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THESIS

APPLICATION OF MOLECULAR TECHNIQUE TO MONITOR MICROBIAL CHANGE IN CHICKEN GASTROINTESTINAL TRACT

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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To understand the effect of intestinal microbiota on healthy chicken, pyrosequencer was applied to determine microbial community according to V6-V8 region of 16s rRNA sequences. In ileum, the dominant bacteria were belonged to lactic acid bacteria group including *Lactobacillus, Leuconostoc* and *Weissella*. While high population of those strictly anaerobic group *Lachnospiraceae Incertae Sedis*, *Subdoligranulum* and *Faecalibacterium* were dominant in cecum. During growing state (28 d) and finisher state (42 d), microbial population in each region were similar. However, pyrosequencing was expensive for poultry industry and limited in quantification assay. Optimization of high resolution melting analysis (HRM) for bacterial identification was studied. This technique well distinguished for only two bacterial groups which was not appropriate to analyze the microbial abundance in intestine. The intestinal microbiota was sensitive to various stress treatments. Two exogenous factors of probiotic and high protein content were proposed in this study. The effect of probiotic strain *Lactobacillus reuteri* KUB-AC5 fed on newborn for first week of broiler chicken were analyzed. This strain significantly enhanced population level and species diversity of lactobacilli in ileum at day 42. In addition, the suppression of *Proteobacteria*, including nonbeneficial bacterial groups were observed. Another factor of high crude protein (CP) from soy bean meal and essential amino acid (EAA) affecting gut microflora at 21 d and 35 d were carried out. These enrichments increased the amount of *C. coccoides–E. rectal* group for two times in ileum compared to the control and suppressed the growth of *Campylobacter* group in cecum as well. Moreover the increasing of lactic acid production in high CP treatment was observed. These changes of gut microflora due to the effect of external factors would be helpful for poultry industry in the future.

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Student's signature Thesis Advisor's signature

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Massalin Nakphaichit

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µl	=	microliter
bp	=	base pair
cDNA	=	complementary DNA
CFU/g	=	colony forming unit per gram
cm	=	centimeter
d	=	day
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyribonucleotise triphosphate
etc.	=	et cetetra (and other)
g	=	gram
h	=	hour
HPLC	=	high performance liquid chromatography
M	=	molar
mg/L	=	milligram per liter
min	=	minute
ml	=	milliliter
mM	=	milimolar
mm	=	millimeter
ng	=	nanogram
nm	=	nanometer
pmol	=	picomole
rDNA	=	Ribosomal DNA
rpm	=	revolutions per minite
sec	=	second
w/v	=	weight per volume

APPLICATION OF MOLECULAR TECHNIQUE TO MONITOR MICROBIAL CHANGE IN CHICKEN GASTROINTESTINAL TRACT

INTRODUCTION

Gastrointestinal (GI) tract microbiota are variable over time because of the influence of various factors related to age, diet, and other growth promoters (Apajalahti *et al.*, 2004; Lu *et al.*, 2003; Montesi *et al.*, 2005; Tanaka *et al.*, 2009). The gut microbial community may play an important role in maintaining conditioned microbiota for competitive exclusion and antagonism against pathogens. This microbial community alters metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production. This activity improves feed intake and digestion and stimulates the immune system (Kabir, 2009). According to their function, gut microflora were directly effect on the health of host.

In chicken, microorganisms of GI tracts have been studied by culture-based methods (Salanitro *et al.*, 1978). Nevertheless, it is well recognized that these methods often fail to characterize populations or type of microorganisms which would be anaerobic and strictly anaerobic bacteria because specific media are needed for cultivation (Schabereiter-Gurtner *et al.*, 2001). These limitations have prompted the development of culture-independent techniques of which those based on polymerase chain reaction (PCR). Comparing to conventional methods, molecular methods are generally faster, more specific, more sensitive and more accurate, allowing a precise study of microbial communities (Gong *et al.*, 2002a).

The molecular techniques for detecting diversity of chicken GI tracts microorganism tended to be denaturant gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) techniques (Gong *et al.*,

2002a; Hume *et al.*, 2003; Pedroso *et al.*, 2006). However above-mentioned techniques are labor-intensive and time-consuming. Because they need to run on gel electrophoresis and spend about 4 hour per run. The new techniques, real time PCR and their application, high resolution melting analysis (HRM), were applied to solve this problem. Real time PCR by group specific primer can quantify amount of target bacteria in study condition. In addition, HRM technique can identify microorganism based on Tm value of target genes (Cheng *et al.*, 2006). Moreover, in comparison with other molecular techniques, sequence-based analyses as pyrosequencing technique have the potential for high discrimination for all microbial typing and for the undisputable detection of new subtypes (Ronaghi and Elahi, 2002).

Since the gastrointestinal tract has a huge microbial ecosystem, particular change in the ecosystem might contribute to development of chicken microflora causing healthy or sickness. In this study the effect of two exogenous biological substances, probiotic and high protein concentration, to chicken microbiota and their performance were studies.

In commercial poultry production, probiotic were applied to develop chicken intestinal microbiota instead antibiotic treatment which affect on human health (Bronzwaer *et al.*, 2004; Timmerman *et al.*, 2006). However the role and action of probiotic in GI tract are poorly understood. *Lactobacillus reuteri* KUB-AC5 isolated from chicken intestine in Thailand was tolerant at wide pH of 2-9, 3% bile salt and high temperature to 50°C as well as exhibited high adherence activity. It was able to produce bacteriocin like inhibition substance "KAC5" against both G+ and G- bacteria, especially various serotypes of *Salmonella* (Nitisinprasert *et al.*, 2011). These findings confirmed probiotic property with antagonism function. To confirm its probiotic potential by *in vivo*, the effect of a single application of *Lactobacillus reuteri* KUB-AC5 over 7d at a relatively low dosage of 10^5 cfu/g of feed to broiler chicken performance and gut microflora was carried out. In addition, crude protein (CP) of feed ingredient is one important factor on chicken development. It also had the effect on intestinal microorganism through remain food from host (Dahiya *et al.*, 2005). The

present study aimed to investigate three feed formulars according to protein concentration affecting broiler chicken performance and their gut microorganism.

OBJECTIVES

1. Optimization of HRM technique to determine microorganisms found in chicken intestine.

2. Intestinal microbiota identification in healthy chicken.

3. Effect of exogenous biological compounds to microbial community of chicken intestines.

LITERATURE REVIEW

The microorganism in gastrointestinal tract contained more than hundred of different species (Apajalahti *et al.*, 2004). These bacteria directly effect to the health of host. Therefore, microbial community analysis techniques were an important tool to investigate the microbe in gut system.

1. Microbial community analysis technique

Previously, microbial communities have been studied by culture-based method (Salanitro *et al.*, 1978). However, it is well-recognized that this method often fails to characterize population and some types of microorganism especially in gastrointestinal tract since they are difficult to culture due to special requirements of nutrient and strictly anaerobic condition (Schabereiter-Gurtner *et al.*, 2001; Ward *et al.*, 1990). These limitations have prompted the development of culture-independent techniques based on molecular methods, which has enabled less laborious and less time-consuming approach to see microbial communities (Apajalahti *et al.*, 2004; Gong *et al.*, 2002a; Hume *et al.*, 2003).

Microbial communities in molecular methods can be characterized base on three fundamental properties encompassing diversity, identity and quantity (Justé *et al.*, 2008).

1.1 Microbial diversity analysis

Microbial diversity technique including %G + C profile, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), have been widely adopted for genetic analysis of microbial communities because they provide a relatively comprehensive description of a given community. More in particular, these techniques are extremely suitable to compare

microbial community compositions between different treatments, environments or situations.

1.1.1 % G + C profile

Total microbial communities may be analyzed using technology based on the difference percentage of guanine (G) and cytosine (C) found within the bacterial chromosomal DNA extracted (Apajalahti *et al.*, 2004). As the proportion of G and C differ between bacterial genera, analysis of the percentage can be used to create a bacterial profile. DNA is extracted from the community of interest using a method that involves several rounds of high speed centrifugation and washing step to extract maximum amounts of bacterial cells. Following lysis, the DNA is purified with several rounds of caesium cholide-ethidium bromide equilibrium-gradient centrifugation. The resulting highly purified DNA is subjected to a caesium chloride-bisbenzimidazole gradient. Bisbenzimidazole is a DNA-binding dye that specifically binds adenine (A) and thymidine (T). Exposure to a bisbenzimidine gradient allows the fractionation of DNA dependent on its AT/GC content. When compared to gradients of known GC content, the GC content of the sample community can be estimated. Apajalahti *et al.* (2004) studies the effect of grain base diets to chicken microbiota using G+C analysis. They found that wheat and corn favored different bacteria group in cecum. However this analysis could not reveal the identity of bacteria. Their result suggested that possible corn favored low G+C bacterial group of clostridia, enterococci and/or lactobacilli while wheat favored high G+C bacterial group of bifidobacteria.

Although G+C analysis is an effective method for determining a bacterial profile from an unknown community, it provides only an overall snapshot of the bacterial community. However no sequence data can be easily obtained from this analysis, so if more detailed information is required regarding the community of interest, more specific techniques must be used.

