Chapter 5

Discussions

5.1 Isolation and identification of probiotic lactic acid bacteria from poultry's coacal swap

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).

The first characteristics observed in the properties of the Lactic Acid Bacteria (LAB) strain were gram positive and the negative reaction to catalase test, while Probiotics bacteria must be resistant to the acidity of the stomach, bile and to antibiotics used as 'growth enhancers' in animal nutrition and in therapy. These characteristics may be observed *in vitro* and can be used for selection of strains (Salminen et al., 1998).

In physiological function, bile salt is an emulsifying agent, which acts to prevent the smaller droplets from reaggregating back into large droplet. On the other hand, bile salt is the detergents, which act on bacterial membranes,

expose to bacterial periplasm and cytoplasm. It is highly toxic substance for microorganism (Thanassi et al., 1997). Hence, the probiotics bacteria were expected to survive in host intestines, consequently, they must resist to bile salts.

Unconjugated bile acids, even at low concentrations, can inhibit the *in vitro* growth of microorganisms (Fuller, 1992). Bile tolerance has been described as an important factor for survival and growth of LAB in the intestinal tract (Gilliland et al., 1990). According to Gilliland et al. (1984), 0.3% is considered to be a critical concentration for screening for resistant strains. Reque et al. (2000) presented that *Lactobacillus fermentum* LPB as probiotic in chickens tolerated 0.3 % bile. Also *Bacillus racemilacticus* and *Bacillus coagulans* strains were tolerant to bile concentrations over 0.3% (w/v) (Hyronimus et al., 2000).

In this study 47.93% of gram positive and catalase negative isolates show the resistance to bile salts. Rapid food passage rate in the duodenum which bile salts involved 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. *Streptococcus (enterococcus)* and *Lactobacilli* which are Lactic acid bacteria could be survive here.

Most bacteria do not survive well at low pH values. The pH values along the gastrointestinal tract in chicken are pH 2 - 9. The severe acidic conditions of the crop

(pH 4 – 6.3), proventriculus (pH 3.17 – 4.80) and gizzard (pH 2.50 – 4.74) could have an adverse effect on the bacteria. Microorganisms from the crop which survive the low pH of the gizzard generally multiply in the small intestine (pH 5.7 – 6.0). Organisms from this organ may be taken in to caeca (pH 5.70 – 8.40) (Fuller, 1992). These pH values in specific areas of the avian gastrointestinal tract selectively allow establishment of a specific microbial population in birds.

Hyronimus et al. (2000) found that different strains of *Sporolactobacillus*, *Bacillus laevolacticus*, *Bacillus racemilacticus* and *Bacillus coagulans* grown in MRS broth were subjected to low pH conditions (2, 2.5 and 3). Anyway, in this study only 16.38% of bile salts tolerance isolates could tolerance to widely pH value 2 – 10.

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. A probiotics was able to use exogenously or endogenously to enhance nutritional status and/or health of the host (Goldin and Gorbach, 1984). These beneficial will improve growth performance. In this study only 3 strains of acid – base tolerance could utilize nutrients as protein, starch and lipid.

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. 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).

Tellez et al. (2001) indicated that a combination of *Lactobacillus acidophilus*, *Streptococcus faecium*, and *Salmonella Enteritidis*, *Salmonella Typhimurium*, and *Salmonella Heidelberg*-Specific antibodies have a beneficial effect in reducing the colonization of *Salmonella* Enteritidis in market-aged broilers. While *Lactobacillus* spp. reduced *Escherichia coli* in feces (Muralidhara et al., 1977), guts and stomachs of small pigs (Barrow et al., 1980), and they suppressed and neutralized *E. coli* toxin in calves (Gilliland et al., 1984). Antibacterial of 3 strains which could utilize

nutrients were examined in this study. All strains could inhibit *E. coli* and *Salmonella Typhimurium*.

