Chapter 3

Materials and Methods

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

3.1.1 Materials

Equipments

4	Microscope	NIKON
4	Incubator	BINDER
4	Analytical Balance; 4 digits	CP 22 4S/SARTORIUS
4	UV-Vis Spectrophotometer	HP HEWLETT PACKARD
4	Microbiological Cabinet	MARK II/DWYER
4	Hot Plate and Magnetic Stirrer	WSAHS2/WELLAB MODEL
4	Autoclave	HVE-50/HIRAYAMA
4	pH Meter	EUTECH INSTRUMENTS
4	Hot Air Oven	OVER MEMRET

4	Water Bath	MEMRET
4	Pipette and Auto pipette	SOCOREX
4	Loop and Needle	
4	Forceps	
4	Eppendroft Tube	
4	Test Tube Rack	

Glass wares

4	Test Tube	PYREX
4	Petri Dish	PETRIQ
4	Beaker	DURAN
4	Flask	DURAN
4	Slide and Cover Slide	
4	Spreader	
4	Stirring Rod	

Media

4	MRS Broth (de Man Rogasa and Sharpe)	HIMEDIA
4	MRS Agar	HIMEDIA
4	Milk Agar	HIMEDIA

- Starch Agar HIMEDIA
- Bile Salt HIMEDIA
- Tributyrin Agar HIMEDIA
- NA (Nutrient Agar) HIMEDIA
- NB (Nutrient Broth) HIMEDIA
- Brain Heart Infusion Agar HIMEDIA

Chemicals

- NaCl (Sodium Chloride) AJAX
- Bromocresol Purple AJAX
- HCl (Hydrochloric Acid) AJAX
- NaOH (Sodium Hydroxide) AJAX
- Iodine Solution AJAX
- 3 % Hydrogen Peroxide Solution AJAX
- Gram Stain BIOTECH
- Alcohol 95 %

Microorganisms

- *Escherichia coli* ATCC25922
- *Salmonella typhimurium*

All bacteria-tested strains were provided by Faculty of Animal Science and Agricultural Technology, Silpakorn University, Petchabury IT Campus.

3.1.2 Methods

3.1.2.1 Sample collections

Samples were collected by coacal swap from 120 healthy antibiotic-free poultry on November, 2006 until November, 2007 from all over regions in Thailand as shown in Table 1.

3.1.2.2 Isolation of probiotic lactic acid bacteria

Serial dilutions of faeces were plated on Man, Rogasa, and Sharpe (MRS) agar (Himedia, India) with 0.02% bromocresal purple (Ajax, Australia) and incubated at 37°C for 24 hours (De Man et al., 1960). The bacterial colonies, which changed the media color around colony to yellow, were selected, picked and purified on MRS agar plates by streak. All colonies were tested for general morphology by Gram staining and Catalase activity. Each of the Lactic Acid Bacteria (LAB) - purified sample were transferred to MRS slant and stored at 4°C (Reque et al., 2000).

Table 1 Sample source of healthy antibiotic-free poultry

Regions	District and Province	Source
Northern region	Sanpatong, Chiang-mai	3 week - Laying Chicken Commercial breed
	Hangdong, Chiang-mai	40 week - Laying Chicken Commercial breed
	Mae-rim, Chiang-mai	Fighting Cock
North-eastern region	Mueng, Mahasarakharm	Broiler Chicken
Middle region	Mueng, Nakorn Prathom	Laying Duck Khaki Campbell
Southern region	Hua Hin, Prajuab Kirikhun	Broiler Chicken Arber Archer Thai Native Crossbred Chicken

3.1.2.3 Probiotics properties examinations

1) Bile salt tolerance

The tolerance to bile salts was performed on MRS agar plates (Hyronimus et al., 2000). Samples of overnight cultures (20 µl corresponding to 10^6 CFU/ml) were steak onto MRS agar containing bile salts (0.3% w/v) (Biomark, India)

(Gilliland et al., 1984). Plates were incubated at 37°C for 3 days. The growth of bacteria was judged from visual examination.

2) Acid - Base tolerance

From 2.1.1, the strains of LAB Cultures which grown on bile salt plated were selected to determine acid – base tolerance. The tolerance to different pH value (pH 2 – 10) (Conway et al., 1987) was performed in MRS broth. Cultures were grown in MRS broth at 37°C overnight, and sub cultured in 10 ml of MRS broth adjusted to different pH values with hydrochloric acid (1.0 M) and sodium hydroxide (1.0 M). The initial bacterial concentration was checked by turbidity examination.