1.1.2 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products. The polymerase chain reaction of environmental DNA can generate templates of different DNA sequence that represent many of the microbial organisms. However, since PCR products from a given reaction are similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences resulting in differential denaturing characteristics of the DNA. During DGGE, PCR products encounter increasingly higher concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Different sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands. Each band theoretically represents a different bacterial population presenting in the community.

DGGE is a popular technique for studying microbial community in chicken. Hume *et al.* (2003) monitored biodiversity in cecum of Leghorn chicks according to their development. Base on DGGE profile, their biodiversity was divided into three main groups, corresponding to chicks at 2 d of age, 5 – 20 d of age and 23 – 32 d of age. DGGE technique used to investigate bacteria community in broiler chicken treated by antibiotics (Pedroso *et al.*, 2006). The bacterial profile and their performance were analyzed. They found that the change of intestinal bacteria profile induction by antibiotic may be related to improvement in growth performance. The suppression of 6 amplicons and presence of 4 amplicons in enramycin treated revealed the best performance in their experiment.

Although, DGGE technique could determine biodiversity base on different patterns of DNA bands. In practically, visualization of some bands might

have been obscured by band comigration. In spite of having different G-C content and primary sequencing, multiple amplicons may migrate to the same denaturant level resulting in an inaccurate indication of genotypic diversity and abundance (Hume *et al.*, 2003). In addition, this technique could not indicate microbial in genus or species level. The PCR cloning and sequencing techniques were required.

1.1.3 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a molecular profiling tool based on the size of restriction fragments from a PCR amplified marker (Terence L, 1999). Briefly, universal or taxon-specific PCR primers are designed for the amplification of the gene of interest. This target gene has traditionally been 16S rRNA due to the wealth of sequence information available, however, any other suitable target genes may be used as well (Terence L, 1999). One PCR primer has a fluorescent molecule coupled to the 5' nucleotide resulting in PCR products fluorescently labelled at one terminus. The labelled PCR amplicons are cleaved with selected restriction endonucleases, resulting in the production of fragments of various lengths depending on product sequence and enzyme specificity. The fragments are separated on a polyacrylamide gel and visualized by ultraviolet excitation or an automated DNA sequencer can be used to provide a fluorescence pattern equivalent to fragment nucleotide length (Saikaly *et al.*, 2005).

The advent of T-RFLP emerged after the innovation of DGGE and TGGE (Temperature gradient gel electrophoresis). However, the use of T-RFLP is becoming more common, perhaps due to several key advantages of the technology (Marsh, 1999). Primarily, sequence data can be generated for the unique terminal restriction fragments (T-RFs), allowing the reference of sequence databases (http://www.trefid.net/ - Rosch and Bothe (2005)). Thus, the unique T-RFs obtained from a digestion can be directly compared with the database in order to obtain phylogenetic information. Secondly, T-RFLP has greater resolution ability than DGGE and TGGE (Terence L, 1999). Lastly, the data of T-RFLP is immediately analyze from gel and resulted digital data.

T-RFLP has been used to assess bacterial diversity in several different microbial communities. Gong *et al.* (2002) characterize diversity and phylogenetic of bacteria in the mucosa of chicken cecum comparison with bacteria in the cecum lumen using T-RFLP and cloning libraries. T-RFLP analysis revealed some pattern difference between mucosa and cecum lumen. The 197 bp fragment generated from *HhaI* was found only in lumen. However most of bacteria were similar. *Fusobacterium prausnitzii* and butyrate-producing bacteria were the largest group in cecum. In addition, T-RFLP was applied to chicken microflora response to dietary composition (Torok *et al.*, 2008). According to T-RFLP profile, microbial diversity in control diet was significant different from exogenous enzyme treated diet in ileum and cecum but it was similar in duodenum and jejunum. The dissimilarity in bacteria community between two diets in ileum and cecum was 73% and 66% of total bacteria, respectively.

1.2 Microbial identification

Some studies require not only the microbial diversity, but also precise identification of the key microorganisms such as discrimination of disease (Amit-Romach *et al.*, 2004) and antibiotic producing bacteria in feed studies (Wise and Siragusa, 2007). The microbial identification techniques were such as clone libraries, pyrosequncing, fluorescence in situ hybridization and high resolution melting analysis.

1.2.1 Clone libraries technique

In order to identify DNA signals obtained with the common community profiling techniques for gel-based approaches such as DGGE, TGGE, the DNA bands can be excised from gels, cloned and sequenced (Lafarge *et al.*, 2004). Alternatively, the PCR-amplified sequences can be directly cloned and sequenced, allowing species identification of individual community members by comparing to the information from Genbank. Moreover, Gong *et al.* (2007) investigated microbial communities in chicken gastrointestinal tracts (GI) from crop to cecum using random cloning library. The 16s rRNA gene from the genomic DNA sample were amplified,

cloned into cloning vector and sequenced. The microbiota mainly were gram-positive bacteria along the GI tract. They found that *Clostridium* was dominant in cecum whereas *Lactobacilli* were predominant in upper GI tract. *L. aviaries* and *L. salivari*us were predominant species among lactobacilli. However this technique is tedious and time-consuming due to the large number of samples that have to be analyzed.

1.2.2 Pyrosequencing by Genome Sequencer

Pyrosequencing is a DNA sequencing technology based on real-time detection of DNA synthesis monitored by bioluminescence. This technique is not based on Sanger DNA technology (dideoxy chain termination technology). It dispenses with the need for labeled primers, labeled nucleotides, and gel electrophoresis. In addition, pyrosequencing eliminates the need of cloning, thus removing the potential for both production of aberrant recombinants and cloning-related artifacts (Speksnijder *et al.*, 2001).

The 4 enzymes included in the pyrosequencing system are the klenow fragment of DNA polymerase I (Klenow *et al.*, 1971), ATP sulfurylase (Segel *et al.*, 1987), luciferase (Deluca, 2006) and apyrase (Komoszynski and Wojtczak, 1996). The reaction mixture contains the substrates of adenosine phosphosulfate (APS), D-luciferin and the sequencing template with an annealed primer to be used as starting material for the DNA polymerase. The four nucleotides are added one at a time, iteratively, in a cyclic manner and a CCD camera detects the light produced (Ahmadian *et al.*, 2006).

The enzymatic reactions exploited in the pyrosequencing technology are the following. The first reaction, the DNA polymerization, occurs if the added nucleotide forms a base pair with the sequencing template and thereby is incorporated into the growing DNA strand.

$$(DNA)_n + dNTP \longrightarrow (DNA)_{n+1} + PPi \text{ (Polymerase)} \quad (1)$$

The inorganic pyrophosphate, PPi, released by the klenow DNA polymerase serves as substrate for ATP sulfurylase, which produces ATP as equation 2:

$$PPi + APS \longrightarrow ATP + SO_4^{2-} \text{ (ATP Sulfurylase)} \quad (2)$$

Through the third and fourth reactions, the ATP is converted to light by luciferase and the light signal is detected. Hence, only if the correct nucleotide is added to the reaction mixture, light is produced.

$$\text{Luciferase + D-luciferin + ATP} \longrightarrow \text{Luciferease-luciferin-AMP + PPi} \quad (3)$$

$$\text{Luciferase-luciferin-AMP} + O_2 \longrightarrow \text{Luciferase+oxyluceferin+AMP+}CO_2\text{+ Light} \quad (4)$$

Apyrase removes unincorporated nucleotides and ATP between the additions of different bases as equation 5 and 6.

$$ATP \longrightarrow AMP + 2Pi \text{ (Apayrase)} \quad (5)$$

$$dNTP \longrightarrow dNMP + 2Pi \text{ (Apayrase)} \quad (6)$$

Currently, pyrosequncing has been used extensively in microbial community analysis such as in soil (Roesch *et al.*, 2007), chicken's cecum (Callaway *et al.*, 2009), human fecal (Nakayama, 2010), fermentation food (Sakamoto *et al.*, 2011) and mine (Edwards *et al.*, 2006). This technology allows to analyse a high number of clonal 16S rRNA genes in the PCR amplicon batch obtained from bacterial community and provides precise information on the relative population of each bacterium.

1.2.3 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is another technique applied for bacterial identification. It combines the simplicity of microscopy observation and the specificity of DNA hybridization (DeLong *et al.*, 1989). FISH is based on the hybridization of labeled DNA probes to taxon-specific regions of the bacterial ribosomes and can be detected by fluorescence microscopy or flow cytometry (Amann *et al.*, 2001; Wallner *et al.*, 1993). FISH help to understand the ecology of complex microbial communities. It is widely used in environmental microbiology (Aminov *et al.*, 2006; Maszenan *et al.*, 2000). This technology reveals the morphology of the target organisms and how abundant they are in a given environment.

Although in theory, FISH could detect single cells, in practice, however, the detection level is often 10^3 cells per ml, rendering this technique in general less sensitive than PCR-based techniques (Hogardt *et al.*, 2000; Moreno *et al.*, 2003; Poppert *et al.*, 2005). Another limitation is the insufficient automation for high sample throughput (Amann *et al.*, 2001). Furthermore, a limited number of probes can be applied in one hybridization run.