In conclusion, there were 3 isolates which named CMU-FP001, CMU-FP002 and CMU-FP003 indicated probiotics properties as described. They were tolerance to 0.3 % bile, growth in widely pH range although utilize starch, protein and fat. They also had antibacterial activity to *Salmonella Typhimurium* and *Escherichia coli*.

All these 3 bacteria isolates were further identified by 16S rDNA sequence. CMU-FP001 showed homology with *Enterococcus faecalis*. CMU-FP002 showed homology with *Lactobacillus plantarum.* And CMU-FP003 showed homology with *Enterococcus faecalis.* Both *Lactobacillus* and *Enterococcus* were Lactic acid bacteria (LAB), which have generally been considered as good probiotic organisms and the genus currently being used in probiotic preparations (Pandey, 1979; Sullivan et al., 1992).

5.2 The effect of selected probiotic bacteria on productive performance and humoral immunity in male broilers

5.2.1 The effect of selected probiotic bacteria on productive performance

In the present experiment, the improvement of body weight was consistent in all probiotics fed groups ($p<0.05$) while feed intake was not differing ($p>0.05$).

Enterococcus faecalis CMU-FP001, *Lactobacillus plantarum* CMU-FP002 and *Enterococcus faecalis* CMU-FP003 show 9.97, 10.39 and 9.01 percent respectively increase in average daily gain as compared to control. This is in agreement with Mohan *et al*. (1995) who reported 15.1 percent increase in body weight gain of broilers as compared with the control diet with supplementing diet with probiotics. Yeo and Kim (1997) observed that supplementation of 0.1% *L. casei* improved the average daily gain of broilers from 0 to 42 days of age, while Mohan et al. (1996) reported that improvement in body weight gain was observed in broilers only after 4 weeks of feeding probiotics. Khaksefidi and Ghoorchi (2006) found body weight gain of birds fed diet supplemented with 50 mg/kg of probiotics were significantly higher during 1 – 21 and 22 – 42 days but no difference was observed among 25 and 50 mg/kg probiotics supplemented group. Aftahi et al. (2006) found significantly better performance on body weight gain of probiotics groups than the control group. Rowghani et al. (2007) found 12.9, 15.6 and 7.7% increase body weight gain when supplement with probiotics, Toxiban (a mixture of aluminosillicate and ammonium proplonate) and Probiotic with Toxiban. While the results contradict with the observations of Hossain (2004), Kwon *et al*. (2002) and Priyankarage *et al*. (2003), found no significant effect of probiotic on live weight gain of broiler.

All 3 probiotics group had no significant on feed consumption ($p>0.05$) which is in agreement with findings of Miazzo *et al*. (2000), Ledoux *et al*. (1999), Hossain (2004) and Mutus *et al*. (2006) found non-significant effect of probiotic on feed consumption of broiler. But not in the line of the findings of Jernigan *et al*. (1985) and Yeo and Kim (1997) who reported that the use of probiotics in broiler chicken diets

significantly improved the daily body weight gain, feed intake and feed efficiency. Also, Aftahi et al. (2006) found probiotics significant effect on feed intake. They said higher feed intake might be due to the increased rate of appetite and enzymatic activity in the digestive tract in their treatments. However, the significant effects of probiotics on feed intake were observed by Kim *et al*. (1988); Erdogan (1999) and Haq *et al*. (1997).

Feed Conversion Ratio and Feed Efficiency had a significant difference ($p<0.05$) in broiler fed with both probiotics group 2 (*Lactobacillus plantarum* CMU-FP002) and probiotics group 3 (*Enterococcus faecalis* CMU-FP003). The improvement of FCR with feeding probiotics were in agreement with the findings of Miazzo *et al*. (2000), Haddadin *et al*. (2001), Pelicia *et al*. (2004) and Papaioannou *et al*. (2005). As same as Jin et al. (1998) whom showed that body weights and feed to gain ratios were improved significantly ($P < 0.05$) when compared to control broilers for broilers fed diets containing 0.05 or 0.10% Lactobacillus culture.

