Chapter 4

Results

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

4.1.1 Isolation of probiotic lactic acid bacteria from poultry's coacal swap

Samples were collected by coacal swap (Figure 5) from 120 healthy antibiotic-free poultry on November, 2006 until November, 2007 from Northern, North-eastern, Middle and Southern regions of Thailand.

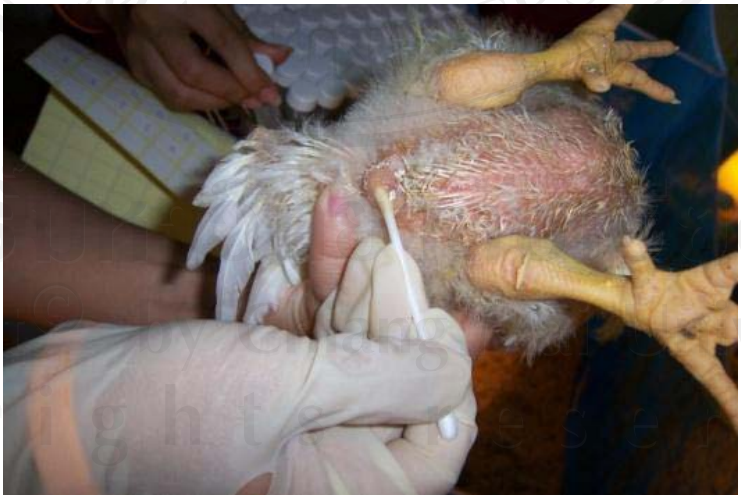


Figure 5 Sample colletion by coacal swap

A total of 1200 representative isolates, changed the media color around colony to yellow (Figure 6), were isolated. 390, 260, 200 and 350 isolates were isolated from Northern region, North eastern region, Middle region and Southern region of Thailand (Figure 7). Only 548 isolates (45.66%) were representative gram positive (Figure 8) and only 242 isolates (20.16%) were catalase negative (Figure 9).

Figure 6 Bacterial isolates change the media color around bacterial colony to yellow

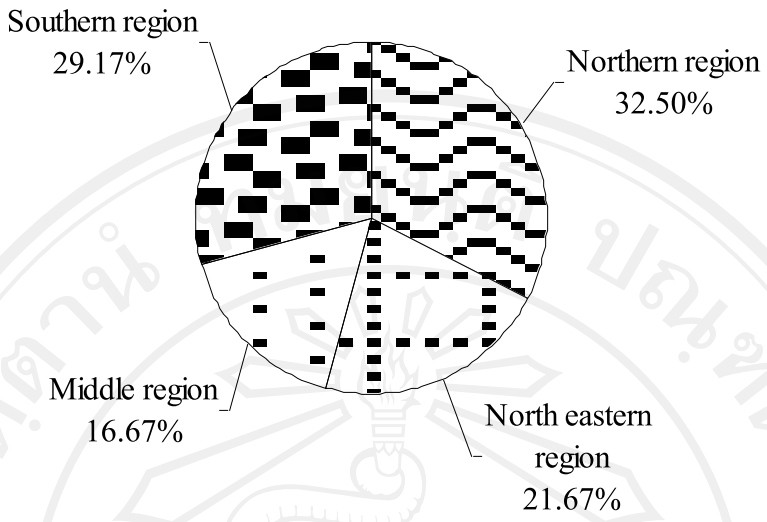


Figure 7 A total of 1200 representative isolates, changed the media color around colony to yellow, divided by source of regions.

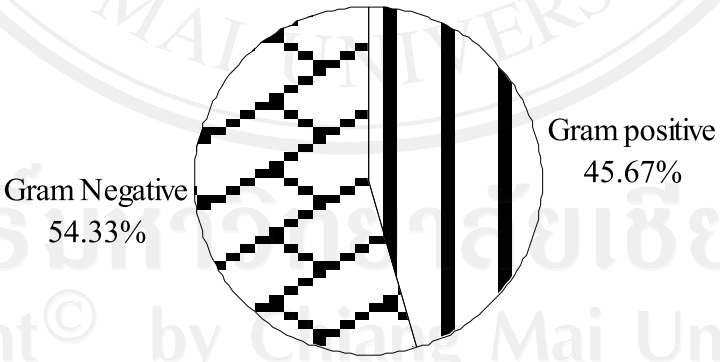


Figure 8 A total of 1200 representative isolates, changed the media color around colony to yellow, divided by gram strain.

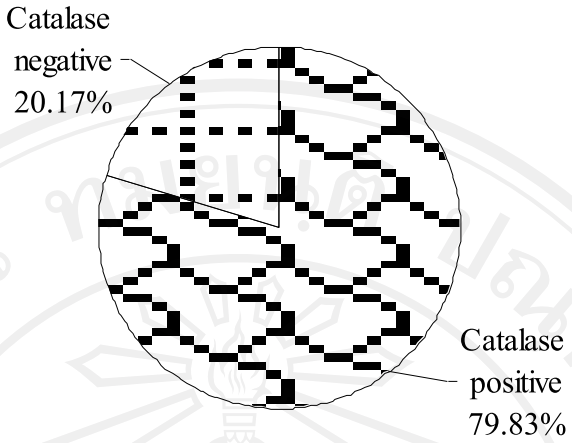


Figure 9 A total of 1200 representative isolates, changed the media color around colony to yellow, divided by catalase activity

4.1.2 Probiotics Properties examinations

4.1.2.1 Bile salt Tolerance

All 242 isolates which represent gram positive and catalase negative were further employed to study the tolerance to 0.3% bile salts (Figure 10). 116 isolates (47.93%) were found relatively resistance to bile salts (Figure 11).

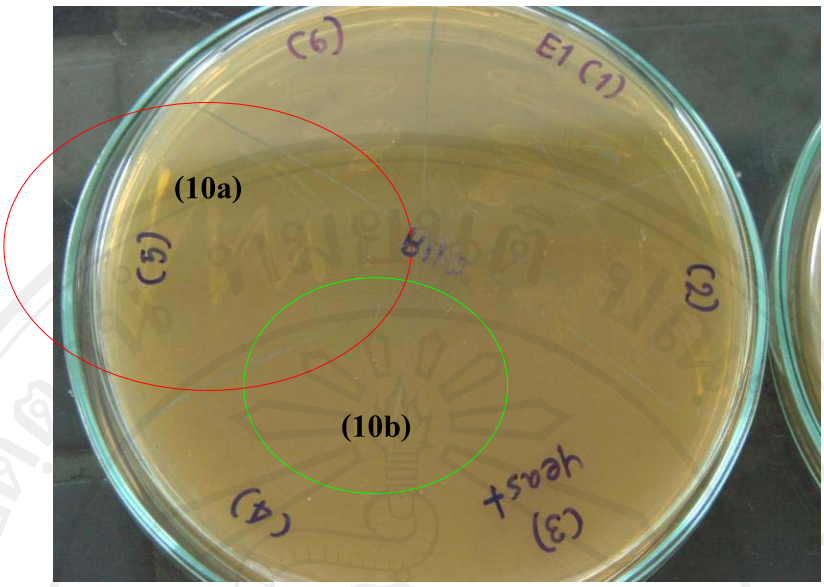


Figure 10 Bile salt tolerance tests; (10a) Bacteria isolates tolerance to 0.3% bile salts and (10b) Bacteria isolates not tolerance to 0.3% bile salts

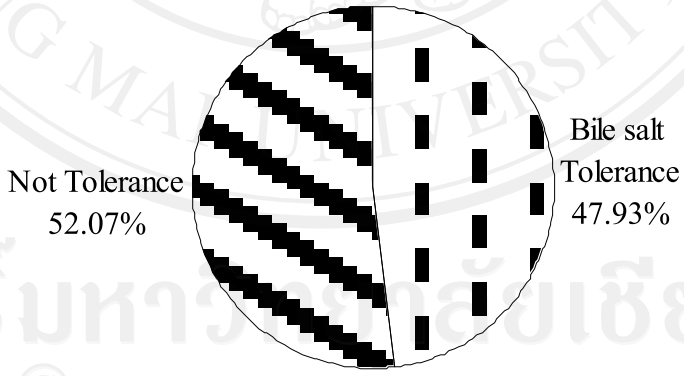


Figure 11 Bile salt tolerance tests of 242 isolates which represent gram positive and catalase negative represent resistance to Bile salts

4.1.2.2 Acid - Base Tolerance

116 isolates of bacterial cultures which were grown on bile salt plated were selected to determine acid – base tolerance (Figure 12). By screening test observed the turbidity, only 19 isolates (16.38%) survival at pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 (Figure 13).

