Chapter 2

Literature review

Domestication of poultry is said to have started in Asia. The earliest record of poultry domestication dated back to about 3200 BC in India. Chickens were bred in capacity in Egypt since about 1400 BC. Domestication of poultry in China also dated back to about 1400 BC. The red jungle fowl, an Asian breed, was assumed to be the ancestor of our modern poultry breeds (Daghir, 1995). At present, poultry production is an increasingly important agricultural industry in the world. Because there are few social or religious taboos concerned with eating poultry products. Poultry are kept for food production in most areas of the world.

The world production of poultry meat has increased throughout the 20th century. Intensive broiler chicken production methods were first developed in the 1950s. Poultry meat production was less than 10 % of the world's total meat output at that time. Poultry meat output has taken a greater share of the expanding world meat market since then and it accounted for over 20% of total meat output by the mid 1980s (Rose, 1997). Poultry meat production continues to expand in developing countries such that in 2001 world output had reached a new record level of around 70 million tons for the first time. In 2002 production was estimated to approach 72 million tons and represent almost 30% of world meat output. Between 1993 and 2001 world production expanded by 22.3 metric tons or 46%. The fastest growth in this period was noted in South America (80% increase). However, while Asia recorded a

near 63% gain in production, the increase in Europe was a much more modest 14%. The corresponding figures for North and Central America, Africa and Oceania were 42%, 36% and 33%, respectively. World chicken slaughtering in 2001 totaled almost 43,000 million with Asia accounting for 15,120 million (35%), North and Central America for 11,039 million (26%) and Europe and South America each accounting for around 16%, respectively (Evans, 2002). These records illustrate that poultry meat is a very important food source worldwide.

Broiler meat was important export product of Thailand. During the period of 1990 to 2000, Thailand's chicken meat exports jumped from 140,000 tons to 241,000 tons (Evan, 2002b). In 2001, production of broiler meat in Thailand was estimated to approach 1,260,000 tons (Evans, 2002a). At that level, Thailand was ranked 5th in poultry meat production in the world. Moreover, Linden (2003) reported that in 2003 Thailand produced an annual 1.32 million tons of chicken. About 66%, or 806,000 tons, was destined for domestic markets and 450,000 tons for export. This year exports are expected to amount to around 460,000 tons of chicken's worth about Thai bath (THB) 42 billion.

Beside greatly expanded over the past several decades, the commercial broiler industry has evolved from backyard flocks into an ingenious mass food production system. Growth and yield of birds have been enhanced with the help of highly specialized diets and through the use of genetics. At the breeder level, superior birds are selected based on attributes desirable to the customer, while enhancing growth, reproductive rates, and an improved immune system of the bird. This ability to adapt

the bird based on consumer preference while improving bird livability has allowed the industry to thrive and succeed. (Bruce, 2002)

The primary focus of the commercial broiler industry is to maximize profits by promoting maximal yield and maintaining the health of the bird. Any hindrance to bird health will decrease profitability. Improvements in technology relating to vaccines or nutrition could save companies money and allow the industry to operate more efficiently by increasing revenue and decreasing overall costs. One of the main expenses faced by the industry is loss associated with poultry diseases, including costs of vaccination, prevention, treatment, reduction in weight gains, and mortality. (Bruce, 2002)

Coccidiosis is a parasitic disease that is responsible for losses in production of food producing animals worldwide. The incidence of coccidiosis in commercial poultry has increased due to higher stocking densities and more intensive husbandry practices. Stocking densities such as 0.7 ft^2 per bird, among other stressors, have favored the spread of this disease in commercial poultry facilities. It has been documented that coccidiosis is the most consistently reported health problem in poultry (Biggs, 1982; Rose et al., 1987; Williams, 1999).

2.1 Coccidiosis

Coccidiosis is an infectious disease caused by protozoan parasite of the genus Eimeria. Coccidia are classified under the subkingdom Protozoa of the phylum

Apicomplexia (Jeurissen et al., 1996; Lillehoj, 1996). As a group, coccidia of the genus *Eimeria* cause the most widespread health problems in the broiler industry and remain one of the most expensive diseases of commercial poultry production (Edgar, 1992; Henken et al., 1994; Yun et al., 2000). 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; McDougald, 2003). Chickens of all ages are susceptible to coccidiosis, but birds that are three to five weeks of age are the most vulnerable (Edgar, 1992; Yun 2000).

The induced tissue damage and change in intestinal function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis. Caecal coccidiosis caused by *Eimeria tenella* may contribute to an increased severity of blackhead disease in chickens. 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).

Chickens are susceptible to at least 11 species of coccidia. Coccidiosis is a self-limiting, infectious disease of the digestive tract caused by host specific intracellular protozoal parasites of the genus *Eimeria*. The most common species are *Eimeria tenella*, which causes the cecal or bloody type of coccidiosis, *E. necatrix*,

which causes bloody intestinal coccidiosis, and *E. acervulina* and *E. maxima*, which cause chronic intestinal coccidiosis (Murray, 2001).

2.1.1 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 (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).

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).