Similar improvements in body weight, feed efficiency have been reported for poultry receiving probiotics (Tortueto, 1973; Mohan *et al*., 1996; Yeo and Kim, 1997). Aftahi et al. (2006) found that feed conversion ratio was not significantly affected ($p>0.05$) during starter period (1-21 days of age) except birds in 5.0 g Yogurt-fedding group, which significantly ($p<0.05$) higher than other groups. Mohan *et al.*, (1996) and Hamid *et al.* (1994) reported that broiler fed different level of *L.acidophilus* culture (probiotic) up to 35 days of age and found improved feed conversion ratio in birds fed 5.0 g probiotic per liter of water, while Hossain *et al*.

(2004) found no significant effect of probiotic groups on feed conversion ratio. Sohail et al. (2002) also noted that addition of Bacillus subtilis C-3102 to hen diets had no significant influence on feed consumption, egg production, egg weight, or body weight of hens.

The reason of variable effect of biological additives may be confounded by variations in gut flora and environmental conditions (Mahdavi *et al*., 2005) and strain of probiotics that add up in feed.

Probiotics maintained a better microbial environment in the digestive tract of birds by reducing the number of pathogenic microbes. This enhanced digestion, absorption and efficiently of utilization of feed. It is generally accepted that the enhanced growth performance of broilers receiving dietary probiotics depends largely on consequent reduction of the undesirable microbial population of the alimentary tract that compete with the host for nutrients (Brzoska et al., 1999).

Maruta et al. (1996) reported that administering *Bacillus subtilis* C-3102 to chickens decreased the presence of intestinal pathogens such as *Salmonella* and *Campylobacter*. Dunham et al. (1993) reported that birds treated with *L.reuteri* exhibited longer ileal villi and deeper crypts suggesting better gut health and more functional surface area for nutrient absorption. Schneitz et al. (1998) reported that probiotic treatment improved the digestibility of organic matter. Jin et al. (1997) reported that undesirable microbial enzyme tends to decrease with the supplementation of *L. acidophilus*.

In animal nutrition, probiotics are viable microorganisms used as a feed supplement, which lead to beneficial effects for the host animal. For most species, a trend towards improved performance has been reported due to the use of probiotics, but statistically significant improvements of weight gain and of feed conversion are rare. This may be because of variations in the individual reactions of the animals.

5.2.2 The effect of selected probiotic bacteria on humoral immunity in male broilers

In this study, there was highly significantly higher HI titer for Newcastle Disease in broiler fed - probiotics *Lactobacillus plantarum* CMU-FP002 which HI titer 7 day post secondly vaccinated ranged from 1:16 to 1:128 with average HI titer 87.11.

This HI titer was higher than Shuaib et al. (2006) who found the HI titers of chickens vaccinated with Lasota strain of Newcastle disease virus through drinking water, 7 day post single vaccination ranged from 1:16 to 1:128 with average HI titer 55.693. This was shown that probiotics – fed – group can stimulate immune system especially humoral immune system.

Shoeib et al. (1997) reported the effect of dietary supplement of pronifer probiotic on immune response in 80 day-old chicks. Haematological profiles showed an increase in total erythrocyte and leukocyte cell count and marked increase in percentage of lymphocytes and monocytes.

Panda et al. (2000) supplemented diet with various level of probiolac probiotic (a commercial probiotic mixture of lactic acid bacteria, *Aspergillus oryzae* and Torulopsis) and observed the significant increase in antibody production at 10 days of post immunization when Sheep red blood cells (SRBC) was injected at 14 days of age and at 5 days of post inoculated when SRBC was injected at 21 days.

Zulkifli et al. (2000) exposed two lines of commercial broilers (Hubbard and Shaver) to heat stress regime and superimposed a Lactobacillus treatment. Birds were given a Newcastle vaccination at 7 and 21 days at which time (21 days) the heat stress treatments were imposed. Hubbard birds fed Lactobacillus had a higher antibody production than Shaver birds suggesting that there may be some strain differences.

Khaksefidi and Ghoorchi (2006) found antibody production against Newcastle disease virus in 50 mg/kg probiotics supplemented group was significantly higher at 10 days of post immunization compared to control.