1.2.4 High resolution melting analysis (HRM)

High resolution melting (HRM) analysis is one application in real-time PCR after PCR amplification step. It is used to characterize DNA samples according to their dissociation behavior as they transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperature (Figure 1).

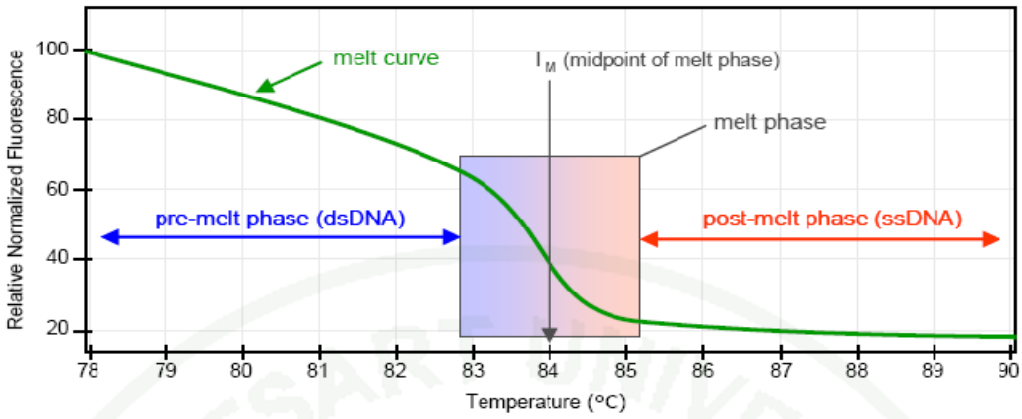

Figure 1 Fundamentals of a typical HRM (high resolution melt) plot. The melt curve plots the transition from high fluorescence of the initial pre-melt phase through the sharp fluorescence decrease of the melt phase to basal fluorescence at the post-melt phase. Fluorescence decreases as DNA intercalating dye is released from double-stranded DNA as it dissociates (melts) into single strands. The midpoint of the melt phase, at which the rate of change in fluorescence is greatest, defines the temperature of melting (T_M) of the particular DNA fragment under analysis.

Source: HRM assay design and analysis Corprotocol™ 6000-1 July 06.

The HRM analysis has been used for detecting sequence variant for genotyping (Wittwer *et al.*, 2003). The targets species-specific genes such as the 16S rRNA, 23S rRNA and 16S-23S rRNA were amplified and identified according to individual Tm value (melting temperature). Cheng *et al.* (2006) identified nine clinically bacteria using HRM technique. Some of clinical bacteria could be separated via the shift of melting plot during heteroduplex formation with a PCR amplicon of a reference bacterial species. Whereas bacteria that show same sequence of PCR region had to do second PCR with more specific primer. Moreover HRM analysis was used to classify *Mycoplasma synoviae* comparison with single strand conformation polymorphism (SSCP) in strain level (Jeffery *et al.*, 2007). Both techniques were capable to detect 1 bp variation in PCR products of approximately 400 bp. Two

exceptional advantages of SSCP are its very high resolving capacity and dispensed nucleotide sequence analysis. This option is not possible for HRM curve analysis. However, both technique of nucleotide sequencing and SSCP are time-consuming procedures and require skill for interpretation of results. In contrast, the HRM curve analysis is rapid and convenient, and all relevant procedures including PCR and melting-curve analysis can be performed in a single tube. An additional advantage of HRM curve analysis is that it can be performed in an automated module, obviating the need for extensive interpretation of results. Furthermore, with each unknown specimen, a library of prototype profiles can be used to facilitate identity of a profile. Jeffery *et al.*(2007) has shown that such a library of prototype PCR products can be reused several times without detectable variation in the melting-curve temperature.

1.3 Microbial quantification

The development of real-time PCR has allowed for the development of detection and quantification assays for various microbial species. It is based on the periodic monitoring of the change in fluorescence as an indicator of product generation during the exponential amplification phase of the PCR (Heid *et al.*, 1996; Suzuki and Giovannoni, 1996). Several chemistries have been developed to permit fluorescence detection of PCR product accumulation including for example, SYBR Green (Molecular Probes Inc. Oregon, USA), Taqman (Heid *et al.*, 1996) and Molecular Beacons (Tyagi and Kramer, 1996). The use of primers specific for microbial species or groups in real-time PCR has proven to be a sensitive method for quantification specific bacterial group in various communities (Dumonceaux *et al.*, 2006; Fortin *et al.*, 2001; Huijsdens *et al.*, 2002; Kosters *et al.*, 2001; Nadkarni *et al.*, 2002).

Quantitative PCR (qPCR) involves the use of standardized samples that contain known numbers of genetic copies. Standard templates can be a plasmid carrying a target gene, PCR product, genomic DNA, cDNA etc. Standard curve is generated by plotting the log of the initial template copy number against the threshold cycle (Ct values) which is a few cycles number that reach a point of the fluorescence

signal first recorded as statistically significant above background. The standard curve should be linear over the whole concentration range covering sample concentrations. The linearity is denoted by the R squared (R^2) value and should be very close to 1. The linear standard curve implies that efficiency of amplification is consistent at various template concentrations. One hundred percent efficiency implies perfect doubling at each cycle (Songjinda, 2007).

Wise and Siragusa (2006) developed q-PCR to enumerate the presence of *Clostridium perfingens* in the broiler chickens gastrointestinal tract. Primers and Taq-man probe were decided from 16s rRNA of *Clostridium* cluster I. The assay could detected 50 fg of *C. perfringen* Genomic DNA or approximately 20 cell in pure culture. However the assay sensitivity was decreased when it applied to quantify *C. perfingens* spiking in ileum and cecum at about 10^2 CFU/g and 10^4 CFU/g, respectively. Consequently, the present of unidentified chemical inhibited in DNA amplification. In addition, Wise and Siragusa (2007) studied the effect of conventional diets and antibiotic-free vegetable-based diets on 13 groups of intestinal bacteria in broiler chicken using q-PCR analysis. They found that the major difference between two types of diet occurred in ileum. However, the antibiotic growth promotants (AGPs) in conventional diets may be important to control *Campylobacter* colonization in cecum. Dumonceaux *et al. (*2006) examined several methods to enumerate various bacterial populations via a *Chaperonin*-60 (*cpn60*) based qPCR protocol and found that the most efficient standard curve was created with a serial dilution of vectors containing a fragment of *cpn60* of the species in question. Amplification of these defined standards results in the production of a reliable standard curve, allowing for extrapolation of the quantity of the bacteria in unknown samples.

The use of qPCR in microbial characterization has many advantages. Many samples can be analyzed at one time and the results are obtained in a timely fashion with relatively low labor cost. However, the equipment required and reagents used are expensive. The presence of PCR inhibitors and fluorescence quenchers extracted in environmental samples may also affect accuracy (Dumonceaux *et al.*, 2006). In addition, previous knowledge of the nucleotide sequence of the bacterial

species is in question required to develop functional primer and probe sets. Thus, the target organism must be selected before analysis can begin. qPCR is an excellent method when one is interested in a small number of specific groups; however, both extensive cost and the requirement for previous knowledge of target organism sequence prohibit this method as a widely used.

2. Chicken

Chicken are an important meat production for human. The production has grown more quickly from 1990 – 2010 during 50 years (Figure 2). Therefore manipulation of chicken production in industries are required such as diet development, environment or chicken's genetic. The important ways to manipulate broiler production efficiency are the understanding and management of gut microflora. Consequently, it is well recognized that the intestinal microbial had a direct impact on the growth, health and welfare of poultry.

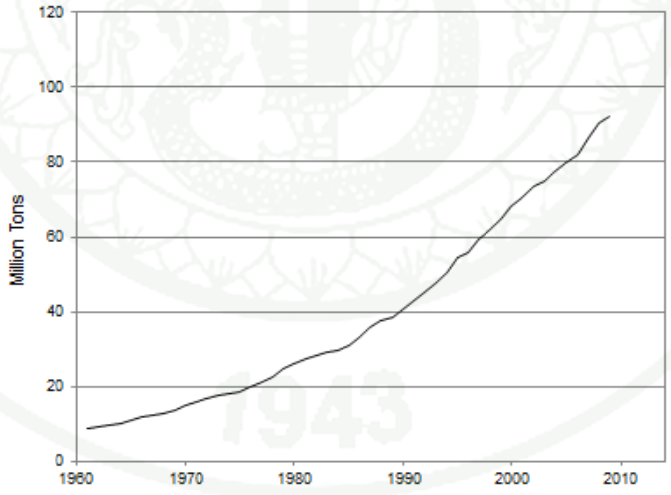

Figure 2 World poultry production from 1961-2009.

Source: Earth Policy Instiute (2011)

2.1 Gastrointestinal tract (GI) microorganism

Different gastrointestinal (GI) tract regions of chickens play different roles in feed digestion, nutrient absorption and intestinal health (Figure 3). These are relative to the health of animal. The crop, proventriculus, gizzard and duodenum of chicken have major functions in feed digestion. The jejunum and ileum is a principle site of nutrient absorption. And extensive fermentation occurred in cecum resulting in further nutrient absorption, detoxification of harmful substance and prevention of pathogen colonization (Gong *et al.*, 2007). The major group of chicken GI tract microorganism belong to gram positive bacteria and mainly include facultative anaerobes from the crop to the terminal ileum, while in cecum the dominant bacteria was strictly anaerobe (Gabriel *et al.*, 2006).

