions other than those in this study, MOS would have positive effect on performance.

In subsequent study, the possible anticoccidial activity of MOS was further investigated in broiler chickens infected mildly with a mixture of sporulated oocysts of *E. acervulina, E. maxima,* and *E. tenella* when they were aged 1 day. Infected and non-infected birds were fed a diet without or with MOS. Growth performance, intestinal lesions, and oocyst excretion were measured at various intervals. The oocysts used to produce the infection were laboratory strains and the dose and the species used simulated commercially

available live vaccines. It was found that the MOS preparation reduced oocyst excretion on days 5 and 12 post-infection and diminished the severity of lesions produced by *E. acervulina*, but not those due to *E. maxima* and *E. tenella* infection. MOS failed to improve body weight or the feed conversions ratio, irrespective of whether the birds were infected or non-infected.

The question addressed was whether the exposure of broiler chickens to EMF could antagonise the negative effect of coccidiosis infection on body weight, feed intake and feed conversion ratio and would reduce the severity of intestinal lesions and reduce oocyst shedding. It was found that exposure of broiler chickens to EMF counteracted the negative effect of coccidiosis infection. In the EMF-treated birds, the infection caused no effect on weight gain and feed intake, whereas the severity of intestinal lesions mediated by *E. acervulina* and *E. maxima* was less than in the infected controls. This study indicates that EMF has anticoccidial activity. However, the practical application of EMF needs to be verified in controlled field trials. Perhaps, EMF exposure could serve as an alternative to the anticoccidial drugs currently used.

Based on the first study with EMF, its potential effect in practice was studied by mimicking nature within the experimental facility. This was achieved by placing the broilers to be infected in new litter seeded with sporulated coccidial oocysts when they were aged 15 days. The chickens will pick up the oocysts, while individual birds will ingest different numbers of oocysts at different times, which mimic a natural progressive infection. The uninfected groups were placed in a clean litter. The infection model was successful as indicated by the oocysts detected in the faeces of the infected birds and by the presence of intestinal lesions. Moreover, the pattern of oocyst shedding was found to agree with the findings in commercial broiler operations. Exposure to EMF counteracted the effect of infection on growth performance. In the infected birds exposed to EMF, the lesion scores related to the three *Eimeria* species were generally lower than in the infected controls. However, EMF treatment had no effect on oocyst shedding. It is concluded that EMF exposure protects against coccidiosis in broiler chickens and that it could serve as an alternative to anticoccidial drugs. The molecular basis underlying the observed antagonistic

activity of EMF on coccidiosis infection in broiler chickens is unknown and needs to be investigated.

A comparison was made between the various methods of infection used in the various experiments. It was expected that this comparison might contribute to the selection of the most suitable model of coccidiosis in broiler chickens and hence speed up the screening of potential anticoccidials. In all studies, there was a successful infection with *Eimeria* species as indicated by the intestinal lesions and the shedding of oocysts. Infection of broilers with low doses at the age of one day will establish 'trickle' infections and enhance immunity of the birds by the combination of an initial parasitic stage and subsequent boosting immunity by multiple re-infections with oocysts shed in the litter. Such an infection model can be used to study the induction of immunity by recycling of the infection in the presence of an agent with coccidiostatic activity. Later challenge through the litter could be done to test for immunity development and to evaluate the anticoccidial under investigation using lesion scores, oocyst shedding, and technical performance as criterions. Infection through a single massive level of inoculum has no similarity with the situations and conditions encountered in the field and may have no practical relevance.

It is suggested that there is a demand for alternatives in the anticoccidial treatment of broiler chickens. To screen and test the efficacy of potential anticoccidials, the experimental model used should mimic the field situation in combination with controlled conditions and high experimental flexibility and a high number of experimental units within the research facility. The present studies show that MOS has anticoccidial potential and is able to decrease the number of the schizonts in the lamina propria and to decrease oocysts shedding. However, the MOS preparation failed to stimulate growth performance. EMF has the potential of anticoccidial activity. Further studies with EMF should focus on its effect under field conditions and on its mechanism of action.

# Samenvatting

Het extensieve gebruik van chemisch gesynthetiseerde coccidiostatica ter preventie en controle van coccidiose bij pluimvee is een belangrijke factor voor het succes van de pluimveehouderij. Deze middelen, wanneer toegepast in zorgvuldige profylactische programma's, zijn efficiënt in de ziektecontrole. Echter, de ontwikkeling van resistentie tegen chemische coccidiostatica en de toegenomen druk van consumenten en overheid om deze stoffen uit de voeding van dieren te weren heeft geresulteerd in het zoeken naar alternatieven.