Rowghani et al. (2007) found blood Newcastle antibody titer was affected ($p<0.05$) by treatments (except feeding Toxiban). The highest value was seen with combination of probiotics and Toxiban followed by probiotics feeding. Combination of probiotic and Toxiban, probiotic and Formaycine increased the Newcastle antibody titer by 22.18, 12.10 and 10.4 percent compared with the control diet.

As feed additive, probiotics has a good impact on the poultry performance (Stavric and Kornegay, 1995). These live organisms after residing intestinal tract and

their metabolites can act as immunomodulatory agent by activating specific and non-specific host immune responses in chicks, which in turn help in prevention and control of various infectious diseases (Fuller, 1992; Koenen *et al*., 2004). The effects of probiotics on immune system of poultry are interesting and complicated. The direct effect might be related to stimulating the lymphatic tissue (Kabir et al., 2004), and indirect effect via changing the microbial population of the lumen of Gastrointestinal Tract (Jin et al., 1998). It is obvious that probiotics are able to enhance the immune response to different antigenic stimulants.

5.3 The effect of selected probiotic bacteria on inhibition of *E.tenella* infection, anti-coccidial antibody and cytokine levels related with *E.tenella* infection

5.3.1 The effect of selected probiotic bacteria on inhibition of *E.tenella* infection

Eimeria tenella undergoes schizogony and gametogony in the cecal wall of broiler. The oocyst, which is shed in the feces, is the mature form of the infective coccidia. Merozoites of the first generation schizonts invade the cecal lamina propria via the crypt epithelial cells. The second generation schizonts develop in the lamina propria within the crypt cells, and the new merozoites invade other cecal cells directly through the connective tissue or via the cecal epithelial cells (Fernando et al., 1983). Generally, the number of oocysts shed is dependent on the number of sporozoites and merozoites that penetrate the enterocytes for a given inoculumdose, and represents the infection-resistant ability of broilers. The susceptibility to Eimeria was also assessed

on the basis of the number of oocysts obtained from droppings collected for four days starting on day 6 day post inoculated (Dalloul et al., 2005).

Chlortetracycline, at a dose of 250–3,000 parts per million, has been patented as an anti-coccidial medication that may be used singly or in a combination of two or more anti-coccidial agents (Tamas and Ostlind, 1996).

Oocyst shedding had a significant difference ($p<0.05$) which broiler fed probiotics slightly differ from control but not differ from Chlortetracycline-fed group ($p>0.05$). In chicken infected *Eimeria tenella*, Chlortetracycline-fed and Probiotics *Lactobacillus plantarum* CMU-FO002 group shed in average 24.44×10^4, 0.61×10^4 and 3.51×10^4 oocysts/birds, respectively. The chickens fed the probiotic showed an 85.63% reduction in the number of oocysts shed compared to the control group.

Lee et al. (2007) found, in *Eimeria tenella*-infected groups, the birds fed probiotics diets shed apparently less oocysts than the infected-control birds, though there was no significant difference between the birds fed regular and probiotics-supplement diets.

Even though the probiotic treatment significantly reduced the fecal oocyst load by 14.37%, the reduction in shedding was similar to Dalloul et al., 2005 which inoculated *Eimeria acervu*lina to probiotics-fed chicken and found 14% reduction. However there was not great as in Dalloul et al. (2003) found Probiotics-fed chicken obtained a 75% reduction (four-fold, $P<0.0001$) in *Eimeria acervulina*. The

differential effect with the 2 Eimeria species tested could be attributed to the species-specific infection sites, where probiotics organisme may favor colonizing 1 site over the other (Lee et al., 2007)

Thus, Dalloul et al. (2005) consider that their prior dose of 10,000 was a very acute challenge, which was met with a high degree of protection by the inclusion of the probiotics in the diet. The present study, therefore, demonstrates double dose than Dalloul et al. (2005) and probiotics *Lactobacillus plantarum* CMU-FP002 continued to protection. These result demonstrated that Probiotics *Lactobacillus plantarum* CMU-FP002 can enhance the resistance of chicken against coccidiosis.