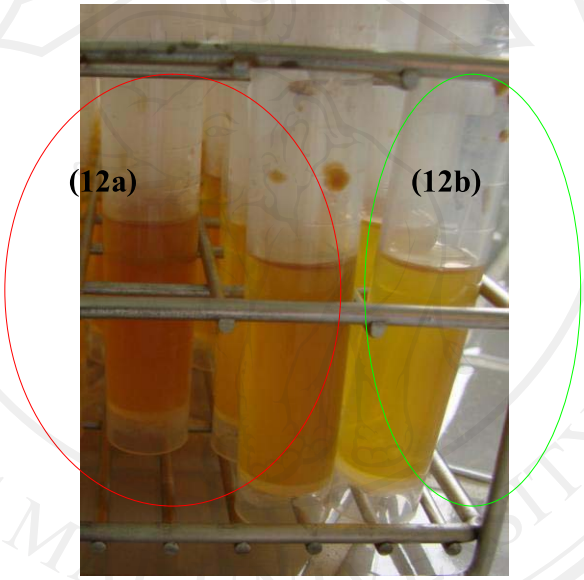


Figure 12 Acid – Base Tolerance test; (12a) Bacteria grow on media with difference pH and (12b) Bacteria did not grow on media with difference pH

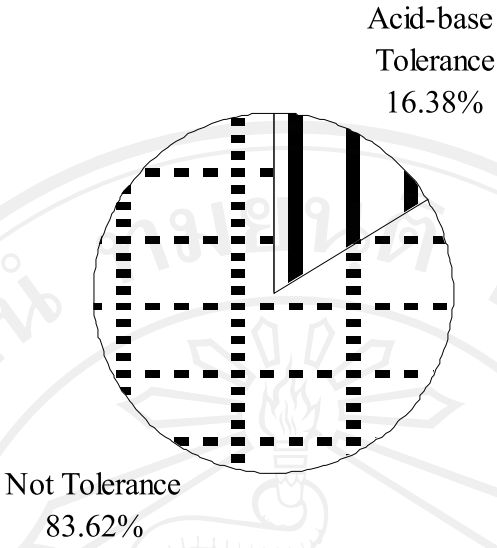
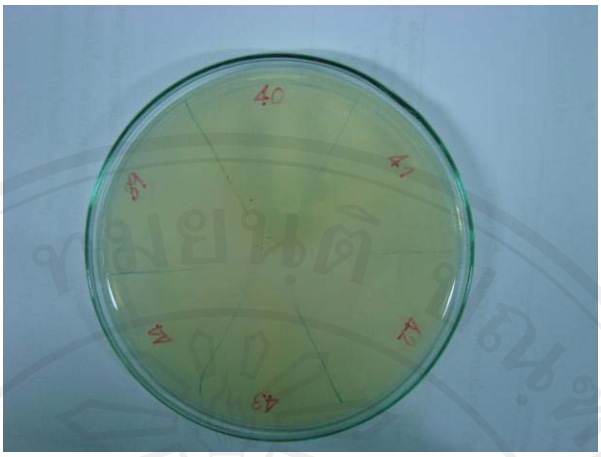
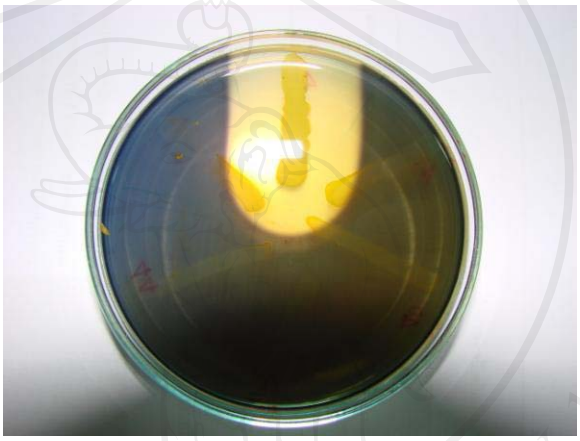


Figure 13 Acid – Base Tolerance test of 116 isolates which grown on bile salt plated.

4.1.2.3 Utilization of protein, starch and fat

19 isolates from 4.1.2.2 that had acid – base tolerance were further determined protein, starch and fat utilization. 15 (78.95%), 3 (15.79%) and 16 (84.21%) isolates had ability to utilized protein, starch and fat, respectively (Figure 14 and 15).

(14a) 

(14b) 

(14c) 

Figure 14 Protein, starch, fat utilization test; (14a) bacteria strain utilized protein, (14b) bacteria strain utilized starch and (14c) bacteria strain utilized fat.

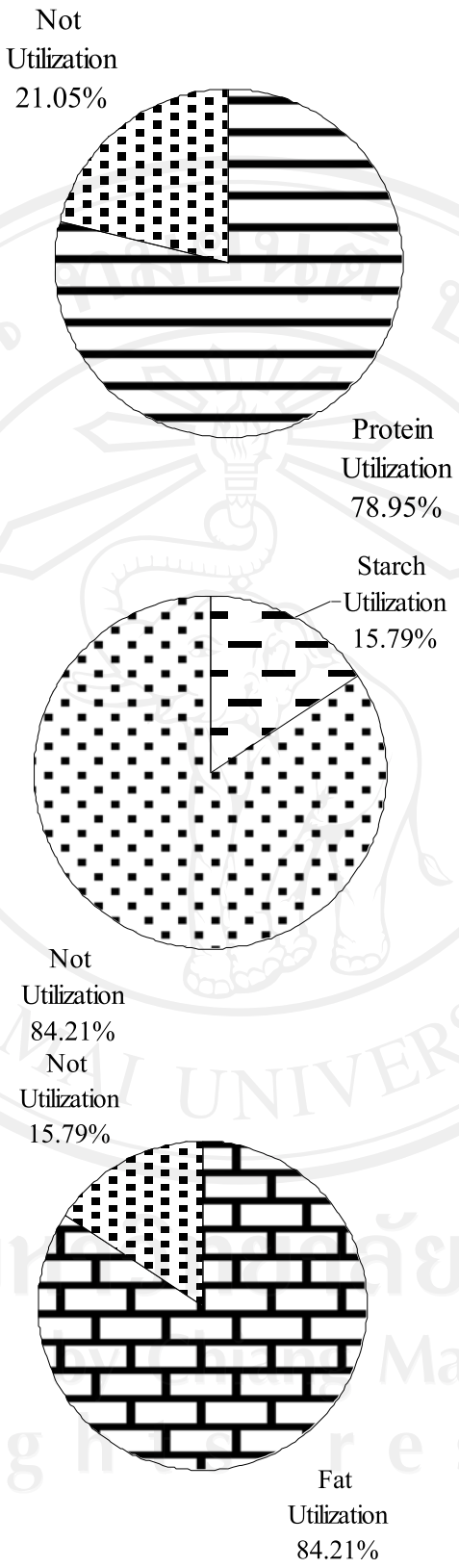


Figure 15 Protein, starch, fat utilization test of 19 isolates which had acid – base tolerance.

4.1.2.4 Antibacterial activity to enteropathogenic bacteria

3 bacterial isolates from 4.1.2.3 which had protein, starch and fat utilization, were further examined antibacterial activity by measuring inhibition zone. All 3 isolates (100%) had antibacterial activity to *Escherichia coli* (Figure 16) and *Salmonella typhimurium* (Figure 17).

Figure 16 Antibacterial activities of bacteria to *Escherichia coli.*

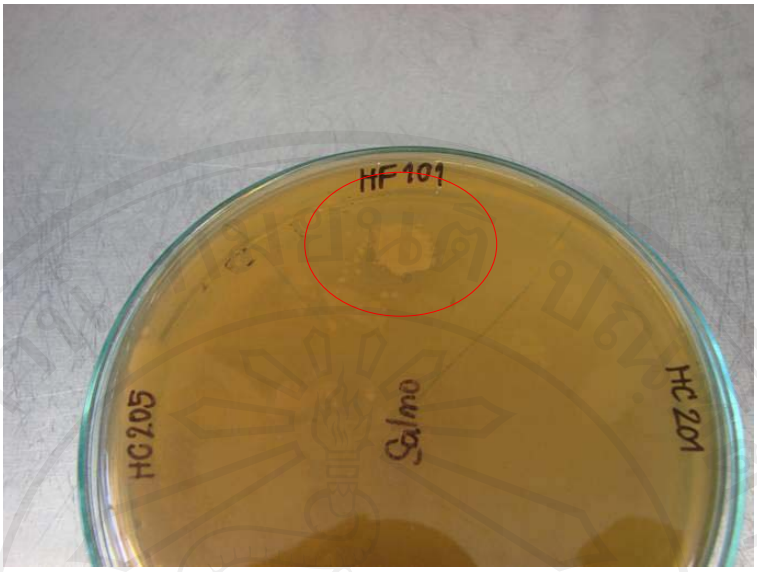


Figure 17 Antibacterial activities of bacteria to *Salmonella typhimurium.*

4.1.3 Identification by 16S-rDNA-sequence analysis

All 3 bacteria isolates from 4.1.2 were further used as CMU-FP001, CMU-FP002 and CMU-FP003. The bacterial genomic DNA was identified by the 16S-rDNA, amplified by PCR and compared to those of the international all GenBank, EMBL, DDBL and PDB catalogue.

The 16S rDNA sequence of CMU-FP001 indicated that the isolate belongs to the genus *Enterococcus*. The sequence homology with *Enterococcus faecalis* group species was 99%.

The 16S rDNA sequence of CMU-FP002 indicated that the isolate belongs to the genus *Lactobacillus*. The sequence homology with *Lactobacillus plantarum* group species was 99%.

The 16S rDNA sequence of CMU-FP003 indicated that the isolate belongs to the genus *Enterococcus*. The sequence homology with *Enterococcus faecalis* group species was 99%.

4.2 The effect of selected probiotic bacteria on productive performances and humoral immunity in male broilers

4.2.1 The effect of selected probiotic bacteria on productive performances

All 3 bacteria isolates from 4.1 were further used as *Enterococcus faecalis* CMU-FP001, *Lactobacillus plantarum* CMU-FP002 and *Enterococcus faecalis* CMU-FP003.