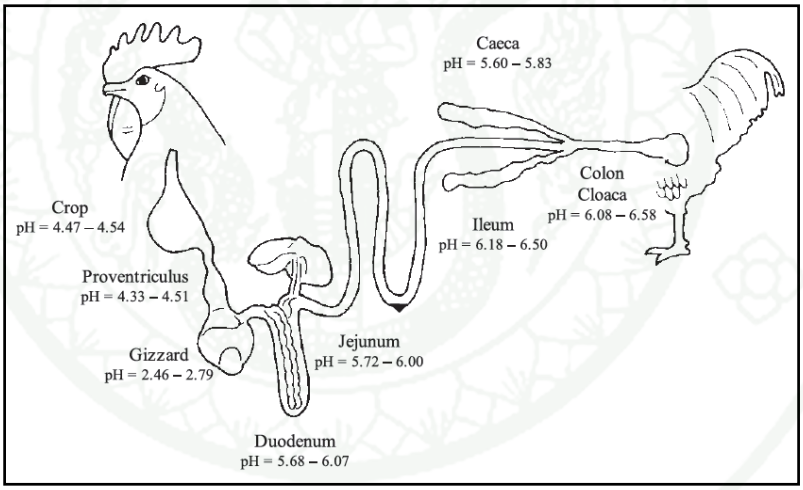

Figure 3 Chicken gastrointestinal tract.

Source: Gabriel *et al.* (2006)

Microbial communities collections recovered from duodenum, jejunum, ileum and cecum were revealed in Table 1 (Dumonceaux *et al.*, 2006; Gong *et al.*, 2002b; Gong *et al.*, 2002a; Gong *et al.*, 2007; Lu *et al.*, 2003; Walter *et al.*, 2001; Zhu

et al., 2002) *Actinobacterium* sp., *Bacillus* sp., *Clostridium* sp., *Enterococcus* sp., *Lactobacillus* sp. and *Streptococcus* sp. are found in all regions. However, bacterial communities in each region of chicken GI tract are different according to their mainly function and circumstance.

Table 1 Overview of microbial communities in duodenum, jejunum, ileum and cecum of 2-7 weeks broiler chickens.

Location	**Bacteria abundance**
Duodenum	*Actinobacterium sp., Bacillus sp., Clostridium sp., Enterococcus sp., Lactobacillus sp., Pediococcus sp., Propionibacterium sp., Staphylococcus sp.,Streptococcus sp., Escherichia coli, Fusobacterium sp., Gemmiger sp.*
Jejunum	*Actinobacterium sp., Arthrobacter sp., Bacillus sp., Clostridium sp., Enterococcus sp., Eubacterium sp., Lactobacillus sp., Macrococcus sp., Pediococcus sp., Ruminococcus sp., Staphylococcus sp.,Streptococcus sp.,Weisella sp., Pseudomonas sp.*
Ileum	*Actinobacterium sp., Bacillus sp., Clostridium sp., Enterococcus sp.,Globicatella sp., Lactobacillus sp., Macrococcus sp., Pediococcus sp., Ruminococcus sp., Staphylococcus sp., Streptococcus sp., Bacteriod sp., Escherichia coli, Fusobacterium sp.*
Cecum	*Actinobacterium sp., Bacillus sp., Clostridium sp., Enterococcus sp., Eubacterium sp., Lactobacillus sp., Megamonas sp., Peptococcus sp., Ruminococcus sp., Sporobacter sp., Sporomusa sp., Sporosarcina sp., Streptococcus sp., Subdoligranulum sp., Weisella sp., Bacteriod sp., Bilophila sp., Escherichia coli, Fusobacterium sp., Prevotella sp., Pseudobutyrivibrio sp.*

2.2 Factors effecting to GI tract microorganism

The bacterial compositions in poultry digestive tract are sensitive to both internal and external stresses treatment.

2.2.1 Chicken age

Hume *et al.* (2003) characterized bacterial community in jejunum, ileum and cecum from young Leghorn chicks during chick development. They observed that large numbers of anaerobic bacteria capable of decomposing uric acid comprise the cecal flora of chicks 3 to 6 hour after hatching. During the first 2 to 4 days postthatch, *Streptococci* and *Enterobacteria* colonize the small intestine and cecum. After the first week, *Lactobacillus* predominates in the small intestine, and the cecum is colonized mainly by anaerobes (*Escherichia coli* and *Bacteroides*) with lower numbers of facultative aerobes (Mead and Adams, 1975). A typical microflora of adult birds in the small intestine is established within 2 weeks; however, it was found that the adult cecal flora, which was mainly obligate anaerobes, took up to 30 day to develop, *Bifidobacteria* and *Bacteroides* as predominate bacteria (Barnes *et al.*, 1972).

2.2.2 Feed composition

Apajalahti *et al.* (2004) studies the effect of wheat and corn based diet on microbial profile in cecum. Their analysis found that microbe in cecum favored different of diet sources. The % G + C analysis revealed that low % G + C microbes (20% - 34%) favored corn based diet whereas high % G + C (65% -69%) favored wheat based diet. The effect of organic acid supplement on intestinal microorganism in chicken was characterized using molecular based method (Nava *et al.*, 2009). They found that organic acid blend DL-2-hydroxy-4(methylthio) butanoic acid, formic and propionic acid induced total bacteria and *Lactobacillus* population colonization in ileum.

In poultry feed, the crude protein (CP) is a main component for body proteins metabolism which are a major impact on chicken broiler in term of growth rate, body weight gain and body consumption (Malheiros *et al.,* 2003). These amino acids are absorbed and metabolized to body proteins. There are 22 amino acids in protein complex which can divide in two groups, essential and non-essential amino

acid. Essential amino acids are amino acid which poultry cannot synthesize and required for meat metabolic production. In poultry industry, the methionine and lysine are the first and second limiting amino acid. Moreover tryptophan and threonine will likely be used more frequently in supplement source. The management of both protein and essential amino acids supplements is an appropriate way to increase the efficiency of protein utilization. Microflora utilizes CP remains in intestine by proteolysis process and generated smaller peptides and amino acid. These products can be either assimilated directly into microbial protein or fermented with the production of ammonia and volatile fatty acid (Macfarlane *et al.*, 1986). Therefore CP has a great influence on microbial ecology in chicken intestine. Two sources of CP in poultry feed used on animal and crop source. Animal source were such as meat meal, feather meal and fish meal. However animal source used for poultry production had limitation because of some effects on broiler health. Meat meal is currently not used in Europe because the problem of Bovine Spongiform Encephalopathy (BSE) contamination had occurred (Leeson and Summers, 2009). Moreover, high-protein diet particularly from fish meal contributed incidence of necrotic enteritis (NE) by *Clostriduim perfringens* in broiler chicken. Dahiya *et al.* (2005) found that high glycine component in fish meal based diet correlated the causative agent of NE. According to these problem, the protein from crop especially soy bean meal is frequently used as crude protein in poultry feed formulations (Palliyeguru *et al.*, 2010). Nevertheless the little is known about the effect of CP from soy bean meal on intestinal microbial in broiler. In addition, percent of CP ingredient directly effecting on feed value need to be carefully concerned because it is a major content in diet ingredient. Therefore the CP quantity had to optimize for poultry utilization efficiency. Jiang *et al.* (2005) tried to optimize feed formula by reducing CP level and supplementing with glycine. These data found that broiler performance in low CP were similar to normal CP.

2.2.3 Chicken growth promoter

In commercial poultry production, the development of chicken intestinal microbiota may be altered by modern practices, such as facility hygiene, routine medication, artificial egg incubation, hatching and chick rearing (Apajalahti *et*

al., 2004). These practices may improve susceptibility to bacterial pathogen colonization. The prophylactic use of many growth promoters, such as dietary antibiotics, has been commonly practiced in commercial poultry production for many years. This practice potentially affects human health due to drug residues and the emergence of antibiotic-resistant strains, which may cause difficulty in the treatment of a variety of bacterial infections. Therefore, the European Union banned the use of such growth promoters in food animal production in 2001 (Bronzwaer *et al.*, 2004). Consequently, probiotics available in a microbial dietary supplement that beneficially affect the host through effects in the intestinal tract have received significant attention in the research community worldwide.

The definition of probiotic by FAO/WHO is “live microorganisms which when administered in adequate amounts confer a health benefit on the host". In broiler, probiotic species belong to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces*. The probiotic has an impact on host through modification of microflora. The mode of action of probiotics in poultry includes: (i) maintaining normal intestinal microflora by competitive exclusion and antagonism ; (ii) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production ; (iii) improving feed intake and digestion ; and (iv) stimulating the immune system (Kabir, 2009). Willis and Reid (2008) found that commercial probiotic contained *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium thermophilus* and *Enterococcus faecium* reduced amount of *Campylobacter jejuni* causing foodborne illness in broiler chicken. Two probiotic strain, *Lactobacillus salivarius* and *Lactobacillus agilis*, contributed to increase abundant of *Lactobacillus* species in jejunum and cecum of chicken under heat stress condition (Lan *et al.*, 2004). This finding suggested that these *Lactobacillus* strain restored the microbial balance and maintained the natural stability of microbiota under stress condition.