Anyway, Lactic acid bacteria have been shown to play an important role in competitive exclusion of pathogenic bacteria by several mechanisms during bacterial infection (Fukata et al., 1999; van der Wielen et al., 2002); these results suggest that the same may also be true for coccidia, including *E. tenella*. Indigenous bacteria occupy gut mucosal surfaces and may prevent the coccidial parasite from attaching to the mucosa, rendering it unable to penetrate the surface epithelial layer. This is one of the mechanisms involved in competitive exclusion (Lan et al., 2004). Some strains of Lactic acid bacteria, particularly *Lactobacilli*, can augment the nonspecific defense mechanism of the host (in rat; Bloksma et al., 1979), or modulate the specific immune response in a different fashion (in humans; Vidal et al., 2002).

Therefore, *E. tenella*-specific antibody secretion plays a key role in *E. tenella* exclusion when the birds are infected. 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. These results will be discussed in next experiment.

5.3.2 The effect of selected probiotic bacteria on caecal tonsil and bursa of fabricius

The Gut associated lymphoid tissue (GALT) is a component of the mucosal immune system, protecting against enteric microorganisms. The avian GALT includes the bursa of Fabricius, the caecal tonsils, Meckel's diverticulum, Peyer's patches and intraepithelial lymphocytes (Lillehoj and Trout, 1996).

1) Cecal tonsils: body weight ratio

Cecal tonsils are the major lymphoid tissue in the cecum and constitute the largest collection of GALT in the chicken. Lymphocytes in cecal tonsils consist of 45±55% B cells and 35% T cells and are thus involved both in antibody production and cell mediated immune functions (Befus et al., 1980). Anatomically, cecal tonsils are located at the ileocecal junction and are not present at the time of hatching but develop shortly afterward. Kitagawa et al. (1998) showed that 45.7% of cecal lymph nodes were distributed in the proximal 7.8% of the cecum. It is probable that the chicken cecum is exposed to backflow from the urodum of the cloaca (Braun and

Campbell, 1989) and, therefore, stimulated continuously with new antigens. Several investigations have indicated that cecal tonsil lymphocytes may be involved in the intestinal immune response to *Eimeria*. For example, during *E. tenella* infection, an increased number of leukocytes (Vervelde et al., 1996) and prominent lymphoid nodules were found in the base of cecal tonsils approximately 3 cm from the ileocecal junction accumulating as dense aggregates of lymphocytes containing irregularly scattered lymphoid tissues and germinal centers (del Cacho et al., 1993).

Yurong et al. (2005) found the density of microvilli and length of cecal tonsils increased after probiotics were administrated. Their immunological maturation and overall size are dependent on the degree of antigenic stimulation in the intestine (del Cacho et al., 1993) and the increased number of lymphocytes appearing as chickens age may be a consequence of their migration into the organ after antigenic stimulation.

Anyway in this study, caecal tonsils in Control group were larger than Chlortetracycline-fed and Probiotics *Lactobacillus plantarum* CMU-FP002 group. This result show that caecal tonsil in control group were stimulated by *Eimeria tenella* while anitibiotic and probiotics *Lactobacillus plantarum* CMU-FP002 groups did not or were less stimulated. It might be confirmed that Lactic acid bacteria have been shown to play an important role in competitive exclusion of coccidia by several mechanisms during infection (Fukata et al., 1999; van der Wielen et al., 2002). Indigenous bacteria occupy gut mucosal surfaces and may prevent the coccidial

parasite from attaching to the mucosa, rendering it unable to penetrate the surface epithelial layer.

2) The bursa of Fabricius: body weight ratio

The bursa of Fabricius is an immunological organ that plays a primordial role in the poultry immunity (Toivanen et al 1987). It is according to its physiological state that will especially depend on the immune status of poultries at the beginning of broiler chickens weight development. The different aggressions of the environment (stress, bad hygiene, vaccination, pathologies) undergone by birds, influential on the anatomical and physiological development of the bursa of Fabricius. It can, therefore, lead to an immunodepression in certain birds (van Denberg et al 2000). .