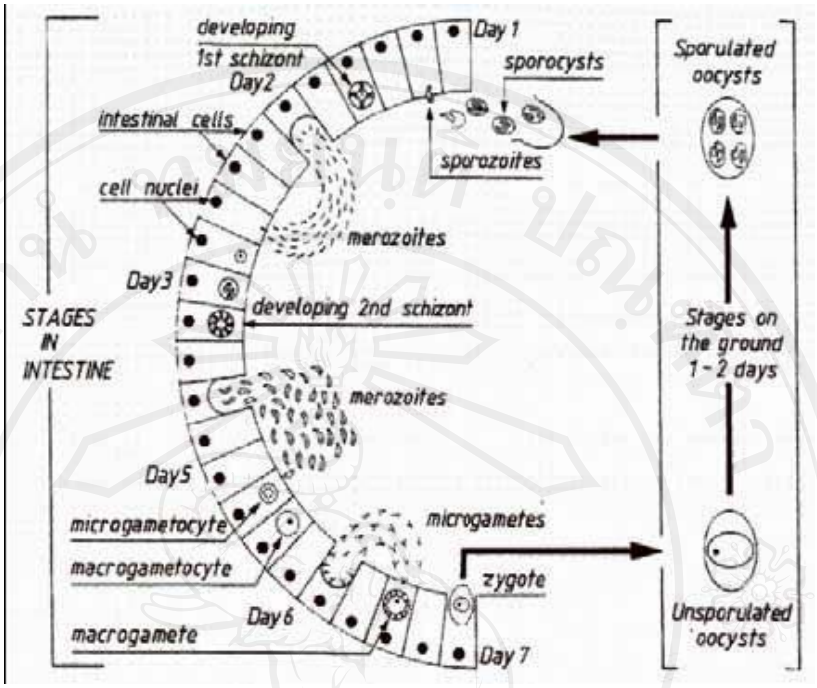

Figure 1 Overview of the various stages of *E. tenella*, which is typical for the genus Eimeria (Elmushalaf et al., 2007)

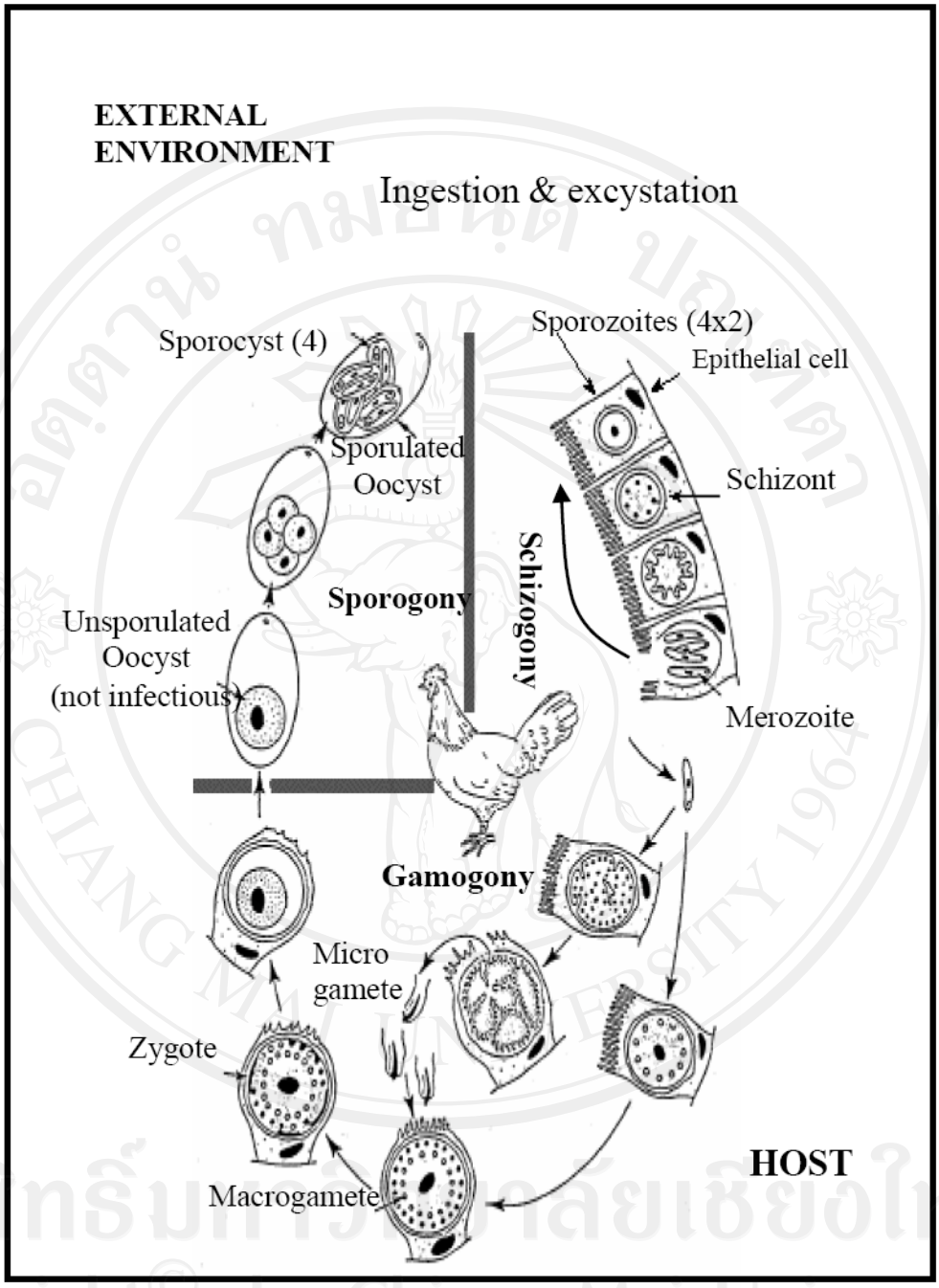

Figure 2 Stages of the life cycle take place in the external environment and in the host. (Elmushalaf et al., 2007)

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 specie (*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).