3) Utilization of protein, starch and fat

LAB strain from 2.1.2 that had acid – base torelance were selected to determine protein, starch and fat utilization. The Protein, Starch and Fat utilization was verified in milk agar (Himedia, India) plate, Starch agar (Himedia, India) and 0.1 % Tributyrin agar (Larpent and Larpent, 1985) and was judged clear zone from visual examination.

4) Antibacterial activity to enteropathogenic bacteria

Antimicrobial activity against pathogenic indicator bacteria, *Escherichia coli* and *Salmonella typhimurium*, were tested by agar spot method

(Raque et al., 2000; Timbuntam et al., 2001). LAB strain from 2.1.3 which had utilization of protein, starch and fat were grown on MRS broth at 37°C, 18 hours, adjusted cell concentration to 10^7 CFU/ml with MRS broth. Spot of 5 microlitres on MRS agar plate were prepared. Each of enteropathogenic bacteria, *Escherichia coli* and *Salmonella typhimurium*, was grown in Brain Heart Infusion (BHI) broth (Himedia, India) for 3 hours, after that adjusted cell concentration to 10^6 CFU/ml in 7 ml. molten (45 – 50 °C) BHI soft agar (0.7% agar). After well mixing, it was over layered onto LAB-spotted plate. After incubated at 37°C, 24 hours, Antibacterial activity was observed by measuring inhibition zone.

5) Identification by 16S-rDNA-sequence analysis

All bacteria strains from 4) were further identified by genomic. 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.

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

3.2.1 Materials

Equipments

4	Microscope	NIKON
4	Incubator	BINDER
4	Analytical Balance; 4 digits	CP 22 4S/SARTORIUS
4	Microbiological Cabinet	MARK II/DWYER
4	Hot Plate and Magnetic Stirrer	WSAHS2/WELLAB MODEL
4	Autoclave	HVE-50/HIRAYAMA
4	pH Meter	EUTECH INSTRUMENTS
4	Hot Air Oven	OVER MEMRET
4	Water Bath	MEMRET
4	Pipette and Auto pipette	SOCOREX
4	V-bottom, 96 well plates	CORNING
4	Loop and Needle	
4	Forceps	
4	Eppendroft Tube	
4	Test Tube Rack	

4 Trays

4 Gutters

4 Chaff

4 Nest

Glass wares

4	Test Tube	PYREX
4	Beaker	DURAN
4	Flask	DURAN

Media

4	MRS Broth (de Man Rogasa and Sharpe)	HIMEDIA

Chemicals

4	NaCl (Sodium Chloride)	AJAX
4	Alcohol 95 %	
4	Newcastle plus Infectious Bronchitis diseases Vaccine	CEVA SANTE ANIMALE
4	1% Chicken Red Blood Cell	
4	1 M Phostphate Buffer Saline	SIGMA

Microorganisms

4 Probiotics Bacteria from 3.2.1

Animals

4 day-old Aber acres chickens

3.2.2 Methods

3.2.2.1 Effect of selected probiotic bacteria on productive performances and humoral immunity in male broilers

A total of 150, day-old Aber acres chickens were randomly assigned to five treatments with three replications as Control (nothing added), Antibiotic (2% Colistin added up in dietary) and Probiotics group 1, 2 and 3 (10^6 CFU/ml of each probiotics added up by oral force feeding) (Jin et al., 1998). Commercial diet was used as basal diet as describe in table 2. Chickens were provided ad libitum access to feed and water. Body weight and feed consume were recorded at the beginning and end of feeding trial at day 38 to further calculated growth performances as average daily gain (ADG), feed intake (FI), feed conversion ratio (FCR) and feed efficiency (FE). Vaccinated with Newcastle plus infectious bronchitis diseases vaccine was given to each chicken in 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).

Table 2 Percentage composition of diets fed to broilers.