In this study, bursa of fabricius: body weight ratio has no significantly difference among group. Similarly, Panda *et al* (2000) also reported that in broilers fed probiotics, there was no significant difference in parameters like body weight, weight of spleen and Bursa, FCR and feed consumption.

The liver, spleen, bursa, and intestines, all play an integral role in the inflammatory immune response as indicated by their increase in weight during such challenges (Roura et al., 1992). These organs are also involved in acute phase protein synthesis, lymphocyte activation, antibody production, and antigen sampling (Abbas et al., 1997). Kabir et al. (2004) demonstrated that the differences in the weight of bursa of probiotics and conventional fed broilers could be attributed to different level

of antibody production. They also were interesting to note that the weights of spleen and bursa were found higher in non-vaccinated broilers as compared to vaccinated broilers. However, in Probiotics CMU-FP002 group seemed to have larger bursa than others. It may be conclude that Probiotics CMU-FP002 stimulated the bursa of fabricius with no difference from other group.

In Chlortetracycline-fed groups seemed to have smallest bursa when compare to the other groups. Franti et al. (1971) showed a decrease in bursa weights in chicks fed bacitracin and chlotetracycline.

Anyway, Shoeib et al. (1997) reported that the bursa of fabricius in probiotic-treated group showed an increase in the number of follicles with high plasma cell reaction in the medulla. Because in this study we did not identify bursa in histopathology to define cell reaction so it will be further note.

5.3.3 The effect of selected probiotic bacteria on anti-coccidial antibody and cytokine levels related with *E.tenella* infection

1) Anti-coccidial antibody in collected serum, intestinal washes and lymphocyte supernatant by ELISA

The role of parasite-specific antibodies has been extensively studied in coccidiosis (Lillehoj and Ruff, 1987; Fayer and Jenkins, 1992; Dalloul et al., 2005). Although humoral immunity to coccidiosis seems to play a minor function (Dalloul

and Lillehoj, 2005, 2006), *Eimeria* infections trigger a significant specific Ab immune response in serum (Dalloul et al., 2005), and immunoglobulins could have a contributory function in the defense of the host against *Eimeria* (Wakelin and Rose, 1990; Lillehoj and Trout, 1996).

All groups had highest Serum anti-coccidial antibody in day 9 but there was no significantly difference between these 3 groups. In Intestinal anti-coccidial antibody of Probiotics group were highest than the other groups in day 3 post inoculated as same as in supernatant of spleen cells stimulated with Con A.

Lee et al. (2000) found *Eimeria* specific antibodies to EtMIC2 antigen were significantly ($P < 0.05$) higher in chickens fed the MitoGrow diets in *E.tenella*-infected birds.

Dalloul et al. (2005) found no clear conclusion can be made as to what extent the probiotic affected humoral immunity. They explained since the parasites would have reached the final stages of their life cycle, and the antibodies detected at this point of infection may not be very indicative of the status of protective immunity against the pathogen.

Anyway, Yun et al., 2000c supported that antigen-specific antibodies of different isotypes including IgA have been detected 8–10 days following *Eimeria maxima* infection. In this study also found serum anti-coccidial antibody were high in days 6, 9 and 12 post inoculated with significantly difference ($p<0.05$) from day 0 and

3, it might because of antigen – specific antibody produced to serum in these day. Except probiotics group *Lactobacillus plantalum* CMU-FP002 had no significantly difference among day and antibody level was still high. It might be conclusion that Probiotics *Lactobacillus plantalum* CMU-FP002 could enhance humoral immune response.