2.1.2 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 though out the world wherever domesticated fowls are reared (Williams, 1998). Davies et al. (1963), Long et al. (1976), 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.
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 and PCR.

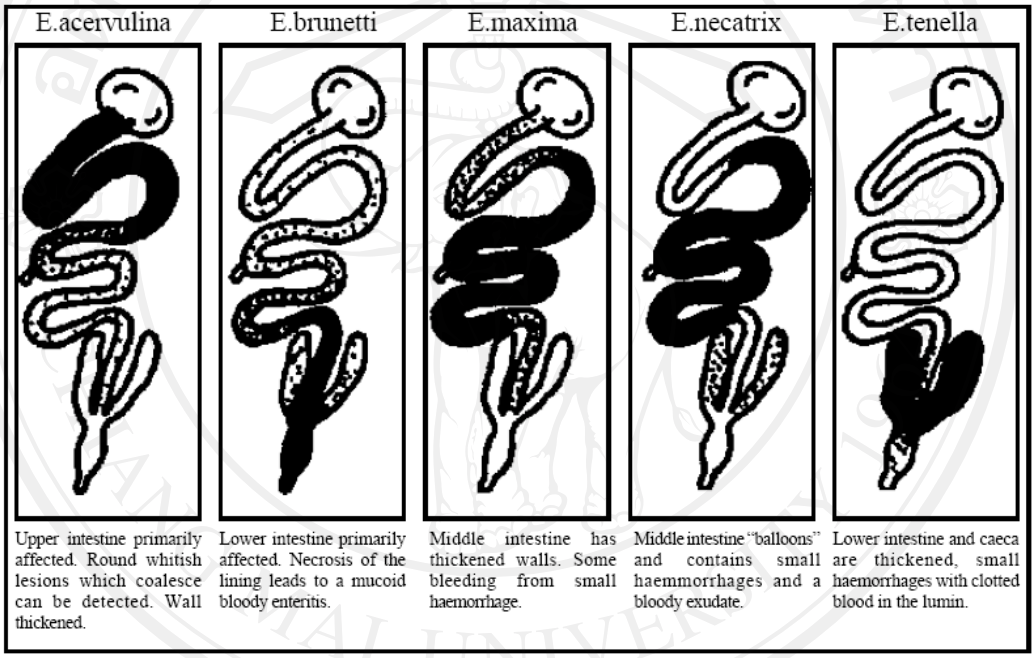

Figure 3 Location of the lesions caused by different *Eimeria* species in the gastrointestinal tract (Yun et al.,2000a).

2.1.3 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 (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 (Johnson and Reid, 1972). 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 *Clostridium perfringens* and coliforms, especially *E. coli*, is stimulated and the growth of *Lactobacillus spp.* is suppressed (Johansson and Sarles, 1948). 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 6th day of *E. acervulina* infection, on the 3rd, 6th, 7th, and 14th days of *E. necatrix* infection, and on the 3rd and 6th 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 8th day in *E. necatrix* infection. However, the faecal coliforms increased in all infections on the 6th 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.

2.1.4 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 gut associated lymphoid tissue 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 (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; 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).

Because the life cycle of *Eimeria* comprises intracellular, extracellular, asexual, and sexual stages, it is not surprising that host immunity is also complex and

involves many facets of non-specific and specific immunity, the latter encompassing both cellular and humoral immune mechanisms (Lillehoj HS., 1998).

Non-specific factors include physical barriers, phagocytes and leukocytes, and the complement system. Specific host immunity is mediated by lymphocytes and their secretions such as antibodies and cytokines. In the natural host, immunity is species specific such that chickens immune to one species of *Eimeria* are nevertheless susceptible to others. Additionally, different species of *Eimeria* demonstrate different tissue and organ specificity in the infected host. Understanding the interplay between host and parasites in the intestine is crucial for design of new approaches against coccidiosis. (Yun et al., 2000)

Infection with one species of *Eimeria* induces protective immunity in the host that is long lasting and exquisitely specific to that particular parasite. While a large number of inoculating oocysts is generally required to generate an immune response against *Eimeria*, some exceptions have been noted, e.g. *E. maxima* is highly immunogenic and requires only a small number of oocysts to induce almost complete immunity.

The early endogenous stages of the parasite life cycle are considered to be more immunogenic than the later sexual stages (Rose and Hesketh, 1976) although Wallach et al. (1990, 1995) showed that immunization with a recombinant gamete associated antigen induced partial protection against challenge infection. Studies using oocysts irradiated to prevent intracellular development, but not invasion,

demonstrated partial protection against challenge infection, thereby suggesting that sporozoites may also be immunogenic (Jenkins, 1991). However, because these immune responses were insufficient to induce full protective immunity, antigens other than those expressed by sporozoites are probably important for induction of complete immune protection.