A total of 150, day-old Aber acres chickens were assigned to five treatments with three replications as Control (nothing added), Antibiotic (2% Colistin added up in dietary) and Probiotics group 1 (10^6 CFU/ml of probiotics *Enterococcus faecalis* CMU-FP001 added up by oral force feeding), Probiotics group 2 (10^6 CFU/ml of probiotics *Lactobacillus plantarum* CMU-FP002 added up by oral force feeding) and Probiotics group 3 (10^6 CFU/ml of probiotics *Enterococcus faecalis* CMU-FP003 added up by oral force feeding).

Ten birds were randomly selected to each treatment. Body weight and feed consume were recorded at the beginning and end of feeding trial at day 38. Their growth performance was calculated, respectively.

1) Average Daily Gain (ADG)

Average Daily Gain (ADG) was shown in Table 3. Average Daily Gain had a significant ($p<0.05$) difference between broiler fed with 10^6 CFU/ml of each probiotics and the group fed with 2% Colistin. However there were not differing to control group. Probiotics group 2 (*Lactobacillus plantarum* CMU-FP02) had highest average daily gain than Probiotics group 1 (*Enterococcus faecalis* CMU-FP001), Probiotics group 3 (*Enterococcus faecalis* CMU-FP003), Control and Colistin-fed group, consecutively.

2) Feed Intake (FI)

Feed Intake (FI) had no significant difference ($p>0.05$) between each group (Table 4). Probiotics group 2 (*Lactobacillus plantarum* CMU-FP02) had highest feed intake than Control, Probiotics group 2 (*Enterococcus faecalis* CMU-FP001), Colistin-fed group and Probiotics group 3 (*Enterococcus faecalis* CMU-FP003), consecutively.

Table 3 Average Daily Gain (ADG) of broilers in 1- 38 day of aged.

Group	Average Daily Gain (gram/day)
Control	59.62 ± 1.77[ab]
Colistin-fed	57.58 ±3.74[b]
CMU-FP001-fed	65.57 ±1.67[a]
CMU-FP002-fed	65.82 ±1.88[a]
CMU-FP003-fed	64.99 ±5.51[a]
p-value	0.0320

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$).

Table 4 Feed Intake (FI) of broilers in 1 - 38 day of aged.

Group	Feed Intake (gram/day)
Control	105.30±5.56[a]
Colistin-fed	100.69±2.75[a]
CMU-FP001-fed	107.35±3.42[a]
CMU-FP002-fed	103.27±4.32[a]
CMU-FP003-fed	100.59±8.79[a]
p-value	0.5023

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$).

3) Feed Conversion Ratio (FCR)

Feed Conversion Ratio (FCR) had a significant difference ($p<0.05$) between broiler fed with 10^6 CFU/ml of probiotics group 2 (*Lactobacillus plantarum* CMU-FP02), probiotics group 3 (*Enterococcus faecalis* CMU-FP003) and Control group, Colistin-fed group (Table 5). Probiotics group 3 (*Enterococcus faecalis* CMU-FP003) had greatest Feed conversion ratio then Probiotics group 2 (*Lactobacillus plantarum* CMU-FP02), Probiotics group 1 (*Enterococcus faecalis* CMU-FP001), Colistin-fed and Control group, consecutively.

4) Feed Efficiency (FE)

Same as Feed conversion ratio, Feed Efficiency had a significant difference ($p<0.05$). Broiler fed with 10^6 CFU/ml of probiotics group 2 (*Lactobacillus plantarum* CMU-FP002) and probiotics group 3 (*Enterococcus faecalis* CMU-FP03) are differ from group Control and also group Colistin-fed. Probiotics group 3 (*Enterococcus faecalis* CMU-FP03) had greatest Feed efficiency then Probiotics group 2 (*Lactobacillus plantarum* CMU-FP002), Probiotics group 1 (*Enterococcus faecalis* CMU-FP001), Colistin-fed and Control group, consecutively.

Table 5 Feed Conversion Ratio (FCR) of broilers in 1- 38 day of aged.

Group	Feed Conversion Ratio
Control	1.76±0.04[a]
Colistin-fed	1.75±0.07[a]
CMU-FP001-fed	1.64±0.01[ab]
CMU-FP002-fed	1.57±0.09[b]
CMU-FP003-fed	1.55±0.10[b]
p-value	0.0110

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$).

Table 6 Feed Efficiency (FE) of broilers in 1 - 38 day of aged.

Group	Feed Efficiency
Control	0.567±0.013[b]
Colistin-fed	0.572±0.022[b]
CMU-FP001-fed	0.611±0.004[ab]
CMU-FP002-fed	0.638±0.037[a]
CMU-FP003-fed	0.647±0.044[a]
p-value	0.0160

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$).

5) Percent Mortality

Percent Mortality (Table 7) in this trial was 5.64%. Probiotics group 3 (*Enterococcus faecalis* CMU-FP03) had higher percent mortality (2.05%) then Colistin-fed group (1.54%), other groups had 0.51% mortality.

Table 7 Percent Mortality of broilers in 1 - 38 day of aged.

Group	Mortality (%)
Control	0.51
Colistin-fed	1.54
CMU-FP001-fed	0.51
CMU-FP002-fed	0.51
CMU-FP003-fed	2.05
Average	5.64

4.2.2 Effect of selected probiotic bacteria on humoral immunity in male broilers

Vaccinated with Newcastle plus infectious bronchitis diseases vaccine was given to each chicken at day 21 and day 31. Serums were collected in day 38 and tested for Newcastle disease titers by Haemagglutination Inhibition (HI) test (Shuaib et al., 2006). The results were presented in Table 8.

Haemagglutination Inhibition Titer had highly significantly ($p<0.01$) which broiler fed with 10^6 CFU/ml of probiotics group 2 (*Lactobacillus plantarum* CMU-FP002) was differ from group Control, Colistin-fed and also Probiotics group 3 but not differ from Probiotics group 1. Probiotics group 2 (*Lactobacillus plantarum* CMU-FP002) had highest HI Titer then Probiotics group 1 (*Enterococcus faecalis* CMU-FP001), Colistin-fed, Control and Probiotics group 3 (*Enterococcus faecalis* CMU-FP03), consecutively.

Table 8 Haemagglutination Inhibition Titer (HI Titer) of Newcastle disease.

Groups /Total No. of Birds	Haemagglutination Inhibition Titer							GMT
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	
Control	0	0	1	0	5	2	1	47.11^{bc}
Colistin-fed	0	0	0	1	3	5	0	48.00^{bc}
CMU-FP001-fed	0	0	0	0	1	8	0	60.44^{ab}
CMU-FP002-fed	0	0	0	1	2	1	5	87.11^{a}
CMU-FP003-fed	0	0	1	2	6	0	0	25.78^{c}
p-value								0.0017

^{a,b,c} Means within a row with no common superscript differ significantly ($P < 0.05$).

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

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

Ninety, day-old male broiler chicks will be randomly assigned to 6 cages; two were fed with broiler diet as controls, other two were fed with the same diet with antibiotic chlortetracycline, lastly two were fed with the same diet with selected probiotics *Lactobacillus plantarum* CMU-FP002.

Then 3 – week – old chickens, 15 chickens from each cage, were selected on mean weight-basis within treatment and transferred to an isolation facility to be challenged with 20,000 *Eimeria tenella* sporulated oocysts.

On days 6, 7, 8, 9, 10 after inoculated, homogenized fecal material ground was taken, diluted, and counted the oocysts microscopically using a hemacytometer. The result was shown in Figure 18.

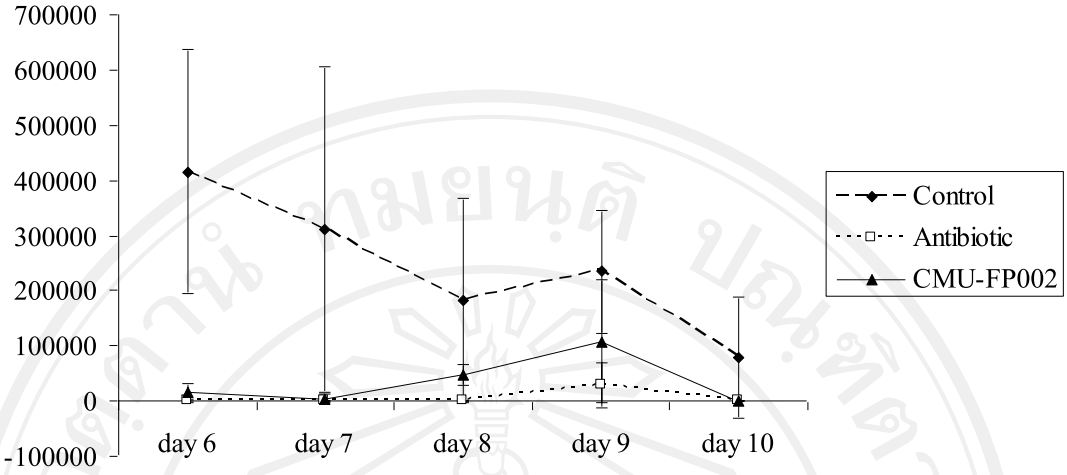


Figure 18 Oocyst shedding in fecal material ground each group on days 6, 7, 8, 9, 10 after inoculated.

Oocyst shedding had a significant difference ($p<0.05$); which broiler fed Probiotics CMU-FP002 slightly differ from Control but not difference from Antibiotic Chlortetracycline (Table 8).