Dit proefschrift beschrijft onderzoek naar een mannanoligosaccharide (MOS) preparaat en een zwak electromagnetisch veld (EMF) als mogelijke alternatieven voor coccidiostatica voor vleeskuikens. Het MOS preparaat wordt bereid uit de celwand van de gist *Saccharomyces cerevisiae*. Het wordt beweerd dat MOS onder praktische condities pathogene bacteriën in de darmmucosa onderdrukt, de darmgezondheid bevordert en de immuunfunctie stimuleert. Zwak EMF wordt opgewekt door een magnetische spoel bestaande uit lussen van elektriciteitsdraad. De spoelen zijn middels een relais verbonden met een versterker, die wordt geregeld door een microprocessor. Het wordt verondersteld dat EMF-signalen een ontstekingsremmend effect hebben, de productie van cytokines stimuleren, de immuunreactie bevorderen, de perifere bloedstroom activeren en de infiltratie van beschadigde weefsels door macrofagen doen toenemen.

Het doel van de studies was om de rol van MOS en EMF als mogelijke alternatieve behandeling van een infectie met coccidiose te evalueren bij vleeskuikens. Bij de kuikens is het effect van MOS en EMF op de technische prestatie, vetvertering, excretie van oocysten en darmlaesies bestudeerd. Bovendien zijn de verschillende toegepaste methoden om een infectie met E*imeria* soorten te induceren met elkaar vergeleken in de verwachting dat de uitkomst zou kunnen bijdragen aan de keuze van het meest geschikte coccidiose-model bij vleeskuikens. Een dergelijk model zou het onderzoek naar stoffen met potentiële coccidiostatische activiteit versnellen.

Het literatuuroverzicht in dit proefschrift beschrijft dat coccidiose, zoals veroorzaakt door de parasiet van de soort *Eimeria,* een ernstige, alomtegenwoordige en complexe ziekte bij pluimvee is. Coccidiose is een ziekte van wereldwijd belang in de pluimveehouderij, welke tot grote economische verliezen kan leiden. De *Eimeria* parasieten vermenigvuldigen zich in het darmkanaal en veroorzaken weefselschade, hetgeen leidt tot depressie van de voeropname, verminderde vertering en absorptie van nutriënten, dehydratie en toegenomen gevoeligheid voor andere ziekteverwekkers. Reeds gedurende vele jaren wordt coccidiose algemeen gecontroleerd door toediening van coccidiostatica via het voer, hetgeen een betrouwbare aanpak wordt geacht. Het succes van coccidiostatische producten wordt echter in negatieve zin beïnvloed door de ontwikkeling van resistentie, toxiciteit door verkeerd gebruik en door een beperkt activiteitsspectrum. Bovendien zou, door de toegenomen bezorgdheid bij de consumenten en het verwachte verbod op de toepassing van chemisch gesynthetiseerde coccidiostatica, de pluimveehouderij gebaat zijn bij alternatieven. Als gevolg wordt onderzoek uitgevoerd om mogelijke alternatieve coccidiostatische behandelingen te identificeren.

Twee verschillende experimenten zijn uitgevoerd om het effect van het commerciële preparaat MOS (Bio Mos®) te evalueren bij vleeskuikens die waren geïnfecteerd met 5000 of 3500 gesporuleerde oocysten van *E. tenella* toen zij respectievelijk 8 en 12 dagen oud waren. Een voeder gebaseerd op maïs en sojameel, met of zonder MOS, werd gebruikt. De haantjes werden gehuisvest in kooien met gaasbodem. Aangetoond werd dat bij de geïnfecteerde kuikens die het MOS preparaat kregen het aantal schizonten was gereduceerd; dit echter zonder verbetering van de groeiprestatie, zonder vermindering van de ernst van laesies in het coecum en zonder beïnvloeding van de vetvertering. Het kan echter niet worden uitgesloten dat onder andere condities dan die tijdens de experimenten er wel een positief effect van MOS op de technische prestatie zal zijn.

In vervolgonderzoek is de mogelijk coccidiostatische activiteit van MOS bestudeerd bij kuikens die een milde infectie kregen met een mengsel van gesporuleerde oocysten van *E.*

*acervulina, E. maxima* en *E. tenella* toen zij 1 dag oud waren. Geïnfecteerde en niet-geïnfecteerde kuikens kregen een voeder met of zonder MOS. De groeiprestatie, de ernst van darmlaesies en de excretie van oocysten werden gemeten. De oocysten gebruikt voor de infectie waren laboratoriumstammen; de dosis en de soorten waren vergelijkbaar met commercieel verkrijgbare, levende vaccins. Het MOS preparaat reduceerde de excretie van oocysten op dag 5 en 12 na inductie van de infectie en verminderde de ernst van de laesies veroorzaakt door *E. acervulina*, maar beïnvloedde niet de laesies door *E. maxima* en *E. tenella*. MOS verbeterde niet de groei of de voerconversie, noch in de geïnfecteerde noch in de niet-geïnfecteerde kuikens.