In Figure 4, Yun et al. (2000a) summarized current understanding of the avian intestinal immune system and its response to *Eimeria* as well as provide a conceptual overview of the complex molecular and cellular events involved in intestinal immunity to coccidiosis.

Chickens infected with *Eimeria* produce parasite specific antibodies in both the circulation and mucosal secretions but humoral immunity plays only a minor role in protection against this disease. Rather, recent evidence implicates cell-mediated immunity as the major factor conferring resistance to coccidiosis (Yun et al., 2000a).

Sporozoites are first seen within intestinal intraepithelial lymphocytes, primarily CD8+ cells and macrophages shortly after invasion, and found later developing inside epithelial cells (Trout and Lillehoj, 1996).

T lymphocytes and macrophages are most likely the source of cytokine production in the chicken intestine (Lillehoj, 1994). Non-specific stimulation of these cells especially within the intraepithelium (Dalloul et al., 2003) would result in their

activation and proliferation early on during an enteric infection and thus leading to early IFN-γ production.

Early cellular immune responses characterized by IFN-γ production are critical to the fight against coccidiosis (Lillehoj et al., 2004). Chicken IFN-γ regulates acquired immunity by activating lymphocytes and enhancing expression of MHC class II antigens (Kaspers et al. 1994). Further, IFN-γ is a common marker of cellular immunity and higher levels have been associated with immune responses to coccidial infections (Lillehoj and Trout, 1996; Lillehoj, 1998; Yun et al.,2000b).

IL-2 is one of the most important T cell growth factors and is a potent immune system modulator affecting nearly every facet of host immune response. It is produced by T helper cells and is typical of immune responses against intracellular infections such as Eimeria parasites (Choi et al., 2000; Lillehoj et al., 1992).