In addition, probiotic supplementation by either single or multiple strains in combination may effect on poultry growth performance with either positive or negative results. For example, Timmerman *et al.* (2006) reported that probiotic

Lactobacillus species could reduce mortality of broilers and promote their growth. While commercial probiotics containing *Lactobacillus acidophilus, Saccharomyces cerevisae* sc-47 and *Saccharomyces boulardii* applied instead of antibiotics in two strains of cockerels had no effect on their growth (Fatufe and Matanmi, 2008). Several factors such as duration of treatment, diet type and age of birds account for this effect. Little benefit is expected from a provision of organisms already present in the flora. The inability to determine which probiotics are capable of improving the flora is a problem that arises due to a lack of knowledge on the microbial community structure of the ideal biota. The role and action of individual microbial species or groups present in the GI tract are also poorly understood.

Nitisinprasert *et al.* (2000) has primary screened for 256 lactic acid bacteria isolates producing antibacterial substances against both pathogens, *Escherichia coli* and *Salmonella* sp. resistant to antibiotic used in Thailand. One of them, the isolate KUB-AC5, was tolerant at wide pH of 2-9, 3% bile salt and high temperature to 50°C as well as exhibited high adherence activity comparing to the commercial LAB. It was able to produce bacteriocin like inhibition substance "KAC5" against both G+ and G- bacteria, especially various serotypes of *Salmonella* (Nitisinprasert *et al.*, 2011). According to the results of morphology, physiology, biochemistry and molecular basis, this strain was identified as *Lactobacillus reuteri*. These findings showed that *L. reuteri* KUB-AC5 has probiotic properties with antagonism function. However, its probiotic potential has not been studied by *in vivo* yet.

2.3 Short-chain fatty acid (SCFA) production in intestine

Short-chain fatty acid (SCFA), such as acetic acid, propionic acid, butyric acid and lactic acid are the major end product of bacterial metabolism in an intestine. They are formed principally from polysaccharide, oligosaccharide, protein, peptide and glycoprotein precursors by anaerobic bacteria (Macfarlane and Macfarlane, 2003; Titus and Ahearn, 1988). The intestinal microflora or lumen fermentation and their end products represent in Table 2.

Table 2 Fermentation properties and their product of ruminal bacteria.

Species	Function[a]	Product[b]
Fibrobacter (Bacteroides) succinogenes	C,A	F,A,S
Ruminococcus albus	C,X	F,A,E,H,C
Ruminococcus flavefaciens	C,X	F,A,S,H
Butyrivibrio fibrisolvens	C,X,PR	F,A,L,B,E,H,C
Clostridium lochheadii	C,PR	F,A,B,E,H,C
Streptococcus bovis	A,S,SS,PR	L,A,F
Ruminobacter (Bacteroides) amylophilus	A,P,PR	F,A,S
Prevotella (Bacteroides) ruminocola	A,X,P,P	F,A,P,S
Succinimonas amylolytica	A,D	A,S
Selenomonas ruminantium	A,SS,GU,LU,PR	A,L,P,H,C
Lachnospira multiparus	P,PR,A	F,A,E,L,H,C
Succinivibrio dextrinosolvens	P,D	F,A,L,S
Methanobrevibacter ruminantium	M,HU	M
Methanosarcina barkeri	M,HU	MC
Treponema bryantii	P,SS	F,A,L,S,E
Megasphaera elsdenii	SS,LU	A,P,B,V,CP,H,C
Lactobacillus sp.	SS	L
Anaerovibrio lipolytica	L,GU	A,P,S
Eubacterium ruminantium	SS	F,A,B,C
Oxalobacter formigenes	HU	F,C
Wolinella succinogenes	HU	S,C

[a]C = cellulolytic; X = xylanolytic; A = amylolytic; D = dextrinolytic; P = pectinoiytic; PR = proteolytic; L = lipolytic; M = methanogenic; GU = glycerol-utilizing; LU = lactate-utilizing; SS = major soluble sugar fermenter, HU = hydrogen utilizer; O = oxalate-degrading.

[b]F = formate; A = acetate; E = ethanol; P = propionate; L = lactate; B = butyrate; S = succinate; V = valerate; CP = caproate; H = hydrogen; C = carbon dioxide; M = methane.

Source: Chiba (2009)

The production of SCFA by intestinal bacteria is correlated with diet composition in host intestine. Figure 4 represented the main route of substrates fermentation in intestine. The metabolism of major intestinal bacteria involve in the glycolytic pathway to derive energy from carbohydrate, which are initially converted to pyruvate and acetyly-CoA. These metabolites are key control point in fermentative metabolism, which can be converted into a wide range of products such as propionate, acetate, butyrate or lactate (Macfarlane and Macfarlane, 2003). For protein catabolism, the enzyme proteinase can digest protein to amino acid. This molecule directs to citric acid cycle and converts to SCFA. The lipid was digested by lipase to glycerol and fatty acid form. The glycerol can convert to glyceraldehyde-3-phospate in glycolysis pathway. The fatty acid can direct to citric acid cycle to produce SCFA. After fermentation, protonated form of SCFA are transported through the intestinal epithelial membrane. Amount of energy obtained from these SCFA is too low for all energy requirements of poultry (Józefiak *et al.*, 2004). However SCFA are necessary for energy sources of colonocytes, prevention of diarrhea, defense against pathogens colonization and pH control within gastrointestinal tract (Meimandipour *et al.*, 2010).

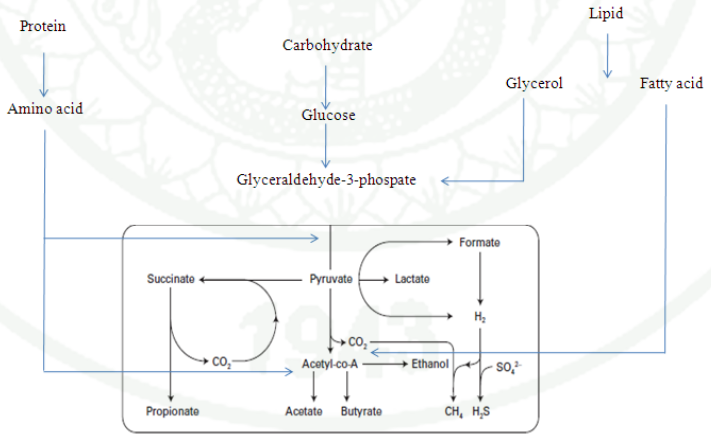

Figure 4 Simplified diagram of carbohydrate, protein and lipid breakdown and main route of fermentation in intestine.

Source: Modified of Macfarlane and Macfarlane (2003)

Comparing to other areas of GI tract, the highest concentration of SCFA are from the cecum according to microbial fermentation (Józefiak *et al.*, 2004). Van Der Wielen *et al.* (2000) investigate SCFA in cecum of broiler chicken affecting on intestinal bacteria during development. High concentration of acetate, propionate and butyrate increased from the age of 1 day to 15 day and then their concentrations were stable. On the other hand, a number of *Enterobacteriaceae* decreased at grower stage. This finding suggested that SCFA response to reduction of *Enterobacteriaceae* in chicken cecum during growth. Meimandipour *et al.* (2010) studied SFCA production induced by *Lactobacillus* supplement in chicken cecum. The resulted showed that lactate produced by *Lactobacillus* in cecum improved the growth of butyric producers, which significantly increased butyrate accumulation. Moreover this butyric acid could inhibit *Samonella* growth in cecum.

MATERIALS AND METHODS

1. Microorganism and culture condition

The bacteria and culture condition used in this study was shown in Table 3.

Table 3 Bacteria and culture condition used in this study.

Name	Medium	Culture condition	Purpose
Lactobacillus crispatus JCM 5810	deMan, Rogosa and Sharpe, MRS (Merck, Darmstadt, Germany)	37°C for 12-15 h.	HRM analysis
Lactobacillus salivarius AC21	MRS	37°C for 12-15 h.	HRM analysis
Leuconostoc citreum JCM 9698	MRS	37°C for 12-15 h.	HRM analysis
Weisella cibaria JCM 12495	MRS	37°C for 12-15 h.	HRM analysis
Weisella confusa JCM 1093	MRS	37°C for 12-15 h.	HRM analysis
Lactobacillus reuteri KUB-AC5	MRS	37°C for 18 h.	Probiotic strain
Escherichia coli TISTR 527	Luria broth, LB (Merck, Darmstadt, Germany)	37°C for 12 h with shaking 250 rpm	q-PCR analysis
Campylobacter jejuni ATCC 33291	Brucella broth (Difco, MD, USA)	37°C for 12-15 h.	q-PCR analysis
Acinetobacter calcoaceticus TISTR 360	Nutrient broth, NB (Pronadisa, Madrid, Spain)	30°C for 12-15 h.	q-PCR analysis

Table 3 (Continued)

Name	Medium	Culture condition	Purpose
Pseudomonas sp. TISTR 1249	NB	30°C for 12-15 h.	q-PCR analysis
Bifidobacterium bifidum JCM 1255	MRS	37°C for 12-15 h.	q-PCR analysis
Bacteriodes fragilis ATCC 25285	Chopped meat broth (BD, New Juesey, USA)	37°C for 12-15 h.	q-PCR analysis
Clostridium perfringens ATCC 13124	Brucella broth	37°C for 12-15 h.	q-PCR analysis
Salmonella Typhimurium TISTR 292	NB	37°C for 12-15 h.	q-PCR analysis

For *Ruminococcus productus* JGD 07421**,** its genomic DNA was purchased from Rigen Bioresource Center (Tsukuba, Japan) and used for q-PCR experiment.