As shown in Table 9, In chicken infected *Eimeria tenella*, Chlortetracycline-fed and Probiotics *Lactobacillus plantarum* CMU-FO002 groups 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. The occyst shedding of Probiotics *Lactobacillus plantarum* CMU-FP002 group and Chlortetracycline-fed group were lower than Control group with highly significant ($P<0.01$).

Table 9 Oocyst Shedding in fecal material ground on days 6, 7, 8, 9, 10 after infection.

Group	Oocyst Shedding (oocyst/bird/day)				
	Day 6	Day 7	Day 8	Day 9	Day 10
Control	415325 ± 220546.6^{a}	311719 ± 294996.1^{ab}	182301 ± 184510.7^{abc}	234375 ± 110485.4^{abc}	78125 ± 110485.4^{bc}
Chlortetracycline-fed	1650 ± 2333.5^{c}	0^{c}	0 ^{c}	28516.5 ± 39716.07 ^{c}	0^{c}
CMU-FP002-fed	15650 ± 17465.5^{c}	5000 ± 7071.1^{c}	46650 ± 18879.7^{bc}	108300 ± 110732.9^{bc}	0^{c}

P value = 0.0449

^{a,b,c} Means within a row and column with no common superscript differ significantly ($P < 0.05$).

Table 10 Average Oocyst Shedding in fecal material ground on days 6 – 10**.**

Group	Average Oocyst Shedding (oocyst/bird/day)
Control	244369 + 127819.5[a]
Chlortetracycline-fed	6033.3 + 12588.78[b]
CMU-FP002-fed	35120 + 44743.61[b]
p-value	0.0008

[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$).

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

Three week-old chickens were assigned to control, chlortetracycline-fed and probiotic-fed group and inoculated by 20,000 *Eimeria tenella*, hasvested their spleen. Spleen lymphocytes were prepared according to the method outline by Dalloul et al. (2002). Collected serum and intestinal wash, collected the ceca (infection site of *Eimeria tenella*), caecal tonsil, bursa of fabricius and intestinal wash were accomplished on days 0, 3, 6, 9 and 12 from 3 chickens in each cage. Each of them was stored at -20 degree celcius.

1) Caecal tonsil: body weight ratio

Caecal tonsil was collected from each chicken in each group, weighted and calculated to caecal tonsil: body weight ratio. The results were shown in Table 11 and Figure 19.

In all group, caecal tonsil: body weight ratio seemed to larger pass each day. In Control group, day 12 post inoculated, caecal tonsil: body weight ratio was largest with significant difference from day 0 and 3 post inoculated. This result was the same as Chlortetracycline-fed and Probiotics *Lactobacillus plantarum* CMU-FP002 groups. Anyway, in day 12 post inoculated, caecal tonsil: body weight ratio in all groups have no significant difference.

Table 11 Caecal tonsil: Body weight ratio on days 0, 3, 6, 9, 12 after infection.

Group	Caecal tonsil: Body weight ratio (percent)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.0232 ± 0.0081^{defg}	0.0239 ± 0.0053^{cdef}	0.0272 ± 0.0044^{bcdefg}	0.0343 ± 0.0078^{abcd}	0.0363 ± 0.0070^{ab}
Chlortetracycline-fed	0.0226 ± 0.0007^{efg}	0.0179 ± 0.0015^{g}	0.0289 ± 0.0073^{abcdefg}	0.0296 ± 0.0072^{abcdef}	0.0384 ± 0.0093^{a}
CMU-FP002-fed	0.0349 ± 0.0106^{abc}	0.0188 ± 0.0071^{fg}	0.0245 ± 0.0068^{cdefg}	0.0274 ± 0.0056^{abcdefg}	0.0303 ± 0.0046^{abcde}

P value = 0.0003

^{a,b,c,d,e,f,g} Means within a row and column with no common superscript differ significantly ($P < 0.05$).

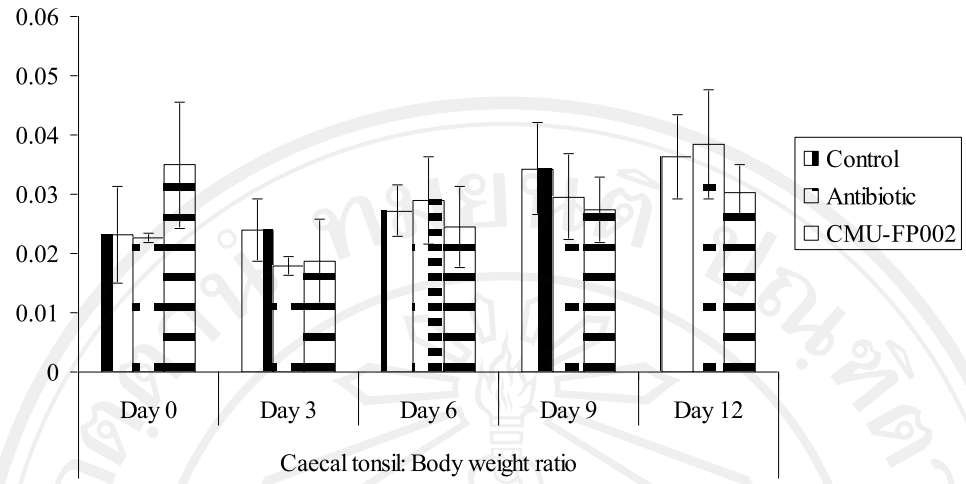


Figure 19 Caecal tonsil: Body weight ratio on days 0, 3, 6, 9, 12 after infection

2) Bursa of Farbricius: body weight ratio

Bursa of Farbricius was collected from each chicken in each group, weighted and calculated to bursa of Farbricius: body weight ratio. There was no significant difference in bursa of Farbricius: body weight ratio. The results were shown in Table 12 and Figure 20.

Table 12 Bursa of Farbricius: Body weight ratio on days 0, 3, 6, 9, 12 after infection.

Group	Bursa of Farbricius: Body weight ratio (percent)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.3479 ± 0.0831	0.3225 ± 0.0583	0.2932 ± 0.0523	0.2506 ± 0.0619	0.3242 ± 0.1065
Chlortetracycline-fed	0.3149 ± 0.0671	0.3247 ± 0.0758	0.2914 ± 0.0407	0.3329 ± 0.0710	0.2454 ± 0.0347
CMU-FP002	0.3226 ± 0.0616	0.4111 ± 0.1547	0.2764 ± 0.0468	0.2967 ± 0.0708	0.2986 ± 0.0825

P value = 0.0841

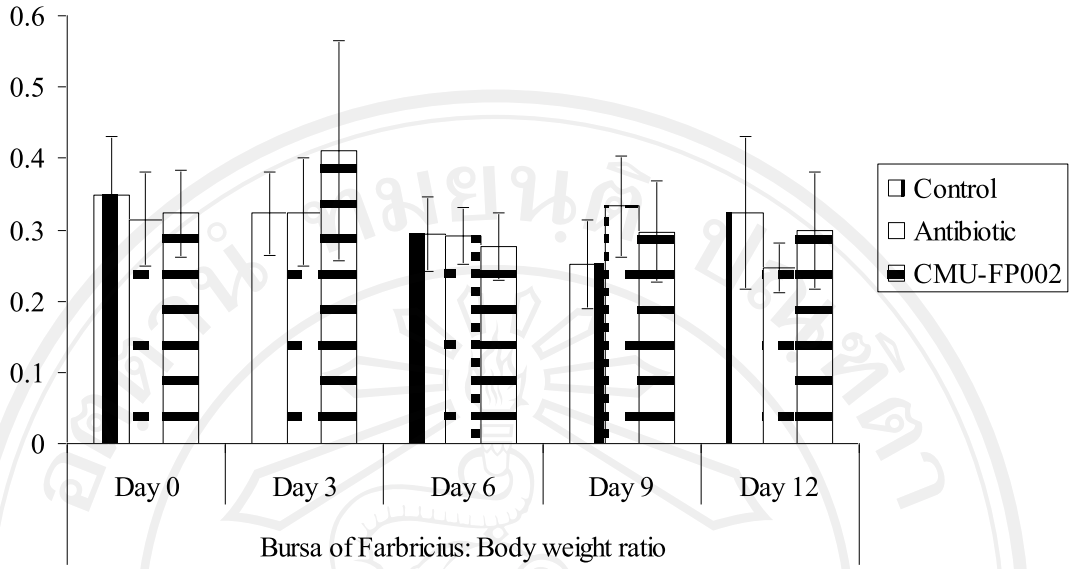


Figure 20 Bursa of Farbricius: Body weight ratio on days 0, 3, 6, 9, 12 after infection

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

Three week-old chicken were assigned to control, chlotetracycline-fed and probiotic groups and inoculated by 20,000 *Eimeria tenella*, hasvested their spleen and spleen lymphocytes prepared according to the method outline by Dalloul et al. (2002), collected serum and intestinal wash, collected the ceca (infection site of *Eimeria tenella*), caecal tonsil, bursa of fabricius and intestinal wash on days 0, 3, 6, 9 and 12 from 3 chickens in each cage. Each of them was stored at -20 degree celcius.

Anti-coccidial antibody, IFN-γ and IL-2 in collected serum, intestinal washes and lymphocyte supernatants were determined as described in Dalloul et al. (2005). The results were shown below.

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

To detect anti-coccidial antibody, microtiter plate wells were coated with Eimeria *tenella* sporulated oocyst coccidial antigen and bound Antibody detected with horseradish peroxidase-conjugated rabbit anti-chicken IgG and substrates 3, 30, 5, 50-tetramethylbenzidine dihydrochloride, and the plates were read at 450 nm.