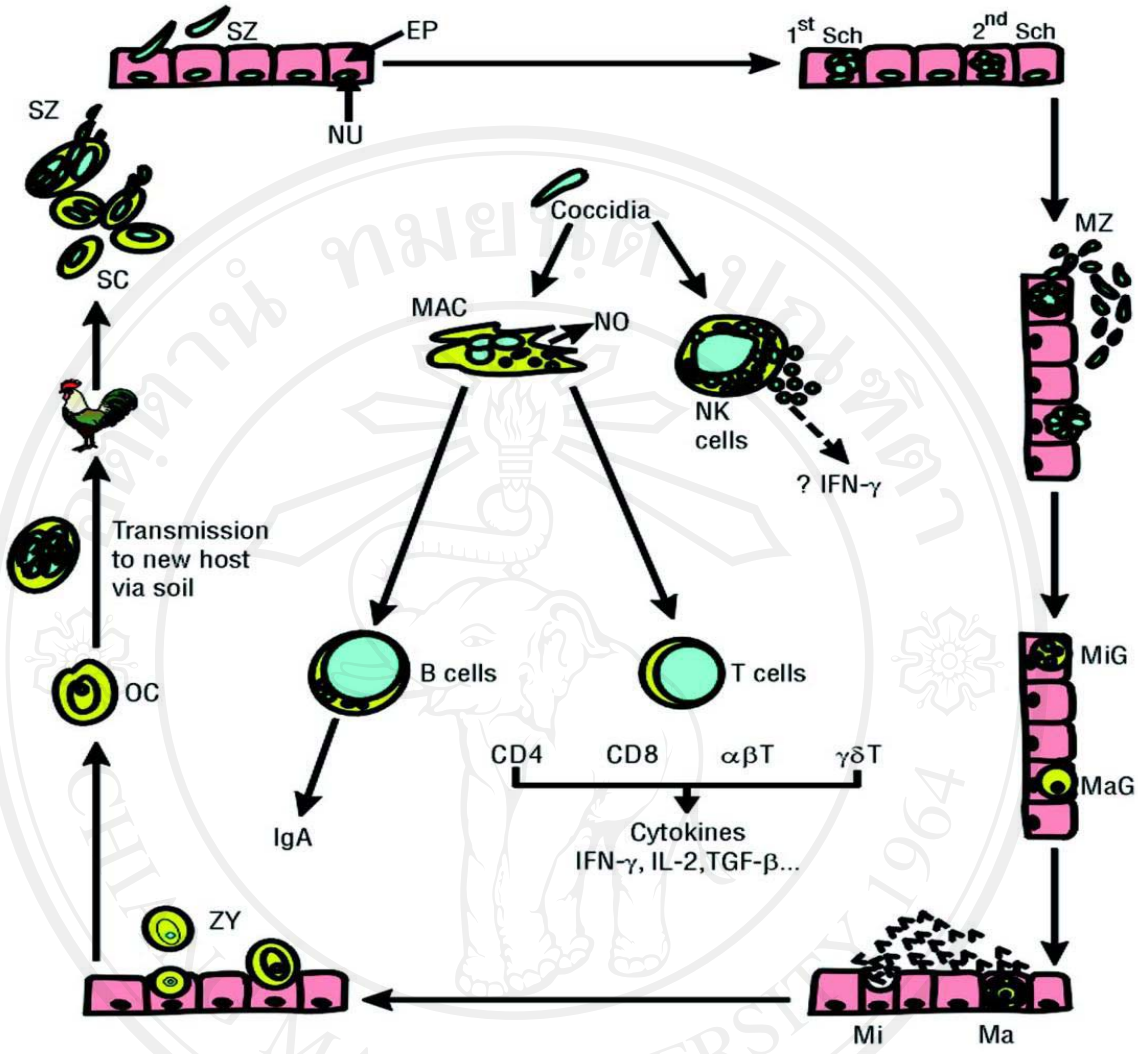

Figure 4 Life cycle of E. tenella and intestinal host immune response to parasites. EP, epithelial cells; NU, nucleus; OC, oocyst; SC, sporocyst; SZ, sporozoite; MZ, merozoite; Sch, schizont; MiG, microgametocyte; MaG, macrogametocyte; Mi, microgamete; Ma, macrogamete; ZY, zygote; MAC, macrophage; NK, natural killer cell; T, thymus-derived lymphocyte; B, bursa-derived lymphocyte; NO, nitric oxide. (Yun et al.,2000a)

2.1.5 Control of coccidia

2.1.5.1 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 (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.

2.1.5.2 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, 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.

2.1.5.3 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 (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 (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.

2.1.5.4 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).

2.1.5.5 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 (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.

2.2 Probiotics

The idea of using microbes to promote a good health and to prevent disease is not new. 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. Initially, several microbes have been used unintentionally in food production such as dairy products and fermented vegetables. In recent years, there has been a renewed interest in microbial uses due to, apart from improving food flavour, their beneficial aspect in health restoration and disease treatment. Several microorganisms, under the name of "probiotics", have been proposed and used in a wide range of clinical trials, ranging from diarrheal disease to cancer prevention (Fuller, 1994; Kaur et al., 2001).

Probiotics are biological products, which can improve the animal's growth performance (Kyriakis et al. 2003) as well as increase the body's resistance to the infectious agents by equilibrating body microflora, stimulating the immune system by increasing the number of antibodies and increasing the effectiveness of macrophages (Goldin and Gorbach,1984 and Francis et al. 2002). Furthermore, they are natural, harmless bacteria and have no drug residues in edible animal products after being fed to the animal.

Usually probiotics contain one or more types of bacteria depending on the media of their growth. The idea of using pure cultures of microorganisms for prophylaxis against gastrointestinal infections appeared during the past century. In 1889, Brudzinski recommended the prophylactic use of coli bacteria cultures, Peretz (1932) curatively used coli bacteria cultured in sterile milk for gastrointestinal infections and named this preparation "Colibacterin". For the first time Metchnikoff (1961) used lactic acid bacteria for suppressing the growth of putrefactive and pathogenic bacteria in the chicken's intestinal tract, the preparations were called "Lactobaciline": fermented milk with *L. bulgaricus* and lactic acid streptococci.

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., 1998; Abdulrahim et al., 1999; Zulkifli et al., 2000). Reque et al. (2000) showed the comparison to the presence and effects of antibiotics, *L. fermentum* LPB implantation resulted in a similar effect as that of antibiotics manifested by feed efficiency in growth of chicks.

2.2.1 Definition of probiotics

The term "probiotic" is derived from Greek and means pro: for and bios: life (for life) in contradiction to antibiotic which means: against life. The term probiotic was first introduced by Lilly and Stillwel (1965) to describe growth-promoting factors produced by microorganisms.

Parker (1974) first specified designation “probiotic”. He defined probiotics as microorganisms or substances, which contribute to the balance of the intestinal microflora.

Crawford (1979) defined probiotics as a culture of specific living microorganisms, primarily Lactobacillus spp. that are implanted in the organism and ensure the rapid and effective establishment of a beneficial intestinal population.

Fuller (1989) discussed the definition given by Parker (1974) and considered it too broad, as cultures, cells, and metabolites are also included in antibiotic preparations. He redefined “probiotic” as a live microbial feed additive, which beneficially affects the animal by improving its microbial balance.

Havenaar et al., (1992) pointed out that the definition of “probiotic” made by Fuller (1989) was restricted to feed supplements, animals, and their intestinal tract. Therefore, they generalized Fuller’s definition of “probiotic” as a mono or mixed culture of living microorganisms, which beneficially affect the host by improving the properties of the indigenous micro-flora.

Through 1989, United States Department of Agriculture (USDA) advised manufactures to use the term: “direct-fed microbial” (DFM) instead of “probiotic” (Miles and Bootwalla, 1991). The USFDA defined DFM as a source of live naturally occurring microorganisms, including bacteria, fungi, and yeast.

Vanbelle et al. (1990) pointed out that most researchers considered “probiotic” for selected and concentrated viable counts of lactic acid bacteria.

Koh et al. (1992) pointed out that as a biological product for newly hatched chicks a bacterial culture producing acetic acid could be used. Such a culture might be supplied to the chicks either through their drinking water or by the feed. For controlling the biological balance in the chicken’s intestinal tract different probiotics may be used.