2) IFN-γ in collected serum, intestinal washes and lymphocyte supernatant by ELISA

Interferon (IFN) - ν plays a central role in resistance against various mammalian (Billiau, 1996; Billiau et al., 1998) and avian (Lillehoj, 1998; Schultz and Chisari, 1999) pathogens. Complex cellular and molecular immunoregulatory mechanisms are mediated by IFN-ν with distinct roles in the protective responses against harmful pathogenic organisms (Sato et al., 1997; Tessitore et al., 1998). IFN-ν is also involved in the regulation, differentiation, maturation and proliferation of hematopoietic cells (Selleri et al., 1995; Lange et al., 1996; Murray et al., 1998), as well as the chemo- and radioprotectants (Gardner, 1998) and as an immunoenhancement against tumors (Ringenbach et al., 1998), bacteria (Sawai et al., 1999), viruses (Fiorelli et al., 1999), and parasites (Luty et al., 1999).

Mammalian and avian IFN-ν has been used as indicators of cell mediated immunity in infected hosts. With regard to the activities of cytokines during avian coccidiosis, IFN-ν appears to play the predominant role (Choi et al., 1999; Lillyhoj and Choi, 1998). Chicken IFN-ν, like its mammalian homologue, regulates acquired

immunity to *Eimeria* by activating lymphocytes and enhancing expression of MHC class II genes (Smith et al., 1994). IFN-ν production in mice (Rose et al., 1994) and chickens (Martin et al., 1994; Yun et al., 2000a) has been used as a measure of T-cell responses to *Eimeria* antigens.

In this study Serum IFN-ν was highly significant difference ($p< 0.01$). Control group was higher than Probiotics CMU-FP002 and Chlortetracycline-fed group on days 0, 3 and 6 but lower than Probiotics CMU-FP002 on days 9 and 12 post inoculated. In day 6, intestinal IFN-ν was highest in Probiotics group. And there were no significantly difference between Probiotics CMU-FP002, Chlortetracycline-fed and Control group in supernatant of spleen cells.

It was seem to similar to previous study as Hong et al. (2006) found IFN-ν mRNA expression was detected in lymphocytes isolated from the intestinal ceaca of *Eimeria tenella* – infected SC strain chickens on day 6 post inoculated. Laurent et al. (2001) found IFN-ν expression in the cecum of White Leghorn chickens increased more than 200-fold at 7 day-primary infection with *Eimeria tenella* as same as Rothwell et al. (2004). Anyway, Chlortetracycline-fed group had highest IFN-ν in day 9.

Dalloul et al. (2003) showed a probiotic influence on the early production of IFN-ν during coccidial infection, as evidenced by higher levels 3 day post inoculated. However, sampling at day 10 did not result in differences in IFN-ν levels.

It was not the same way as Dalloul et al. (2005) found intestinal INF-ν in control birds was constant thought the sampling period except at day 6 post inoculated when a significant ($p<0.05$) increase occurred. Probiotic-fed-birds had a significantly higher IFN-ν at 3 day post inoculated, which then declined until 12 day post inoculated. No difference was observed between the two treatments from day 6 on. Dalloul et al. (2005) also found Serum IFN-ν levels were similar ($P > 0.05$) among and within treatments throughout the sampling period and reported supernatants of the ConA-stimulated lymphocytes from the Control group had higher ($P < 0.05$) IFN-ν level than Probiotics birds regardless of the ConA concentration used. By the way, Dalloul et at. (2005) studied in *Eimeria Acervulina*, the difference among species may occur.

T lymphocytes and macrophages are most likely the source of cytokine production in the chicken intestine (Lillyhoj, 1994). Non-specific stimulation of these cells especially within the intraepithelium (Dalloul et al., 2003b) would result in their activation and proliferation early on during an enteric infection and thus leading to early IFN-ν production. An early higher IFN-ν level following coccidial challenge demonstrates the protective effect of probiotics on cell-mediated immunity to the parasite.

Infection by Eimeria has been shown to increase IFN-ν production in vivo. Lymphocytes from Eimeria-infected chickens produced more IFN-ν when stimulated

with ConA than did lymphocytes from uninfected chickens (Martin et al., 1994 and Dimier-Poisson et al., 1999).