1) Anti-coccidial antibodies in serum

Anti-coccidial antibodies in serum were shown in Figure 21. There were highly significant (P value < 0.01) which slightly difference between Probiotics *Lactobacillus plantarum* CMU-FP002 group, Chlotetracycline-fed and Control groups (Table 12).

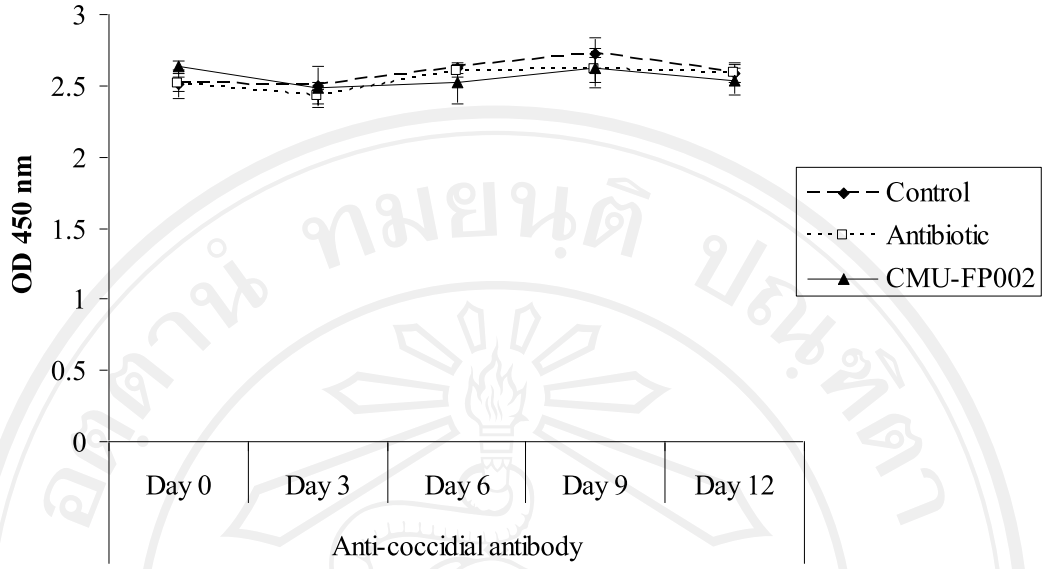


Figure 21 Anti-coccidial antibody in serum on days 0, 3, 6, 9, 12 after infection

As shown in Table 13, anti-coccidial antibody of Control group were highest in day 9 post inoculated with significant difference from day 0 and 3 post inoculated but not difference from day 6 and day 12. Same as Control, anti-coccidial of Chlotetracycline-fed group were highest in day 9 with significant difference from day 3 but not difference from day 6 and day 12 post inoculated. Probiotic group also had highest anti-coccidial antibody in day 9 which is not different from days 0, 3, 6 and day 12. By the way, there was no significant difference between these 3 groups in day 9.

Table 13 Anti-coccidial antibody in serum on days 0, 3, 6, 9, 12 after infection.

Group	Anti-coccidial antibody (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	2.51 ± 0.053^{bc}	2.50 ± 0.017^{bc}	2.62 ± 0.029^{ab}	2.73 ± 0.112^{a}	2.58 ± 0.06^{ab}
Chlotetracycline-fed	2.51 ± 0.09^{bc}	2.42 ± 0.052^{c}	2.60 ± 0.033^{ab}	2.61 ± 0.891^{ab}	2.59 ± 0.068^{ab}
CMU-FP002	2.63 ± 0.04^{ab}	2.49 ± 0.141^{bc}	2.52 ± 0.142^{bc}	2.61 ± 0.137^{ab}	2.53 ± 0.91^{bc}

P value = 0.0039

^{a,b,c} Means within a row and column with no common superscript differ significantly ($P < 0.05$).

2) Anti-coccidial antibodies in intestinal wash

Anti-coccidial antibodies in intestinal wash were shown in Figure 22. There were highly significant (P value < 0.01) which Probiotics *Lactobacillus plantarum* CMU-FP002 group was slightly higher than Chlotetracycline-fed and Control groups (Table 14).

As shown in Table 13, anti-coccidial antibody in Control group was highest in day 12 post inoculated with significant difference from day 0 and day 3 but not difference from day 6 and day 9. Chlotetracycline-fed group had highest anti-coccidial antibody in day 6 with significant difference from day 0 and day 3 but not difference from day 9 and 12. In Probiotics group and anti-coccidial chlortetracycline-fed group were highest in day 9 with significant difference from day 0 but not difference from days 3, 6, 9 and day 12. There were no significant difference in days 6, 9 and 12 in all 3 groups.

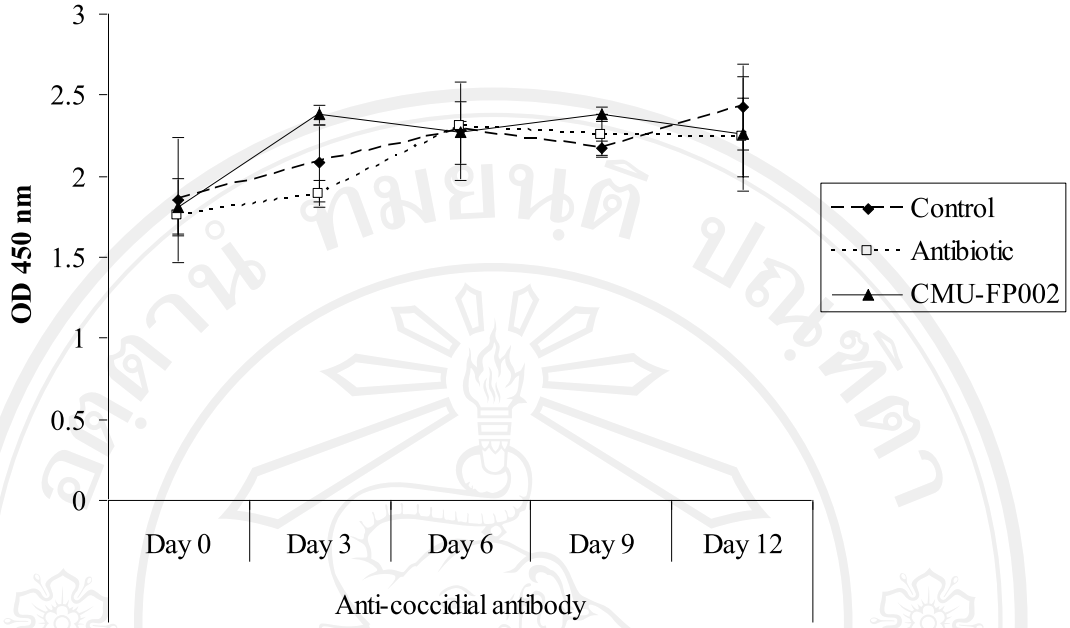


Figure 22 Anti-coccidial antibody in intestinal wash on days 0, 3, 6, 9, 12 after infection

Table 14 Anti-coccidial antibody in intestinal wash on days 0, 3, 6, 9, 12 after infection.

Group	Anti-coccidial antibody (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	1.849 ± 0.388^{cd}	2.082 ± 0.234^{bc}	2.279 ± 0.302^{ab}	2.176 ± 0.041^{ab}	2.432 ± 0.266^{a}
Chlotetracycline-fed	1.752 ± 0.102^{d}	1.894 ± 0.081^{cd}	2.311 ± 0.024^{ab}	2.255 ± 0.134^{ab}	2.243 ± 0.245^{ab}
CMU-FP002-fed	1.807 ± 0.178^{d}	2.357 ± 0.063^{ab}	2.276 ± 0.192^{ab}	2.381 ± 0.042^{a}	2.258 ± 0.353^{ab}

P value < 0.0001

^{a,b,c,d} Means within a row with no common superscript differ significantly ($P < 0.05$).

3) Anti-coccidial antibody in lymphocyte supernatant

Anti-coccidial antibody in lymphocyte supernatant with Con A 12.5 mg/ml were shown in Figure 23. There were significantly difference between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 15).

Anti-coccidial antibody in lymphocyte supernatants with Con A 12.5 mg/ml in all 3 groups were lowest in day 6 ($p<0.05$). Control group had highest anti-coccidial antibody in day 9 with significant difference from day 6 but not difference from days 0, 3 and day 12. In Antibody group, anti-coccidial antibody was highest in day 3 but not difference from days 0, 4 and day 12. Probiotic group had highest anti-coccidial antibody in day 3 with significant difference from day 6 but not difference from days 0, 9 and day 12.