2.2.2 Selection for Probiotics

In the selection of microbial strains for probiotic use, several criteria must be considered, which include bio-safety aspects, production and processing aspects, the method of administering the probiotic, the location on/in the body where the microorganisms of the probiotic product must be active, survival and/or colonization in the host, the tolerance for bile, the tolerance to low pH/ gastric juice, antimicrobial activity, and viability during storage (Gilliland and Walker, 1990; Fuller, 1992; Reque et al.,2000)

In vivo evaluations of Probiotic properties are time-consuming, labor-intensive and require large numbers of animals for the selection of candidate probiotic lactic acid bacterial strains. Hence, many in vitro assays have been developed (Morelli, 2000; Tuomola et al., 2001). These include selection for gut and stomach conditions such as acid and bile tolerance and adhesion to gut mucus or intestinal cell

lines (Caco-2 and HT-29) (Blum and Reneiro, 2000). Also selection methods have been described for probiotics that express antimicrobial activity (Blom and Mortxedt, 1991; Abee et al., 1994) growth inhibition of unwanted flora elements (Jacobsen et al., 1999) and competitive exclusion (Tuomola et al., 1999). Also an in vitro gut model (Macfarlane et al., 1998; Van Der Werf and Venema, 2001) has been described to evaluate gut microbial ecology.

Lactic acid bacteria (LAB) have generally been considered as good probiotic organisms and the genus currently being used in probiotic preparations are *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (*Enterococcus*) (Pandey, 1979; Sullivan et al., 1992).

2.2.3 Microbial community in gastrointestinal tract:

It is well established that the gastrointestinal normal microflora plays an important role in the health and well-being of poultry. Various pathogenic microbes, such as *Escherichia coli,* have been implicated to reduce the growth of poultry. Possible mechanisms for this reduction of growth are: toxin production, utilization of nutrients essential to the host, and suppression of microbes that synthesize vitamins or other host growth factors. Avians possess the same basic structures for nutrient extraction as other vertebrate, a tubular intestine, but specific variation within the avian gastrointestinal tract (GIT) includes a crop for storage of feed, proventriculus (simple stomach), gizzard, and paired ceca (Duke, 1986). The pH values of specific sections of the chicken gastrointestinal tract are: crop 4.5, proventriculus 4.4, gizzard

2.6, duodenum 5.7 to 6.0, jejunum 5.8, ileum 6.3, colon 6.3, ceca 5.7, and bile 5.9 (Farner, 1942). These pH values in specific areas of the avian gastrointestinal tract selectively allow establishment of a specific microbial population in birds.

Animal microbial populations, which grow anaerobically, can be differentiated into two groups: autochthonous (normal) and allochthonous (transient) microbes (Dubos et al., 1965). Peristalsis of gastrointestinal tract drives unidirectional flow of materials through the lumen of the upper and middle gastrointestinal tract and prevents microbial communities from developing unless they attach to underlying epithelial structures (Savage, 1983). Some microbes adhere to the epithelial cells of gastrointestinal tract, but others may colonize in secretions of intestinal line, principally mucins. Some gram-negative bacteria, such as *E. coli,* grow to express the type 1 fimbria and adhere efficiently to the crop epithelium, lamina propria, and apical surfaces of intestinal villi. The adhesion can be inhibited by manna oligosaccharide (Edelman et al., 2003)

Within a few hours of feeding, the pH of the crop falls to about 5.0 due to the microbial production of lactic acid. Few types of bacteria are present in the crop, with *Lactobacilli* being the predominant microbes, especially *Lactobacillus salivarius* (Sarra et al., 1985), which produces lactic acid and subsequently reduces pH value. *Lactobacilli* attach to the epithelial surface of the crop, forming an almost complete layer with two to three cells thick and remaining there throughout the bird's lifetime. The ability of these microbes to adhere to the crop epithelium is restricted to avian strains; those from other species fail to attach (Fuller, 1973). *Lactobacilli* are capable of controlling populations of *E. coli* in the crop, and their effects are both

bacteriostatic (depressing growth of other bacteria by their secretions) and bacteriocidal (killing other bacteria by secretions) (Fuller, 1977). Strict anaerobes, such as *Bacteroides* spp., do not appear in the crop due to its unfavorable oxidation-reduction (redox) potential (essential for bacteria to oxidize nutrients), the predominance of *Lactobacilli*, and possible absence of a suitable growth substrate such as glucose. But the relatively aerotolerant anaerobe, *Clostridium perfringens*, is sometimes found in the crop, as are micrococci, staphylococci, and yeasts (Mead, 1997). Feed can be retained in the crop for as long as 20 hours, but if the organ fails to empty, microbe activity continues and can lead to a condition known as "sour crop". Microbial populations in the crop are also susceptible to other dietary factors. For example, when turkeys are given a high-glucose diet, yeasts become predominant and produce abundant gas, which cause the crop to become large and pendulous (Jayne-Williams et al., 1971).