2. Chicken

The broiler chickens used in three experiments of healthy chicken, probiotic and CP effect were belonged to Ross strain. All chickens were reared from 1 day old under control management without anticoccidial drugs in experiment farm except the experiment of healthy chicken analysis were reared at industrial farm. Body weight, feed intake and feed conversion in broiler chickens were determined every week.

There were 43,554, 500 and 900 birds for the experiment of healthy chicken, probiotic effect and CP effect, respectively. In probiotic effect experiment, the birds were randomly divided into two groups containing 5 floor pens (50 chicks/pen) per group. For CP effect experiment, there were three groups consisting of 10 floor pen (30 chicks/ pen) per group.

3. Diet and feeding program

The birds in healthy chicken experiment were fed with commercial corn and soy bean based diet. Under NRC recommendation (1994), two feed types containing 22% and 20% of CP were fed to chicken at starter (1-28 d) and grower stage (29- 42 d), respectively.

In probiotic effect experiment, chicks were fed with commercial corn and soybean meal (CENTACO, Thailand). Two feed types of starter and grower stage containing 21% and 17% of CP were fed to chicken age of 1-21 d and 22-42 d, respectively. While fat and fiber were constant at 3% and 5%, respectively. For control group, chicks in 5 pens were fed with commercial feed both starter and grower diets. For probiotic-treated group, chicks in other 5 pens were fed with a probiotic-supplemented commercial feed from only day 1 to 7 and then fed the same as control group.

For CP effect experiment, three diets were formulated to provide similar nutrients content according to the broiler's nutrients requirement suggested by NRC (1994), except protein and essential amino acid (EAA) levels (Table 4). These diets consisted of two feeding program, starter (1-21 d) and finisher (22-35 d). At starter stage, the control, high CP and EAA treatment were fed with crude protein of 22%, 28% and 22%, respectively. While EAA diet formula was modified from control diet by adding extra of methionine, lysine and threonine to final concentration similar to high CP treatment. After 22d, the diets in three treatments were changed to the same diet formular (Table 4). All birds had access to feed and water *ad libitum*.

Table 4 Diet composition for crude protein studies at 0-35 d.

Ingredients (g/kg)	Starter (0d-21d)			Finisher (22d-35d)
	Control	High CP	EAA	
Corn	61.8	42.4	60.8	43.1
Wheat	-	-	-	20
Palm oil	-	2.58	-	3.43
Soy bean meal	33.75	50.93	33.75	29.63
Salt	0.4	0.4	0.258	0.271
Mono dicalcium	1.8	1.66	1.8	0.923
Calcium carbonate	1.48	1.42	1.48	1.56
DL-Methionine	0.183	0.15	0.242	0.163
L-Lysine 98.5%	0.158	0.017	0.6	0.03
L-Threonine 98.5%	-	-	0.267	0.0166
Premix (vitamin/mineral)	0.415	0.415	0.615	0.876
Analyzed Composition (%)				
Energy (kcal/kg)	2900	2900.8	2902.7	3050
Crude protein	22.06	28.74	22.64	20
Fat	2.36	4.6	2.33	5.8
Fiber	2.628	2.87	2.61	2.5
Calcium	0.947	0.94	0.947	0.9
Phosphorus	0.73	0.77	0.73	0.57
Salt	0.51	0.458	0.51	0.38
Sodium	0.22	0.22	0.22	0.17
Total Lysine	1.252	1.598	1.598	1.05

Table 4 (Continued)

Ingredients (g/kg)	Starter (0d-21d)			Finisher
	Control	High CP	EAA	(22d-35d)
Total Methionine	0.50	0.546	0.558	0.45
Total Threonine	0.813	1.08	1.072	0.75
Total Trytophan	0.253	0.351	0.253	0.24
Linoleic acid	1.31	1.31	1.30	1.77

4. Probiotic preparation

Lactobacillus reuteri KUB-AC5 was used as a probiotic strain in this study. It was cultured was cultured in 1 liter of MRS broth at 37°C for 18 h. The bacterial cells were harvested by centrifugation at 15,000 xg for 15 min at 4°C, washed once with sterile saline (0.85% NaCl) and then resuspended in a 15% w/v skim milk solution to a volume of 334 ml. The cell suspension was spray-dried with a laboratory spray dryer (Seiko, Japan). The air temperatures at the inlet and outlet of the spray dryer were adjusted to 130°C and 70-72°C, respectively. The dried cell powder was mixed with a commercial chicken feed to achieve the desired concentration of 10^5 CFU per gram of feed.

5. Sample collection and microbial cell preparation

For healthy chicken experiment, 12 chicks of 28 d and 42 d were randomly selected and killed by cervical dislocation. The microbial samples were collected from ileum and cecum using Cytobrush® Plus (CooperSurgical, Berlin, Germany), suspended in 900 µl phosphate-buffered saline (PBS, pH 8.0) in 1.5 ml collection tube and stored at -20°C until genomic DNA extraction. Five hundred microliter of each microbial sample were centrifuged at 15,000 xg for 10 min at 4°C to obtain microbial cell. These cell pellets were further washed twice by 1 ml PBS, suspended in 900 µl of PBS buffer and stored at -20°C until use.

For probiotic effect experiment, one bird was randomly selected from each of the 5 pens per treatment (5 birds per treatment) at 21 and 42 days posthatch. The bird was killed by cervical dislocation, and the ileum was removed from the body. One gram of the ileum was suspended in 9 ml phosphate-buffered saline (PBS, pH 8.0) using stomacher blender (Seward Medical, London) for 2 min. One aliquot of each sample was kept by freezing at -20°C or further processed to collect the microbial cell pellets. To collect the cell pellets, 1 ml of sample was centrifuged at 100 xg for 10 min at 4°C to remove all digesta and tissues. Its supernatant containing bacterial cells was further centrifuged at 15,000 xg for 10 min at 4°C and washed by the same method as healty chicken experiment to obtain the cell suspension solution which were kept at -20°C for DNA extraction.

For CP effect experiment, two birds were randomly selected from each of the 10 pens per treatment (20 birds per treatment) at 21 and 35 days posthatch. The bird was killed by carbon dioxide asphyxiation, jejunum ileum and cecum were collected. The tissues were steriled by 70% ethanol and washed twice by sterile water. The sterile intestines were kept at -20°C or further processed to collect the microbial cell pellets by the same method as probiotic effect experiment.

6. Villi measurement

The jejunum and ileum samples were cut by the size of 1.5 × 1.5 cm^2, flushed through with saline solution, fixed on foam using pins and placed into 100 g/l buffered formalin for further analysis. Paraffin-embedded tissues were sectioned to a thickness of 5 μm. Tissue slides were stained using 0.02% toluidine blue for light microscope measurement. The villous height was measured from the base of the lamina propria to the apex of the villous. All reported villi values were an average of 5 measurements per tissue. Assessments were made only on cleanly sectioned, perpendicular villi as previously described by Sun *et al*. (2005).

7. Genomic DNA extraction

The genomic DNA was extracted using a combination of QIAamp DNA Stool Mini Kit (Qiagen, Germany) and the bead-beating method (Sakamoto *et al.*, 2011). At first, the suspension was transferred to 2 ml screw-capped tube containing 0.3 g of zirconium beads (0.1 mm in diameter; As One Corporation, Osaka, Japan). Three hundred microliter of phenol-chloroform–isoamyl alcohol (25:24:1) was added and beaten at 2,700 rpm for 180 sec (Multi-beads Shocker; Yasui Kikai, Osaka, Japan). The bacterial DNA were collected from upper layer of mixture solution by centrifuged at 20,000 xg for 2 min and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to manufacturer's instructions. The genomic concentration was measured using a micro-photometer (NanoDrop 1000, Nanodrop Technologies, Wilmington, DE, USA). The samples were stored at -20 °C until further use.

8. HRM analysis

8.1 Oligonucleotide primers used and specificity test

Five bacteria including, *L. crispatus, L. salivarius, Leuconostoc citreum, W. cibaria and W. confusa* were applied to optimize HRM technique. The specific primer and expected PCR product size were shown in Table 5. The minimum PCR product size appropriated for HRM analysis was not more than 250 bp (Corprotocol^{TM} 6000, Corbett Life Science, Germany). Therefore reverse primers on 16s rRNA gene for *L. salivarius* and *Leuconostoc citreum* were designed shorter from 322 and 1,298 bp to 140 and 119 bp, respectively. For *W. confuse* and *W. cibaria*, the specific primers were designed manully based on 23s rRNA gene and interspacer of 16s-23s rRNA from NCBI database, respectively (http://www.ncbi.nlm.nih.gov).