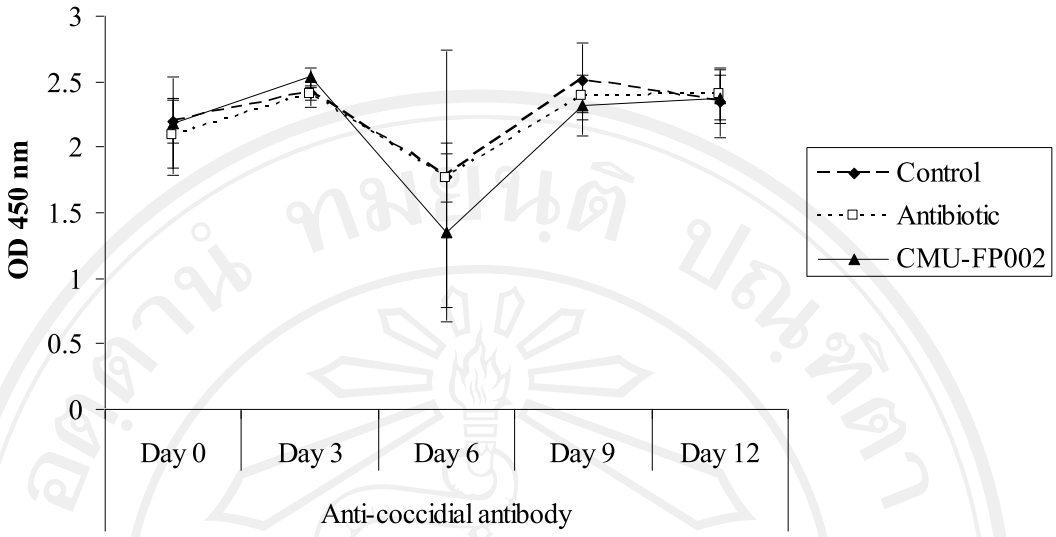


Figure 23 Anti-coccidial antibody in lymphocyte supernatant with Con A 12.5 mg/ml on days 0, 3, 6, 9, 12 after infection

Table 15 Anti-coccidial antibody in lymphocyte supernatant with Con A 12.5 mg/ml on days 0, 3, 6, 9, 12 after infection

Group	Anti-coccidial antibody (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	2.196 ± 0.166^{ab}	2.415 ± 0.054^{ab}	1.767 ± 0.183^{bc}	2.505 ± 0.290^{a}	2.242 ± 0.269^{ab}
Chlotetracycline-fed	2.08 ± 0.293^{ab}	2.404 ± 0.094^{ab}	1.76 ± 0.987^{bc}	2.384 ± 0.127^{ab}	2.401 ± 0.192^{ab}
CMU-FP002-fed	2.188 ± 0.348^{ab}	2.53 ± 0.079^{a}	1.35 ± 0.678^{c}	2.313 ± 0.232^{ab}	2.369 ± 0.183^{ab}

P value = 0.0135

^{a,b,c,d} Means within a row and column with no common superscript differ significantly ($P < 0.05$).

Anti-coccidial antibody in lymphocyte supernatant with Con A 25 mg/ml were shown in Figure 24. There were no significant difference between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 16).

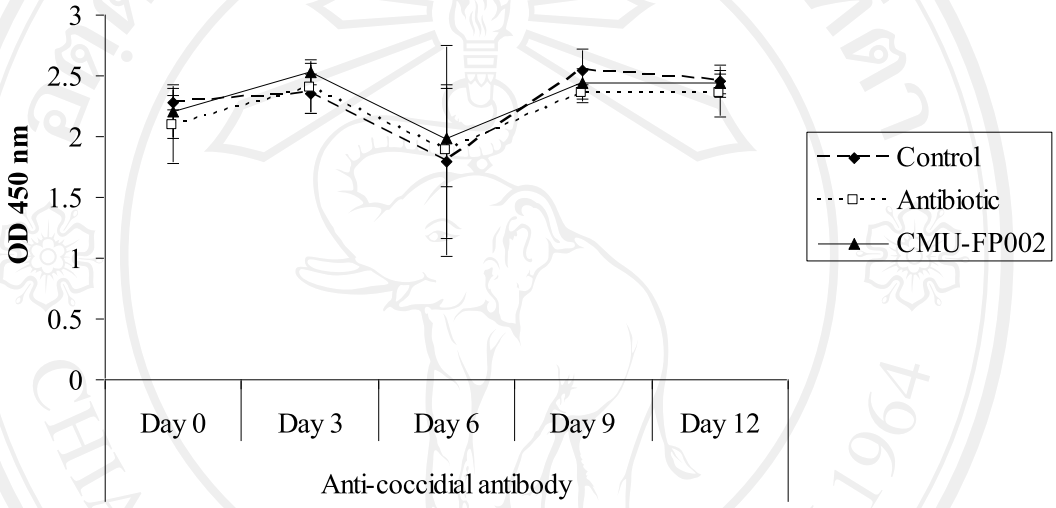


Figure 24 Anti-coccidial antibody in lymphocyte supernatant with Con A 25 mg/ml on days 0, 3, 6, 9, 12 after infection

Anti-coccidial antibody in lymphocyte supernatants with Con A 25 mg/ml in all 3 groups were lowest in day 6 without significant difference ($p>0.05$). As same as lymphocyte supernatants with Con A 12.5 mg/ml, Control group had highest anti-coccidial antibody in day 9. In Antibody group, anti-coccidial antibody was highest in day 3 and Probiotic group also had highest anti-coccidial antibody in day 3.

Table 16 Anti-coccidial antibody in lymphocyte supernatant with Con A 25 mg/ml on days 0, 3, 6, 9, 12 after infection

Group	Anti-coccidial antibody (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	2.284 ± 0.058	2.349 ± 0.165	1.800 ± 0.633	2.546 ± 0.176	2.449 ± 0.132
Chlotetracycline-fed	2.090 ± 0.314	2.399 ± 0.201	1.880 ± 0.866	2.354 ± 0.078	2.353 ± 0.186
CMU-FP002-fed	2.207 ± 0.226	2.530± 0.106	1.99 ± 0.400	2.435 ± 0.123	2.435± 0.082

P value = 1.651

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

IFN-γ were quantified using a direct binding ELISA bound Antibody detected with Mouse anti-chicken IFN-γ mAbs, horseradish peroxidase-conjugated goat anti-mouse IgG mAb and substrates 3, 30, 5, 50 - tetramethylbenzidine dihydrochloride (TMB), and the optical density (OD) read at 450 nm by an automated microtiter plate reader.

1) IFN-γ in serum

IFN-γ in serum was shown in Figure 25. There were highly significant (P value < 0.01) which Control group was higher than Probiotics *Lactobacillus plantarum* CMU-FP002 group and Chlotetracycline-fed group on days 0, 3 and 6 but lower than Probiotics *Lactobacillus plantarum* CMU-FP002 on days 9 and 12 post inoculated (Table 17).

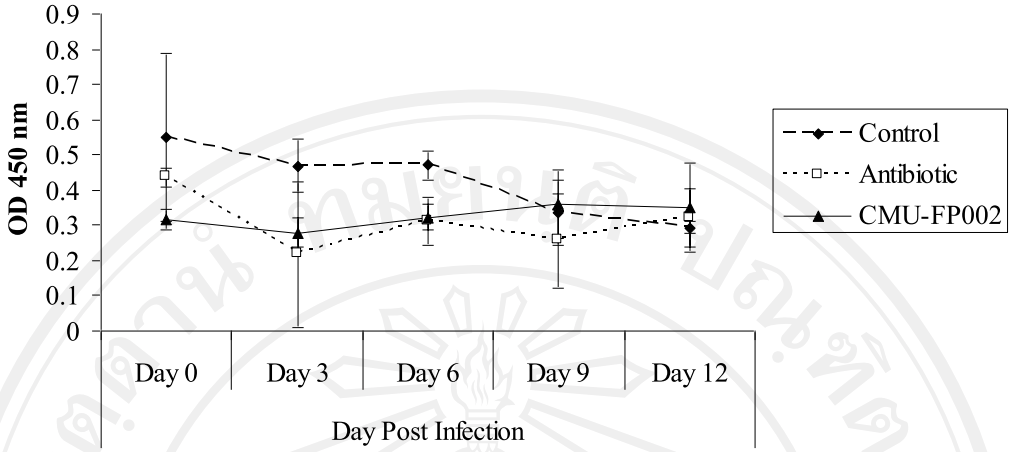


Figure 25 IFN-γ in collected serum on days 0, 3, 6, 9, 12 after infection

Table 17 IFN-γ in collected serum on days 0, 3, 6, 9, 12 after infection

Group	IFN-γ (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.549 ± 0.237[a]	0.469 ± 0.076[ab]	0.470 ± 0.042[ab]	0.337 ± 0.092[cde]	0.294 ± 0.015[de]
Chlotetracycline-fed	0.436 ± 0.028[abc]	0.218 ± 0.206[e]	0.313 ± 0.067[cde]	0.257 ± 0.134[de]	0.322 ± 0.083[cde]
CMU-FP002-fed	0.316 ± 0.029[cde]	0.279 ± 0.041[de]	0.323 ± 0.037[cde]	0.358 ± 0.097[bcd]	0.352± 0.126[bcd]

P value < 0.0001

[a,b,c,d,e] Means within a row with no common superscript differ significantly ($P < 0.05$).

2) IFN-γ in intestinal wash

IFN-γ in intestinal wash was shown in Figure 26.