The proventriculus and gizzard of the chicken are harsh habitats for most microbes, with pH values of 1 to 4. However, Smith (1965) reported the presence of *Lactobacilli* (10^8/g content) with low numbers of *E. coli*, enterococci/streptococci, and yeasts. Rapid food passage rate in the duodenum makes it an unfavorable area of the gastrointestinal tract for microbes to attach and colonize. However, Salanitro et al. (1978) reported that aerobic and anaerobic counts from the duodenum and ileum of the chicken were similar, with *Streptococcus (enterococcus)*, *Staphylococcus*, *Lactobacilli*, and E. *coli* being the predominant bacteria present. However, obligate anaerobes were also present in both the duodenum and ileum, including anaerobic cocci, *Eubacterium*, *Propionibacterium*, *Clostridium*, *Gemmiger* and *Fusobacterium*

spp., comprising 9% to 39% of the total number of strains isolated with the greatest diversity being evident in the duodenum. 70% of 16S rRNA (species specific) sequences from the ileum were related to *Lactobacillus*, with the majority of the rest 11% *Clostridiaceae*, 6.5% *Streptococcus*, and 6.5% *Enterococcus* (Lu et al., 2003).

The ceca in poultry provide a relatively stable environment for microbes. Bacteria are the predominant microbes, specifically obligate anaerobes, which are found in the lumen at up to 10^{11} / g wet weight, while yeasts, molds, and protozoa are not normally present in significant numbers. Lu et al. (2003) observed that in the ceca *Clostridiaceae*-related consisted of 65% with the remainder as *Fusobacterium* (4%), *Lactobacillus* (8%) and *Bacteriodes* (5%).

2.2.4 Microbe-microbe interaction

Beneficial or competitive interactions exist among different microbial populations. Dofing et al. (1988) reported beneficial interactions among microbial communities with interspecies acetate transfer as an electron carrier within an anaerobic environment, where these microbes cooperatively play respective roles on oxidizing substrates. When available nutrients become limiting, competition for the carbon and energy sources develops among various members of the microbial community (Veldkamp et al., 1986). Competition among microbial communities may be influenced by environmental factors such as concentrations of carbon and energy substrates, oxygen, nitrite, sulfate, sodium chloride, antibiotics, temperature, osmotic strength, and pH (Dofing et al., 1997). Indirect and direct antagonisms are utilized by

microbes to inhibit other microbial growth. Indirect antagonisms include deconjugation of bile acids (Bricknell et al., 1969), induction of host immunologic processes (Freter, 1974), and stimulation of peristalsis in the gastrointestinal tract. Direct antagonisms between microbes include depletion of essential substrates, competition for receptor sites, creation of a restrictive physiologic environment, and secretion of antibiotic-like substances. Wilson et al. (1988) observed that an unidentified organism(s) more efficiently competed for monomeric glucose, Nacetylglucosamine, and sialic acid compared to *C. difficile* in a continuous flow culture model of mouse cecal bacteria. Some by-products of bacterial metabolism such as hydrogen ion concentration, oxidation-reduction potential, hydrogen sulfide, and volatile fatty acids (VFAs), create inhibitory physiologic environments. Low pH may be the major mechanism by which lactic acid bacteria (primarily *Lactobacillus*, *Bifidobacterium* and *Streptococcus*) inhibit growth of various facultative and anaerobic bacteria *in vivo* and *in vitro* (Tannock, 1984). Low rodox potential in the gastrointestinal tract is critical for protection against enteric infection by pathogens whose growth requires more oxygen (Meynell, 1963). Byrne et al. (1979) reported that short chain VFAs which are fermented by gastrointestinal microbes, inhibited the growth of other gastrointestinal microbes with the undissociated form of VFAs in lower pH. Inside the cell, VFAs inhibit bacterial growth by uncoupling oxidative-phosphorylation and inhibiting adenosine triphosphate – inorganic phosphate exchange. Bacteriocins, which are also produced by some bacteria to inhibit the growth of others, play a role in regulating the microbial population of the gastrointestinal tract (Rusch, 1980).

Chicks are immunologically naive and prone to rapid and persistent colonization by beneficial and pathogenic bacteria, with anaerobic bacteria dominating the first 3 to 4 weeks of life (Barrow et al., 1988). The beneficial gastrointestinal microbes present in the gastrointestinal tract are important in protecting the host against the invasion of pathogens. Most components of the gastrointestinal microbes are poor inducers of immunoglobulin (Foo et al., 1974), because of the close antigenic similarities between intestinal microorganisms and tissues of the animal host. Proteins, which pathogens may secrete into the medium, include enzymes (proteases, nucleases, lipases, and carbohydrases), toxins, and hemolytic and cytotoxic proteins (Lory, 1992). Pathogenic microbes also secret enterotoxic proteins to maintain their survival and to cause disease to the host. ToxR is a transcription regulatory protein produced by *Vibrio cholerae* which causes cholera. Skorupski et al. (1997) observed higher expression of the ToxR regulon at pH 6.5 and 30 ºC but reduced expression at pH 8.5 and 37 ºC. *In vitro*, toxin production in a pure culture is increased under osmolar condition similar to that in mucus, and in the presence of amino acids likely to be present in mucosal secretions. These results suggested that environmental factors, including pH, temperature, osmolarity, and certain amino acids, could increase the activity of ToxR and result in more virulent bacteria.