Ingredient	Percentage in diets	
	(0-3 week)	(4-6 week)
Protein	≥ 21%	≥ 19%
Fat	≥ 4%	≥ 4%
Fiber	≥ 5%	≥ 5%
Moisture	≥ 13%	≥ 13%

3.2.2.2 Haemagglutination inhibition (HI) test

1) Determination of 4 Haemagglutination units (4 HAU)

According to Shuaib et al. (2006), 1000 doses vial of Newcastle plus Infectious Bronchitis diseases vaccine was suspended in 5 ml of sterile physiological saline solution. 2-fold serial dilution of the vaccine was prepared micro titration plate with 25 σl of sterile physiological saline solution in each well. 25 σl of washed chicken RBC (1%) were added into each well. After gentle mixing, the plate

was left at room temperature for 30 minutes. The highest dilution causing haemagglutination (HA) was taken as one HA unit. The dilution resulted after dividing the one unit dilution of the virus by 4, was prepared with the ratio to that of 4 HA unit.

2) Haemagglutination inhibition test (Grimes, 2002)

Dispense 25 µL of PBS into each well of the plates. Shake each serum sample and dispense 25 µL into the micro well plate. Make two-fold serial dilutions along the row until the second last well from the end. Add 25 µL of the 4HA dilution of antigen to each well. Gently tap the sides of the micro well plates to mix the reagents. Allow to stand for 30 minutes at room temperature. Add 25 µL of 1 percent washed red blood cells to each well. Mix well. Allow to stand at room temperature for 45 minutes. Record the pattern observed in each well.

3.2.3 Statistical Analysis

Data were analyzed in a completely randomized design using the Analysis of Variance and Duncan's New Multiple's Range Test of SAS® (SAS, 2000)

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

3.3.1 Materials

Equipments

4	Microscope	NIKON
4	Incubator	BINDER
4	CO_2 Incubator	BINDER
4	Analytical Balance; 4 digits	CP 22 4S/SARTORIUS
4	Hot Plate and Magnetic Stirrer	WSAHS2/WELLAB MODEL
4	Autoclave	HVE-50/HIRAYAMA
4	Hot Air Oven	OVER MEMRET
4	Water Bath	MEMRET
4	-20 degree Celsius Refrigerator	
4	Pipette and Auto pipette	SOCOREX
4	Automated microtiter plate reader	LABSYSTEM
4	Flat-bottom, 96 well plates	CORNING
4	24-well cell culture plates	CORNING
4	Forceps	
4	Eppendroft Tube	

4 Test Tube Rack

4 Trays

4 Gutters

4 Chaff

4 Nest

Glass wares

4	Test Tube	PYREX
4	Beaker	DURAN
4	Flask	DURAN

Chemicals

4	NaCl (Sodium Chloride)	AJAX
4	Alcohol 95 %	
4	Trypan blue	AJAX
4	Concanavalin (Con) A	BIOCHROME
4	RPMI-1640 complete media	SIGMA
4	Fetal Calf Serum	SIGMA
4	Aprotinin	SIGMA
4	Phenylmethylsulfonyl fluoride	SIGMA
4	Sodium carbonate buffer	SIGMA

- Bovine Serum Albumin (BSA) SIGMA
- Horseradish peroxidase-conjugated goat anti-mouse IgG mAb SIGMA
- Horseradish peroxidase-conjugated rabbit anti-chicken IgG SIGMA
- Tween-20 AJAX
- 3,30, 5,50-tetramethylbenzidine dihydrochloride (TMB) SIGMA
- EDTA AJAX
- Sodium azide AJAX
- Mouse anti-chicken IFN-ν mAbs AbD SEROTEC
- Mouse anti-chicken IL-2 mAbs AbD SEROTEC
- 1 M Phostphate Buffer Saline SIGMA
- 2M Sulfuric acid AJAX

Microorganisms

- *Eimeria tenella* sporurated oocyst
- Probiotics Bacteria from 3.2.1

Eimeria tenella sporulated oocyst was provided by Faculty of Animal Science and Agricultural Technology, Silpakorn University, Petchabury IT Campus.

3.3.2 Methods

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

One hundred and eighty, 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 immunoenhancer probiotics. Body weights and feed consumption will be recorded weekly.

Then 3 – week – old chickens will be selected on mean weight-basis within treatment and transferred to an isolation facility to be challenged with *Eimeria tenella*. Two groups from the control, other two from antibiotic group and another from Probiotic fed group will be inoculated with 20,000 *Eimeria tenella* sporulated oocysts. On day 6, 7, 8, 9, 10 after infection, homogenized fecal material ground was taken, diluted, and counted the oocysts microscopically using Hemacytometer.