As detailed above, IFN-ν has been reported to be crucial to the cellular immunity to coccidia. Because there was no significantly difference between Probiotics *Lactobacillus plantalum* CMU-FP002, Chlortetracycline-fed and Control group in supernatant of spleen cells, but Intestinal IFN-ν was highest in Probiotics group. One logical explanation of this result would be that Probiotic *Lactobacillus plantalum* CMU-FP002 enhanced mucosal immune responses in birds thus delaying the systemic immune reactions such as spleen lymphocyte proliferation.

3) IL-2 in collected serum, intestinal washes and lymphocyte supernatant by ELISA

IL-2 is a major cytokine of cell-mediated immunity. Chickens infected with Eimeria develop a robust cell mediated response against parasite (Miyamoto et al., 2005).

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 and Lillyhoj, 2000 and Lillyhoj et al.,1992) IL-2 has both direct and indirect effects on cells of the immune system. IL-2 binds directly with the receptors for IL-2 to stimultethe growth of T-cells (Gillis et al.,

1978). In an indirect manner, IL-2 was shown to stimulate production of granulocyte-macrophage colony stimulating factor (Wong et al., 1989).

In this study, serum IL-2 had highly significant difference ($p<0.01$) between Probiotics CMU-FP002, Chlortetracycline-fed and Control group. Chlortetracycline-fed group had highest IL-2 in day 3 post inoculated with significantly difference to other group. While Probiotics group were similar level on day on and seem to higher than Control group. Probiotics CMU-FP002 also had highest intestinal IL-2 than Chlortetracycline-fed and Control group. There were no significantly difference ($p>0.05$) between Probiotics CMU-FP002, Chlortetracycline-fed and Control group in supernatant of spleen cells stimulated with 12.5 mg/ml Con A while 25 mg/ml Con A had significantly difference ($p<0.05$) which Chlortetracycline-fed group were highest.

Dalloul et al. (2005) found higher intestinal IL-2 in Probiotics birds only day 3 post inoculated plus a lack of response in Control birds until day 3 post inoculated suggest the probiotic facilitated an earlier, more effective response to eimerian infection.

Miyamoto et al. (2002) found serum IL-2 in infected chickens increased significantly compared with control chickens at day 7 post-primary infection ($p<0.01$). They also found IL-2 levels in supernatants of spleen lymphocytes from infected chickens stimulated with 12.5 mg/ml Con A were significantly increased at day 7 post infection compared with non-infected controls ($p<0.05$). They concluded that serum IL-2 was likely derived from spleen lymphocyte. In this study seem to

support to Miyamoto et al. (2002) that there was similar way of IL-2 level in serum and spleen lymphocyte supernatant but it was not clarify. It seem to had lowest IL-2 level in day 6 both in serum and spleen lymphocyte but in serum IL-2 level are resemble to constant.

As describe, Probiotics *Lactobacillus plantalum* CMU-FP002 had highest intestinal IL-2 than Chlortetracycline-fed and Control group and there was similar way of IL-2 level in serum and spleen lymphocyte supernatant. The effect of Probiotics *Lactobacillus plantalum* CMU-FP002 on IL-2 level, may be concluded that Probiotics *Lactobacillus plantalum* CMU-FP002 can enhance cell-mediated immunity especially in mucosal immunity as an important role of IL-2 in protective immunity to Eimeria tenella in the caecum.

In conclusion, the effect of Probiotics *Lactobacillus plantalum* CMU-FP002 on anti-coccidia antibody, the humoral immunity, was not clarified but it might enhance the humoral immunity in serum, the systemic immunity, and intestinal, the mucocal immunity. In addition, IFN-ν level and IL-2 level were high in intestinal as well as serum and supernatant from spleen lymphocyte so it may be concluded that Probiotics *Lactobacillus plantalum* CMU-FP002 can also enhance cell-mediated immunity especially in mucosal immunity as an important role of these two cytokines in protective immunity to Eimeria tenella in the caecum.