2.2.5 Probiotic and Immunity

The focus of the present review is on the potentials of probiotics to stimulate the immune system of the chicken. However, there are few studies of probiotics on

avian specific immunity. Immunity resulting from gut exposure to a variety of antigens, such as pathogenic bacteria and dietary protein, is important in the defense of young animals against enteric infections (Perdigon et al., 1995).

Several studies have demonstrated the role of intestinal microbiota in promoting endogenous host defense mechanisms, improving non-immunologic intestinal defenses, modulating host innate and acquired immune responses and down-regulating hypersensitivity reactions (Sutas et al., 1996; Perdigon et al., 2003).

Tortuero (1973) demonstrated the antagonism between Lactobacilli and enterobacteria and showed that lactobacilli reduced the severity of clinical signs in *E. tenella* infection.

Dunham et al. (1993) reported that birds treated with L. reuteri exhibited longer ileal villi and deeper crypts, which are a response associated with enhanced T cell function and increased production of anti-Salmonella IgM antibodies.

Nahanshon et al. (1994) found that Lactobacillus supplementation of layers diets increased cellularity of Peyer's patches in the ileum indicating a stimulation of the mucosal immune system that responded to antigenic stimuli by secreting immunoglobulin (IgA).

Yasui et al. (1992) indicated that probiotic Lactobacillus can significantly influence the immune responses of host animals by the stimulation of antibody production.

Orally feeding with L. casei, L. acidophilus, and yogurt enhanced the number of IgA-producing cells in the small intestine. This effect was most pronounced after L. casei treatment (Perdigon et al. 1995).

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).

Koenen et al. (2004) found the lactobacillus showed modulating effects on the immune system of layer- and meat-type chickens. In meat-type strain chickens the lactobacilli had a stimulating effect when the chickens were young (up to 3 weeks) and the dose was relatively high, whereas in layer-type chickens a lower effective dose and discontinuous administration was also effective. Immunoprobiotic lactobacilli can have a positive effect on humoral and cellular immune responses in layer- and meat-type strain chickens, but the lactobacillus strain to be used, the age of the animals and effective dose of lactobacilli to be administered need to be optimised.

Yurong et al. (2005) determine the effect of oral administration of probiotics on the intestinal mucosal immune response and ultrastructure of cecal tonsils and

found probiotics enhanced immunglobulin (Ig)A, the IgG-forming cells, IgM-forming cells in the Peyer's Patch, IgA-forming cells, IgG-forming cells, IgM-forming cells in cecal tonsils and T lymphocytes in cecal tonsils. They also found the density of microvilli and length of cecal tonsils increased after probiotics were administrated. With chicken ageing, the efficiency of probiotics would decrease. These results suggested that probiotcs enhance intestinal mucosal immunity of chicken at the early age. They also suggested increasing the content of IgA and antibody-forming cells in the intestine of chickens, which received probiotics, were possibly due to strengthening the ability of Peyer's Patches to identify antigens. Probiotics are harmless bacteria. They enter Peyer's Patch across the overlying modified epithelium and are presented to lymphoid cells, activate B lymphocytic cells, and B lymphocytic cell are transformed to plasma cells. The IgA-, IgG- and IgM-forming cells arising from this interaction enter the draining lymphatics and gut-associated lymphoid tissue. These cells continue their migration into the thoracic duct and blood circulation, and then they exit in the intestinal lamina propria, where they secrete immunoglobulins which initiate their original production in Peyer's Patches.

Based on immunologic properties Koenen (2004) developed an in vitro system for rapid pre-selection of LAB with immunomodulating properties. For T-cell proliferation following mitogenic stimulation the activation of accessory cells is required. The pre-selection assay was based on a concanavalin A (ConA) mitogen-induced lymphocyte proliferation assay in which enhancement or inhibition of the response was the result of the immunomodulating properties of LAB for which either T-cells or accessory cells may be sensitive. Since chickens do not have lymph nodes,

spleen cell suspensions were used that include all relevant cell types. Spleen cells were incubated with LAB and a suboptimal concentration of ConA to allow positive or negative modulation of the responses. To validate this assay typical LAB strains selected in the in vitro assay were evaluated in two in vivo experiments. Anyway, Immune stimulation in vitro correlated well with the in vivo situation in two experiments and no false negative results occurred. Therefore this assay is an appropriate selection tool for immunomodulating properties of lactic acid bacteria in chicken.

Ogawa et al. (2006) indicated that L. casei subsp. casei together with dextran induced an effective increase in humoral immune response to mixed inactivated vaccines against Newcastle disease and avian infectious bronchitis, and the treatment may be advantageous in protecting against these infectious diseases in chickens in actual application. These results suggest that dietary supplementation of *L. casei* subsp. casei with dextran leads to immunomodulation of humoral immune responses.

Dalloul et al. (2005) examined cytokine and oocyst production under similar conditions using a commercial Lactobacillus-based probiotic. Results showed small but significant ($P<0.05$) differences in cytokine levels and oocyst production but not antibody levels between the probiotictreated and control groups. They suggest a positive impact of the probiotic on cellular immune responses of infected broilers as compared to control chickens resulting in enhanced resistance to *E. acervulina* as shown in reduced fecal oocyst shedding. The results showed an immunoregulatory effect of probiotic diets on the local cell-mediated immunity in poultry.

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.