Other three week-old chicken were assigned to control, antibiotic and probiotics 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 day 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 supernatant by ELISA will be determined as described in Dalloul et al. (2005). Weight collected caecal tonsil and bursa of fabricius. Collected ceca will be sectioned for histopathology.

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

1) Spleen lymphocyte stimulation

Following the scarification of chicken by cervical dislocation, their spleens immediately harvested, and spleen lymphocytes prepared according to the method outlined by Dalloul et al. (2005). Cell viability was determined to be at R 95% by tryphan blue. The splenocytes were stimulated by incubating 1 ml of single cell suspension (10^7 lymphocytes) with equal volume of zero (negative control), 12.5 or 25.0 mg/ml concanavalin (Con) A in RPMI-1640 complete media supplemented with 10% Fetal Calf Serum (FCS). Each sample was incubated in triplicate wells of 24-well cell culture plates at 40 degree Celsius and 5% CO_2 for 60 hours. Following incubation, supernatants were collected from each well and stored at -20 degree Celsius for later analysis.

2) Collection of sera and intestinal washes

According to the method outlined by Dalloul et al. (2005), on each of 0, 3, 6, 9, and 12 day post infection, chickens were sampled for serum and intestinal washes. For sera, blood samples were obtained from individual birds (3 ml/bird), allowed to clot overnight at 4 degree Celsius, and the sera collected. Following blood sampling, each bird was killed by cervical dislocation and the caecum, infection site of *Eimeria tenella*, was removed quickly, cut longitudinally, and incubated in 10 ml of ice-cold PBS containing 0.05 trypsin inhibitory units/ml of aprotinin, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and 0.02% sodium azide for 4 h on ice. The tissues were then discarded and the washes collected. All intestinal washes and sera were individually clarified by centrifugation and stored at -20 degree Celsius until analysis.

3) Preparation of antigen

According to Talebi et al. (1995), water-soluble oocyst antigen was prepared by homogenising 2 X 10^7/ml purified sporulated oocysts in PBS for 20 min while cooling on ice. The solution was frozen at — 20°C, and then it was thawed and homogenized again for 10 min. This step was repeated twice more in order to break down all parts of the oocysts. The solution was centrifuged in a Sorvall superspeed refrigerated centrifuge using a SM-24 rotor at 5000 *g* for 10 min and the supernatant was collected and stored at -70°C until used.

4) Antibodies, IFN-ν and IL-2 ELISA

Individual samples (each chick) of sera, intestinal washes, and lymphocyte supernatants were tested for IFN-ν, IL-2 levels and for antibodies to a recombinant coccidial antigen using ELISA as described (Dalloul et al., 2005). IFN-ν and IL-2 were quantified using a direct binding ELISA where flat bottom micro titer plates were coated with 100 ml of sample in 100 ml of 0.2M sodium carbonate buffer, for 18 h at 4 degree Celsius, and washed three times with PBS containing 0.05% Tween-20 (PBS-T). Blocking followed using PBS-2% BSA for 1 h at room temperature, and plates washed three times with PBS-T. Mouse anti-chicken IFN-ν or IL-2 mAbs were added to each well (100 ml) and allowed to incubate for 1 h at room temperature on a plate shaker. The plates were washed with PBS-T; 100 ml horseradish peroxidase-conjugated goat anti-mouse IgG mAb were added to each well and allowed to incubate for 30 min at RT with shaking. All plates were washed again and 100 ml of the substrates 3, 30, 5, 50-tetramethylbenzidine dihydrochloride (TMB) and 2M sulfuric acid added, and the optical density (OD) read at 450 nm by an automated microtiter plate reader.

To detect anti-coccidial Ab, microtiter plate wells were coated with 2 mg/well of the *Eimeria tenella* sporulated oocyst coccidial antigen in 100 ml of 0.1 M carbonate buffer, for 18 hours at 4 degree Celsius. The plates were washed, wells blocked with PBS-2% BSA for 1 hour at room temperature, and washed again. Serum and intestinal samples (100 ml) were added and incubated for 1 hour at room temperature with continuous gentle shaking. The wells were again washed three times

with PBS-T, and bound Antibody detected with HRP-conjugated rabbit anti-chicken IgG and TMB as above, and the plates were also read at 450 nm.

3.3.3 Statistical Analysis

Data were analyzed in a completely randomized design using the Analysis of Variance and Duncan's New Multiple's Range Test of SAS® (SAS, 2000)