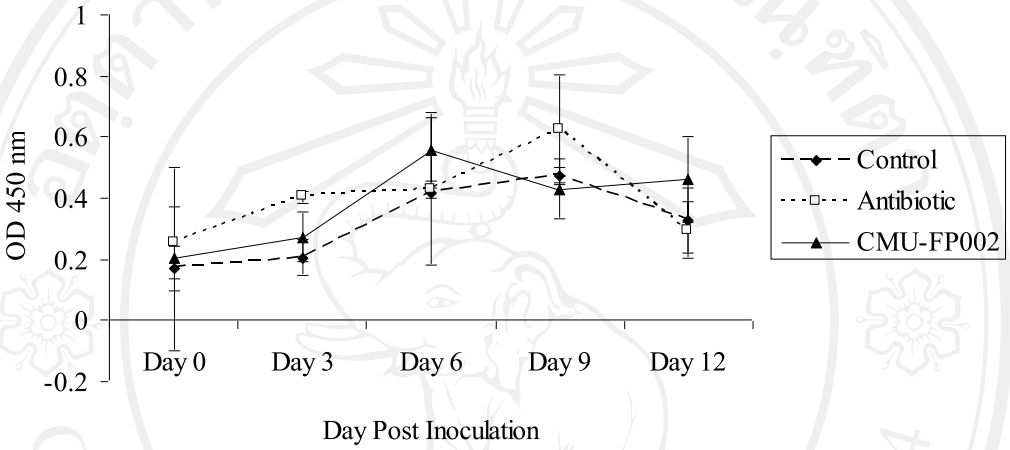


Figure 26 IFN-γ in intestinal wash on days 0, 3, 6, 9, 12 after infection

Probiotics *Lactobacillus plantarum* CMU-FP002 seemed to had intestinal IFN-γ higher than Control group and Chlotetracycline-fed group on days 6 and 12 post inoculated with significant (P value < 0.01) (Table 18).

Table 18 IFN-γ in intestinal wash on days 0, 3, 6, 9, 12 after infection

Group	IFN-γ (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.171 ± 0.007^{f}	0.206 ± 0.057^{f}	0.415 ± 0.015^{bcd}	0.473 ± 0.029^{bc}	0.326± 0.106^{cdef}
Chlotetracycline-fed	0.256 ± 0.117^{ef}	0.404 ± 0.018^{bcde}	0.429 ± 0.250^{bcd}	0.627 ± 0.173^{a}	0.295 ± 0.091^{def}
CMU-FP002-fed	0.203 ± 0.300^{f}	0.273 ± 0.083^{def}	0.559 ± 0.103^{ab}	0.429 ± 0.098^{bcd}	0.461± 0.140^{bc}

P value < 0.0001

^{a,b,c,d,e,f} Means within a row with no common superscript differ significantly ($P <$ 0.05).

3) IFN-γ in lymphocyte supernatant

IFN-γ in lymphocyte supernatant with Con A 12.5 mg/ml were shown in Figure 27.

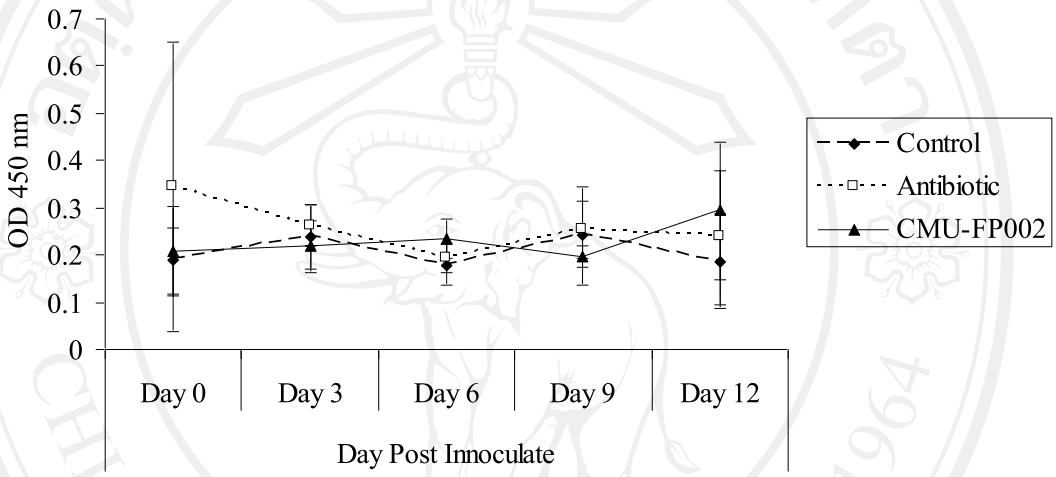


Figure 27 IFN-γ in lymphocyte supernatant with Con A 12.5 mg/ml on days 0, 3, 6, 9, 12 after infection

There was no significant difference between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 19).

Table 19 IFN-γ in lymphocyte supernatant with Con A 12.5 mg/ml on days 0, 3, 6, 9, 12 after infection

Group	IFN-γ (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.188 ± 0.070	0.237 ± 0.067	0.178 ± 0.044	0.241 ± 0.104	0.186± 0.100
Chlotetracycline-fed	0.344 ± 0.305	0.262 ± 0.043	0.193 ± 0.031	0.254 ± 0.059	0.237 ± 0.142
CMU-FP002-fed	0.208 ± 0.094	0.217 ± 0.055	0.235 ± 0.041	0.196 ± 0.024	0.293 ± 0.145

P value = 0.9222

IFN-γ in lymphocyte supernatant with Con A 25 mg/ml were shown in Figure 28.

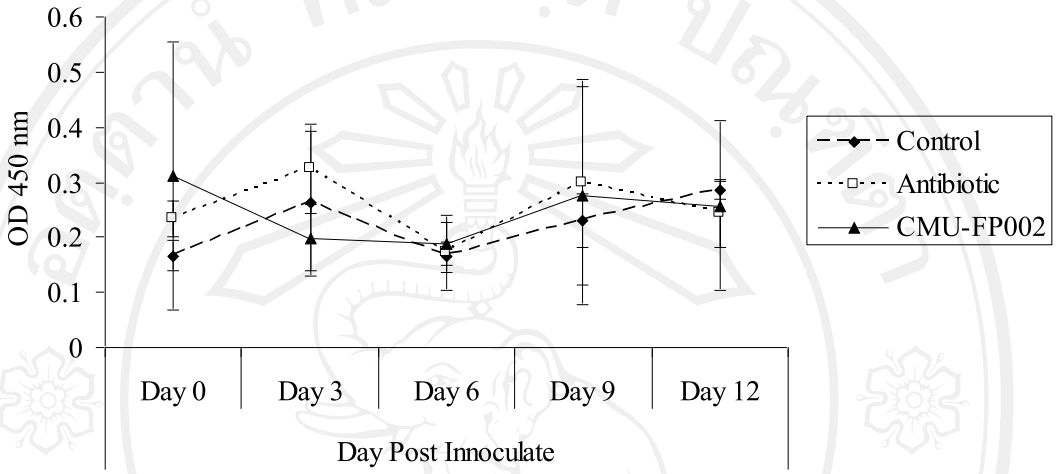


Figure 28 IFN-γ in lymphocyte supernatant with Con A 25 mg/ml on days 0, 3, 6, 9, 12 after infection

There were no significant difference between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 20).

Table 20 IFN-γ in lymphocyte supernatant with Con A 25 mg/ml on days 0, 3, 6, 9, 12 after infection

Group	IFN-γ (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.167 ± 0.026	0.261 ± 0.131	0.164 ± 0.028	0.229 ± 0.048	0.286± 0.019
Chlotetracycline-fed	0.234 ± 0.033	0.324 ± 0.082	0.172 ± 0.068	0.299 ± 0.186	0.242 ± 0.061
CMU-FP002-fed	0.311 ± 0.242	0.198 ± 0.061	0.188 ± 0.039	0.277 ± 0.197	0.257 ± 0.153

P value = 0.8027

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

Same as IFN-γ, IL-2 were quantified using a direct binding ELISA bound Antibody detected with Mouse anti-chicken IL-2 mAbs, horseradish peroxidase-conjugated goat anti-mouse IgG mAb and substrates 3, 30, 5, 50 - tetramethylbenzidine dihydrochloride (TMB), and the optical density (OD) read at 450 nm by an automated microtiter plate reader.

1) IL-2 in serum

IL-2 in serum was shown in Figure 29. There were highly significant (P value < 0.01) between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 21).

Chlotetracycline-fed group had highest IL-2 in day 3 post inoculated with significant difference to other group. While Probiotics group were similar level on day on and seem to higher than Control group.

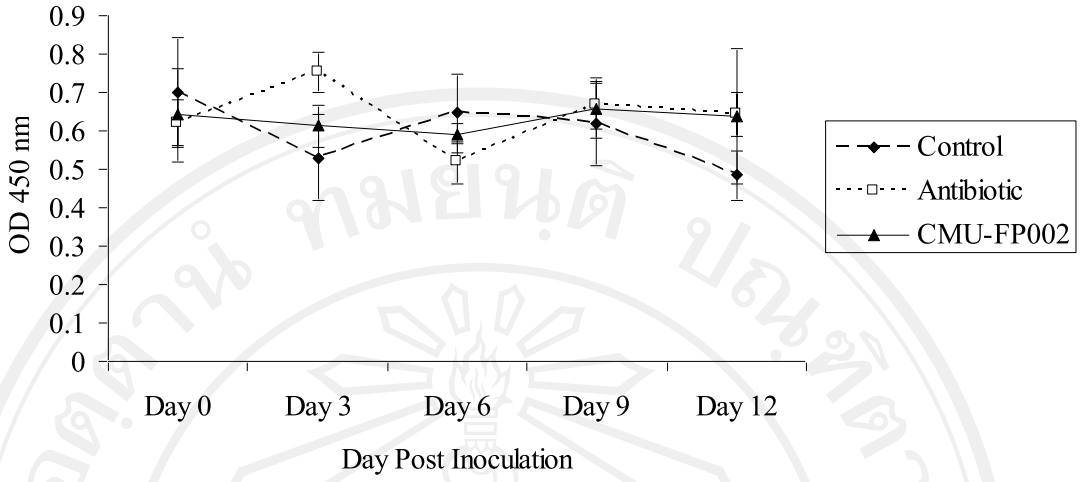


Figure 29 IL-2 in serum on days 0, 3, 6, 9, 12 after infection

Table 21 IL-2 in serum on days 0, 3, 6, 9, 12 after infection

Group	IL-2 (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.702 ± 0.140^{ab}	0.529 ± 0.113^{bcd}	0.646 ± 0.102^{a}	0.617 ± 0.106^{b}	0.484± 0.063^{bcd}
Chlotetracycline-fed	0.618 ± 0.062^{cde}	0.754 ± 0.052^{e}	0.517 ± 0.054^{de}	0.665 ± 0.061^{b}	0.642 ± 0.055^{ab}
CMU-FP002-fed	0.641 ± 0.122^{b}	0.612 ± 0.054^{bcd}	0.591 ± 0.027^{ab}	0.659 ± 0.078^{bcd}	0.637 ± 0.174^{bc}

P value < 0.0001

^{a,b,c,d,e} Means within a row with no common superscript differ significantly ($P < 0.05$).