De vraag werd gesteld of de blootstelling van kuikens aan EMF de negatieve invloed van coccidiose op het lichaamsgewicht, de voeropname en voerconversie zou kunnen tegengaan en ook de darmlaesies en excretie van oocysten zou verminderen. Er werd inderdaad vastgesteld dat EMF de negatieve effecten van een coccidiose-infectie tegenging. Bij de EMF-behandelde kuikens veroorzaakte de infectie geen effect op groei en voeropname, terwijl de ernst van de laesies veroorzaakt door *E. acervulina* en *E. maxima* minder was dan bij de geïnfecteerde controledieren. Het onderzoek wijst op een coccidiostatisch effect van EMF. Gecontroleerde praktijkproeven zijn noodzakelijk om de mogelijke toepassing van EMF te verifiëren. Misschien kan EMF fungeren als een alternatief voor de nu in de praktijk gebruikte chemisch gesynthetiseerde coccidiostatica.

In vervolg op het eerste onderzoek met EMF werd het potentiële effect in de praktijk onderzocht door nabootsing van een natuurlijke infectie in de experimentele faciliteit. Dit werd bereikt door kuikens te plaatsen op zaagsel dat besprenkeld was met een mengsel van gesporuleerde oocysten toen zij 15 dagen oud waren. De kuikens pikken de oocysten op, terwijl individuele dieren verschillende aantallen oocysten op verschillende tijdstippen opnemen, hetgeen een natuurlijke, progressieve infectie simuleert. De niet-geïnfecteerde dieren werden in schoon zaagsel geplaatst. Het infectiemodel bleek succesvol op basis van de aanwezigheid van oocysten in de excreta van de geïnfecteerde kuikens en de aanwezigheid van darmlaesies. Bovendien kwam het

uitscheidingspatroon van de oocysten overeen met waarnemingen in de commerciële vleeskuikenhouderij. Blootstelling aan EMF ging het effect van de infectie op de groeiprestatie tegen. Bij de geïnfecteerde, EMF-behandelde kuikens was de ernst van de darmlaesies veroorzaakt door de drie *Eimeria* soorten geringer dan bij de geïnfecteerde controledieren. EMF had echter geen invloed op de excretie van oocysten. Geconcludeerd werd dat blootstelling aan EMF bescherming biedt tegen coccidiose bij vleeskuikens en dat het zou kunnen dienen als een alternatief voor de nu gebruikte coccidiostatica. De moleculaire basis voor het antagonistische effect van EMF op coccidiose-infectie bij vleeskuikens is onbekend en dient bestudeerd te worden.

De verschillende methoden die in de verschillende experimenten werden toegepast om een infectie te induceren zijn met elkaar vergeleken. Het was de verwachting dat deze vergelijking zou kunnen bijdragen tot de keuze van het meest geschikte model voor coccidiose bij vleeskuikens en daarmee het onderzoek naar potentiële coccidiostatica zou kunnen versnellen. In alle proeven was er een succesvolle infectie met de *Eimeria* soorten, hetgeen wordt onderschreven door de darmlaesies en de excretie van oocysten. Infectie van eendagskuikens met lage doseringen zal 'trickle'-infecties induceren en zal de immuniteit van de dieren versterken door een combinatie van een initieel parasitair stadium en daaropvolgende oplading van de immuniteit door meervoudige her-infecties door de uitgescheiden oocysten in de bodembedekking. Een dergelijk infectie-model kan worden gebruikt om de inductie van immuniteit door "recycling" van de infectie te bestuderen in de aanwezigheid van middelen met coccidiostatische activiteit. Een latere "challenge" middels de bodembedekking kan worden uitgevoerd om de ontwikkeling van de immuniteit te testen en om het coccidiostaticum te evalueren op basis van laesie-scores, excretie van oocysten en de technische prestatie als criteria. Een infectie veroorzaakt door een eenmalige hoge dosis van oocysten komt niet overeen met de praktijkcondities en is mogelijk niet praktisch relevant.

Het wordt gesteld dat er een behoefte is aan alternatieven voor de controle van coccidiose bij vleeskuikens. Om potentiële alternatieve coccidiostatica te kunnen identificeren en evalueren,

zou het experimentele model de praktijkcondities moeten simuleren in combinatie met gecontroleerde condities, een hoge experimentele flexibiliteit en een hoog aantal experimentele eenheden binnen de onderzoeksfaciliteit. Het beschreven onderzoek laat zien dat MOS coccidiostatische potentie heeft en het aantal schizonten in de lamina propria en ook de excretie van oocysten kan verlagen. Het MOS preparaat had echter geen invloed op de groeiprestatie. EMF heeft cocciostatische activiteit. Verder onderzoek met EMF zou zich moeten richten op het effect ervan onder praktijkcondities en op het werkingsmechanisme.

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# Curriculum vitae

Mohammed Ali Elmusharaf Mukhtar was born on February 6th 1966 in Khartoum, Sudan. He obtained his DVM at the Faculty of Veterinary Science, University of Khartoum, in 1991. He continued his studies and was granted a M.Sc. degree in 2002 on the thesis topic of "Mapping Malaria Risk in Western Kenya" from the Department of Geo-Information Science, and the Department of Entomology of Wageningen University The Netherlands. In 2003, he started his PhD program under the supervision of Prof. Dr. Ir. Anton C. Beynen at the Department of Nutrition, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.