The primers specificity tests were analyzed by using pure and mixture template DNA in PCR amplification process. The pure template DNA was genomic DNA from one bacteria whereas mixture template DNA was genomic DNA from five bacteria. The mixture template DNA was prepared by mixing 10 ng/µl of each

genomic DNA at equal volume. The PCR amplification and HRM analysis were performed using LightCycler® 480 (Roche, Germany). The PCR reaction mixture contained 10 µl of 2x High Resolution Melting master mix (Roche, Germany), 0.8 µl of 10 pmol forward and reverse primers, 2 µl of 25 mM $MgCl_2$, 2 µl of DNA template (10 ng) and dH_2O added to obtain the final volumn of 20 µl. Amplication program included an initial denaturation at 95°C for 10 min, followed by 45 cycles consisting of denaturation at 95°C for 10 sec, annealing temperature according to the Tm of primers for 15 sec and extension at 72°C for 5-7 sec. Afterwards melting temperature analysis was done and dissociation curve was created in the following cycles: a denaturation step at 95 ^{o}C for 1 min, decreased to 40°C for 1 min and continuously increased from 65 ^{o}C to 95 ^{o}C every 1 sec signal measurement.

Table 5 The list of primers used for HRM analysis

Target	Sequence (5'-3')	Annealing Temp (°C)	Size (bp)	Reference
L. crispatus	F: AGCGAGCGGAACTAACA GATTTAC	55	154	Byun *et al.*, 2004
	R: AGCTGATCATGCGATCT GCTT			
L. salivarius	F: CGAAACTTTCTTACACCG AATGC	55	140	Byun *et al.*, 2004
	R: GCGATCCTTAGAGATATA CGG			This study
Leu. citreum	F: AAAACTTAGTATCGCATG ATATC	48	119	Lee *et al.*, 2000
	R: CGGCTATGCATCATCGT			This study
W. confusa	F: GCGTAATAGCGCACTAG TCG	55	126	This study
	R: CGGTCTGACTTCATCGCC CTTAG			

Table 5 (Continued)

Target	Sequence (5'-3')	Annealing Temp (°C)	Size (bp)	Reference
W. cibaria	F: CGGAGGTTCGAGTCCTCTC R: CAACCCAAAGGTTGTAAT GGAG	55	122	This study

8.2 Multiplex PCR obtimization

The primer pairs in each species were combined to perform duplex, triplex and multiplex primers at final concentration of 10 pmol/ primer. The melting temperatures in each PCR products were observed comparing to one primer pair.

9. Pyrosequencing

9.1 Sample preparation

The universal primers Q-968F (WACGCGARGAACCTTACC) and Q-1390R (TGACGGGCGGTGWGTAC) were used to amplify around 422 bp of the 16S rRNA gene segment corresponding to the V6-V8 region (Nakayama, 2010). A 10 ng of extracted DNA was applied for a first PCR reaction. The first PCR was performed in a 25 µl solution containing 10 ng of extracted DNA, 2.5 µl of 10X Ex *Taq* buffer, 2 µl of 2.5 mM dNTP, 0.5 µl of 10 pmol of each universal primers, 0.125 µl of 5 U/µl Takara Ex Tag^{TM} HS (Takara Bio, Shiga, Japan) and dH_2O added to obtain the final volume of 25 µl. PCR reactions were performed by initial denaturation at 98°C for 2.30 min, followed by 15 cycles of denaturation at 98°C for 15 sec, primer annealing at 50°C for 30 sec and extension at 72°C for 20 sec with a final elongation step at 72°C for 5 min. The PCR amplicon was labeled by a barcode-sequence tag in a second PCR with the primers Q-968F-# (5′-CWSWSWWSHTWACGCGARGAACCTTACC-3′) and Q-1390R-# (5′-CWSWSWWSHTTGACGGGCGGTGWGTAC-3′) (# indicates a series of 128 barcode sequence tags underlined in the sequence) (Nakayama, 2010). The second

PCR was performed in a 50 µl containing of 0.5 µl of the first PCR amplicon, 5 µl of 10X Ex *Taq* buffer, 4 µl of 2.5 mM dNTP, 1 µl of 10 pmol of each primer, 0.25 µl of 5 U/µl Takara Ex Tag^{TM} HS and dH_2O added to obtain the final volume of 50 µl. The PCR reactions were followed by initial denaturation at 98°C for 2.30 min, 15 cycles of 98°C for 15 sec, 54°C for 30 sec and 72°C for 20 sec with a final extension at 72°C for 5 min. The PCR amplicons were purified using a Qiagen PCR purification kit (Qiagen, Germany) according to the manufacturer's protocol. The purified PCR amplicons were measured using a micro-photometer (NanoDrop 1000, Nanodrop Technologies, Wilmington, DE, USA). Prior to pyrosequencing, equal amounts of each amplicon from different samples (100 ng) were pooled together and purified by ethanol precipitation. The purified amplicon mixture was dissolved in 50 µl of EB buffer (10 mM Tris-Cl, pH 8.5, Qiagen, Germany) and applied to the Genome Sequencer FLX System (454 Life Sciences, Germany).

9.2 Data analysis

The sequence data obtained from the pyrosequencing were sorted in each sample according to their barcode tag and the primer sequences were then removed from each sequence in Pipeline Initial Process at the RDP (Ribosome Database Project, USA, http://rdp.cme.msu.edu/). The sequence data were converted to the bacterial composition at hierarchical levels from genus to phylum using RDP Classifier (Ribosomal Database Project, USA, http://rdp.cme.msu.edu/classifier /classifier.jsp) at the 80% confidence threshold. For determination of the species, the seqmatch algorithm was used to find the 20 closest 16S rRNAs of the cultured strain deposited in the RDP database (http://rdp.cme.msu.edu/seqmatch/seqmatch_ intro. jsp). Seqmatch Q400 algorithm was therefore used to convert the result of RDP seqmatch to species-level population data (Nakayama, 2010). Seqmatch Q400 program, the species showing the best match was assigned to the query sequence, and if more than 2 species showed the same best score, the one with the highest count in the top 20 list was selected.

10. Quantitative real time PCR

10.1 Primer and DNA standard used for real time PCR

The oligonucleotide primers, optimal annealing temperature and their PCR products size are summerrized in Table 6. To obtain a standard curve for absolute quantification, the modified method of Wise and Siragusa (2007) was used. Standard curves of total bacteria, *Lactobacillus* group, *Campylobacter*, *Acinetobacter*, *Psedomonus*, *Bifidobacterium*, *Bacteriod*es – *Prevotell* - *Porphyromonas* group, *C. coccoides–E. rectal* group, *C. perfringens* group and *Enterobacteriaceae* quantification were constructed using specific primers to amplify the genomic DNA of *Escherichia coli* TISTR 527, *Lactobacillus salivarius* AC21, *Campylobacter jejuni* ATCC 33291, *Acinetobacter calcoaceticus* TISTR 360, *Pseudomonas sp.* TISTR 1249, *Bifidobacterium bifidum* JCM1255, *Bacteriodes fragilis* ATCC 25285, *Ruminococcus productus* JGD 07421, *Clostridium perfringens* ATCC 13124 and *Salmonella* Typhimurium TISTR 292. Each PCR products were cloned into pGEM-T Easy vector according to manufacturer's instructions (Promega, Madison, WI). Then the recombinant plasmids were diluted by serial 10-fold dilution until concentration of 10^9 was reached. The set of serial dilution series in each group were used as a template for the standard curve. The standard curves were created by LightCycler® 480 software using second derivative maximum (Roach Applied Science, Mannheim, Germany). The slope of the standard curve is used to determine reaction efficiency in which accurate quantification require the efficiency of 1.8-2 ($E = 10^{-1/slope}$). In addition, the error value is a measure of the accurancy of the quatification result base on the standard curve (an acceptable value should be < 0.2).

Table 6 The list of primers used for quantitative real-time PCR

Target	Primer	Seq	Tm	Size	Ref.
Total bacteria	HDA1	ACTCCTACGGGAGGCAGC AGT	61	200	Tannock *et al.* 2000
	HDA2	GTATTACCGCGGCTGCTGG CAC			
Lactobacillus group	LbF	AGCAGTAGGGAATCTTCC A	53	341	Walter *et al.* 2001
	LBR	CACCGCTACACATGGAG			Heiling *et al.* 2002
Campylobacter spp.	CamF	GGATGACACTTTTCGGAG	54	246	Rinttila *et al.* 2004
	CamR	AATTCCATCTGCCTCTCC			
Acinetobacter	AcF	TTTAAGCGAGGAGGAGG	52	240	Vanbroek--horen *et al.* 2004
	AcR	ATTCTACCATCCTCTCCC			
Pseudomonas	PseF	GGCGACGATCCGTAAC	57	180	Khan *et al.* 2004
	PseR	CCTTCCTCCCAACTT			
Bifidobacterium spp.	BifF	TCGCGTC(C/T)GGTGTGAA AG	62	243	Rinttila *et al.* 2004
	BifR	CCACATCCAGC(A/G)TCCA C			
Bacteriod – Prevotell-Porphyromonas	BacPF	GGTGTCGGCTTAAGTGCCA T	57	140	Rinttila *et al.* 2004
	BacPR	CGGA(C/T)GTAAGGGCCGT GC			
C. coccoides– E. rectal group	ClosF	CGGTACCTGACTAAGAAG C	51	429	Rinttila *et al.* 2004
	ClosR	AGTTT(C/T)ATTCTTGCGAA CG			
C. perfringens group	PerfF	ATGCAAGTCGAGCGA(G/T) G	51	120	Rinttila *et al.* 2004
	PerfR	TATGCGGTATTAATCT(C/T) CCTTT			