2) IL-2 in intestinal wash

IL-2 in intestinal wash was shown in Figure 30. There were highly significant difference (P value < 0.01) which Probiotics *Lactobacillus plantarum* CMU-FP002 was higher than Chlotetracycline-fed and Control groups (Table 22).

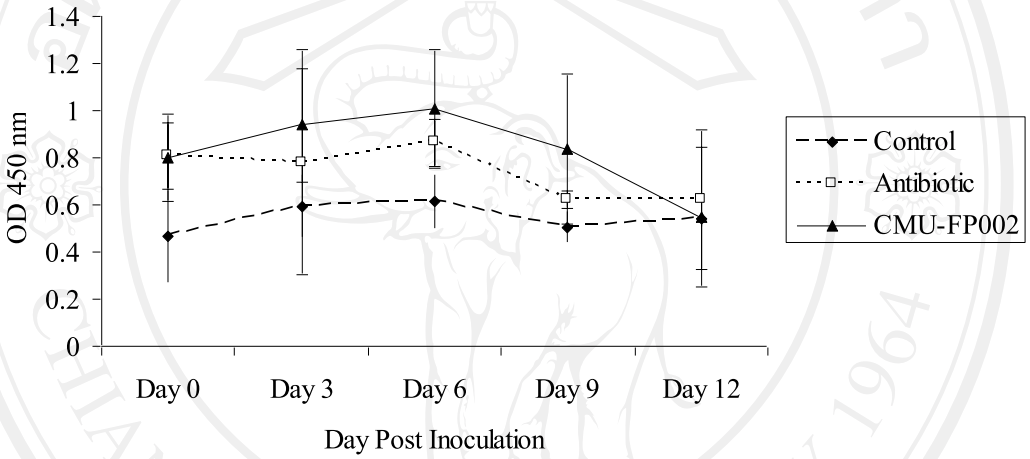


Figure 30 IL-2 in intestinal wash on days 0, 3, 6, 9, 12 after infection

Table 22 IL-2 in intestinal wash on days 0, 3, 6, 9, 12 after infection

Group	IL-2 (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.467 ± 0.201^{d}	0.595 ± 0.137^{d}	0.615 ± 0.116^{abc}	0.506 ± 0.071^{bcd}	0.539± 0.034^{a}
Chlotetracycline-fed	0.809 ± 0.139^{bcd}	0.778 ± 0.478^{cd}	0.863 ± 0.101^{ab}	0.621 ± 0.039^{abc}	0.621 ± 0.295^{ab}
CMU-FP002-fed	0.800 ± 0.182^{b}cd	0.938 ± 0.239^{abc}	1.007 ± 0.255^{bcd}	0.839 ± 0.319^{a}	0.549 ± 0.294^{cd}

P value < 0.0001

^{a,b,c,d} Means within a row with no common superscript differ significantly ($P < 0.05$).

3) IL-2 in lymphocyte supernatant

IL-2 in lymphocyte supernatant with Con A 12.5 mg/ml were shown in Figure 31. There were no significant difference between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 23).

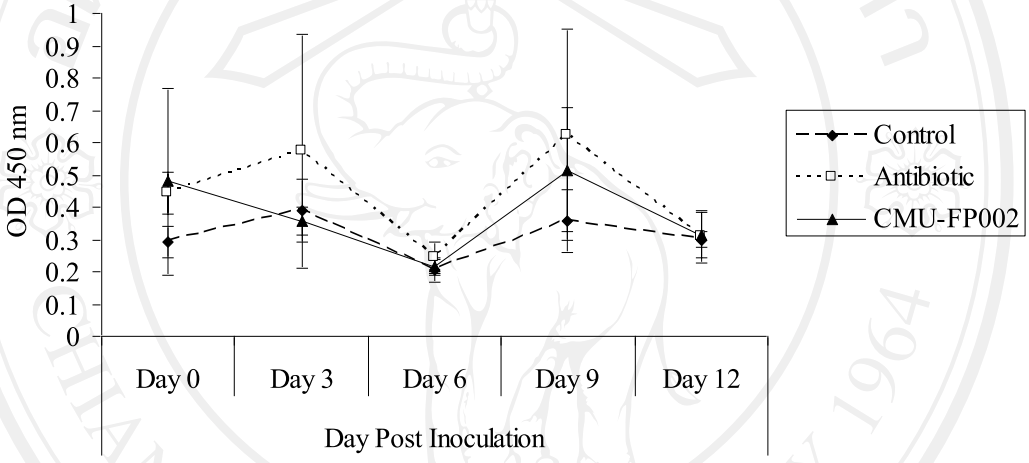


Figure 31 IL-2 in lymphocyte supernatant with Con A 12.5 mg/ml on days 0, 3, 6, 9, 12 after infection

Table 23 IL-2 in lymphocyte supernatant with Con A 12.5 mg/ml on days 0, 3, 6, 9, 12 after infection

Group	IL-2 (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.292 ± 0.050	0.390 ± 0.095	0.204 ± 0.036	0.356 ± 0.098	0.299± 0.023
Chlotetracycline-fed	0.443 ± 0.065	0.574 ± 0.362	0.242 ± 0.049	0.623 ± 0.326	0.308 ± 0.080
CMU-FP002-fed	0.478 ± 0.291	0.354 ± 0.043	0.214 ± 0.026	0.515 ± 0.192	0.312 ± 0.070

P value = 0.0793

IL-2 in lymphocyte supernatant with Con A 25 mg/ml were shown in Figure 32. There were significantly difference (P value< 0.05) between Probiotics *Lactobacillus plantarum* CMU-FP002, Chlotetracycline-fed and Control groups (Table 24).

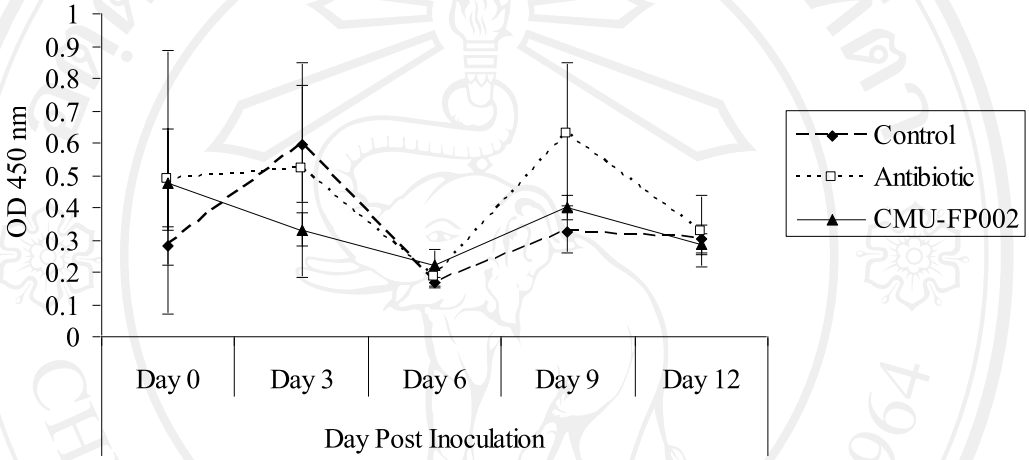


Figure 32 IL-2 in lymphocyte supernatant with Con A 25 mg/ml on days 0, 3, 6, 9, 12 after infection

Table 24 IL-2 in lymphocyte supernatant with Con A 25 mg/ml on days 0, 3, 6, 9, 12 after infection

Group	IL-2 (OD 450 nm)				
	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.280 ± 0.060^{bcd}	0.596 ± 0.181^{ab}	0.166 ± 0.016^{d}	0.323 ± 0.065^{abcd}	0.301± 0.043^{bcd}
Chlotetracycline-fed	0.486 ± 0.156^{abcd}	0.517 ± 0.333^{abc}	0.182 ± 0.025^{d}	0.626 ± 0.223^{a}	0.326 ± 0.110^{abcd}
CMU-FP002-fed	0.478 ± 0.406^{abcd}	0.332 ± 0.052^{abcd}	0.220 ± 0.051^{cd}	0.399 ± 0.037^{abcd}	0.287 ± 0.030^{bcd}

P value = 0.0312

^{a,b,c,d} Means within a row with no common superscript differ significantly ($P < 0.05$).