Table 6 (Continued)

Target	Primer	Seq	Tm	Size	Ref.
Enterobacteriaceae	EnF	CATTGACGTTACCCGCA GAAGAAGC	57	195	Bartosch *et al.* 2004
	EnR	CTCTACGAGACTCAAGC TTGC			

10.2 Real time PCR using SYBR green

Real time PCR using SYBR technology quantified target group of microorganism by LightCycler® 480 (Roche, Germany). The reaction mixture contained 10 µl of 2x SYBR Green I master mix (Roche, Germany), 0.8 µl of 5 pmol forward and reverse primers, 2 µl of DNA template (50-100 ng) and dH_2O added to obtain the final volumn of 20 µl. Amplication program included an initial denaturation at 95°C for 5 min, followed by 45 cycles consisting of denaturation at 95°C for 10 sec, annealing temperature according to the Tm of primer for 10 sec and extension at 72°C for 5-18 sec. To confirm specific amplification of the target DNA, dissociation curve was created in the following cycles: a denaturation step at 95 ^{o}C for 5 sec, decreased to 65°C for 1 min and continuously increased from 65 ^{o}C to 97 ^{o}C every 12 sec signal measurement. For statistic analysis, in the case where the incidence of detection within treatment group was less than 100%, the remaining undetection replicates were considered to be at the theoretical limit of detection for purpose of calculating the mean and standard devitation.

10.3 Real time PCR using Taqman probe

To determine the number of *L. reuteri* species in probiotic effect experiment, Taqman technology with primers Freut (ACCGAGAACACCGC GTTATTT), Rreut (CATAACTTAACCTAAACAATCAAAGATTGTCT) and probe Preut (ATCGCTAACTCAATTAAT) was used for quantitative real-time PCR

(Haarman and Knol, 2006). Mx300P® QPCR system (Stratagene, CA, USA) was used for the real time PCR. The reaction mixture contained 12.5 µl of Taqman® Universal master mix II (Applied Biosystems, Netherlands), 1.5 µl of 10 pmol each primer, 1 µl of 10 pmol Taqman probe, 2.5 µl of template DNA and dH_2O added to obtain the final volumn of 25 µl. PCR reactions were amplified as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles consisting of denaturation at 95°C for 15 sec and primer-specific and probe binding at 60°C for 1 min. For standard curve (R^2 = 0.994), a set of serial dilution series of the pGEM-T Easy carrying the partial 16S rDNA of *L. reuteri* was used as a template.

11. Short chain fatty acid (SCFA) analysis

11.1 Standard preparation

The standard solutions were prepared from lactic acid, acetic acid, propionic acid or butyric acid at concentration of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% v/v. Three volume of each concentration of acid mixture were added into 1 volumn of internal standard (0.2% w/v tartaric acid) to obtain the injection samples which were filtered through 0.2 µm PVDF Syringe filters (Verical, Bangkok, Thailand) prior to injection. Two injections were made for each solution.

11.2 Sample preparation

According to modification method of Zeppa *et al.* (2001), the one gram of intestinal digesta were diluted in 4 ml of 0.008 M H_2SO_4 and homogenized by vortex every 10 min for 1 h at 4 °C. The supernatant contained SCFA were collected by centrifugation at 15,000 xg for 10 min at 4 °C. The injection sample preparation was followed the method of standard preparation.

11.3 Apparatus and operating condition

The high-performance liquid chromatography (HPLC) belong to Breeze 2 System (Walter, USA) including binary pump model 1525 (Walter, USA), 6 port injector model 7725 (Rheodyn, USA), UV/visible detector model 2489 (Walter, USA) and computer with Breeze software sytem. The SCFA separation was achieved using 300 × 7.80 mm of Rezex ROR-Organic Acid column (Phenomenex, USA). The flow rate of mobile phase (0.008 M H_2SO_4) was 0.6 ml/min. An injection sample volumn was 20 µl. Column temperature was maintained at 60 °C and UV detection was carried out at 210 nm.

12. Statistic analysis

All results were analyzed by one way Anova test (SPSS program version 12, Munich, Germany). The group difference in healthy chicken experiment and probiotic effect experiment were determined for significance at $p \leq 0.05$ using Mann-Whitney U test. Whereas the group difference in crude protein effect experiment was determined by LSD and Duncan method. Differences were quantified for their significance at $p \leq 0.05$.

RESULTS

1. Intestinal microbiota identification in healthy chicken

The gut microflora is an essential component of healthy and functional gastrointestinal system in chicken. The microbial community in gut plays an important role of the host through changes in the morphology of gut, nutrition, immune response, pathogenesis of enteric diseases and alterations in colonization resistance (Gong *et al.*, 2007; Shakouri *et al.*, 2009; Torok *et al.*, 2008). Understanding and management of gut microflora system has become a crucial part of broiler chicken production.

The twenty four healthy chicks from poultry industry providing good quality meat products, feed conversion ratio of 1.72 and high survival rate at 97% during six weeks were randomly sampling at grower stage (28 d) and finisher stage (42 d). Microbial communities in ileum and cecum region were analyzed using pyrosequence base on V6-V8 region of 16s rDNA and real time PCR.

1.1 Total amount of bacteria in ileum and cecum of healthy chicken at 28 d and 42 d

Total amount of bacteria in ileum and cecum expressed as copy number of 16s rDNA by real time PCR (Figure 5). The total bacteria of ileal from chicken aged 28 d were 4.95 log 16s rDNA gene copy which were significantly greater than the one from 42 d (4.66 log 16s rDNA gene copy) ($p \leq 0.05$). In cecum, their bacterial concentrations were about 5.5 log 16s rDNA gene copy at both ages of 28 d and 42 d. Comparing between these two regions, a number of 16s rDNA gene copy in ileum sample was significant lower than cecum ($p \leq 0.05$). This would be concluded that cecum provided suitable condition to support microbial growth than ileum (Gong *et al.*, 2002a).

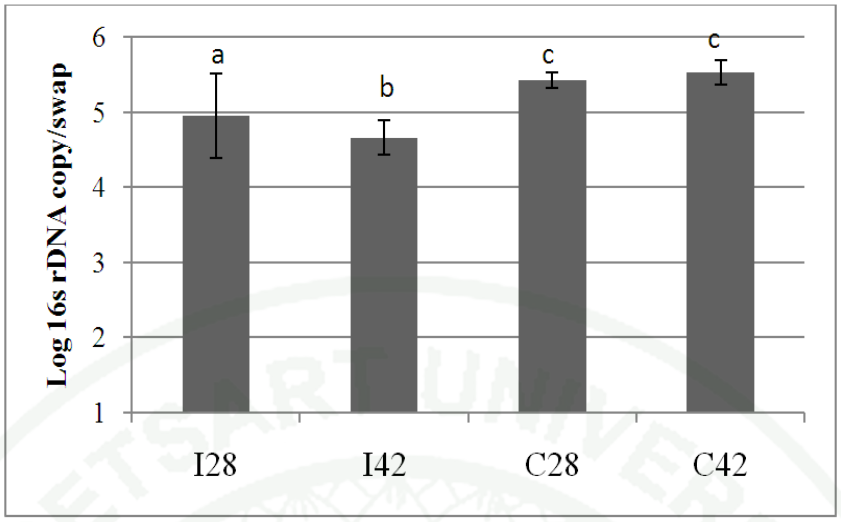

Figure 5 Total bacterial amounts (16S rDNA copy number) in the ileum and cecum of chicks from d 28 to d 42. 16S rDNA copy number was quantified by q-PCR using the total DNA as a template. Standard derivation bars were calculated from 12 chickens. The a, b and c shown significant difference at $p \leq 0.05$.

1.2 Microbial communities found in chicken's ileum at 28 d and 42 d

1.2.1 Phylum level analysis

The microbial composition of ileum samples was profiled by pyrosequencing of 16S rDNA amplicons. The pyrosequence data generated 11,592 and 10,195 sequences obtained from 24 chickens at 28 d and 42 d, respectively. The composition data were obtained by average twelve chickens in each group and represented by a pie chart of phylum composition (Figure 6). Five phyla, i.e., *Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Proteobacteria* and *Firmicutes*, were found from chickens' ileum. In both 28 d and 42 d, phylum *Firmicutes, Proteobacteria* and *Actinobacteria* were the first, second and third most dominant microorganism. More than 78% of total biota belonged to phylum *Firmicutes*. At 42 d, the amount of *Firmicutes* and *Actinobacteria* increased to 83% and 3% respectively, while amount of *Proteobacteria* and *Deinococcus-Thermus* decreased to 13% and

undetectable at pyrosequening limit. Phylum *Bacteroidetes* was stable at 28 d and 42 d. These results suggested that good quality of chicken contained abundant of phylum *Firmicutes* and low level of pathogenic group in phylum *Proteobacteria* and *Deinococcus-Thermus*.

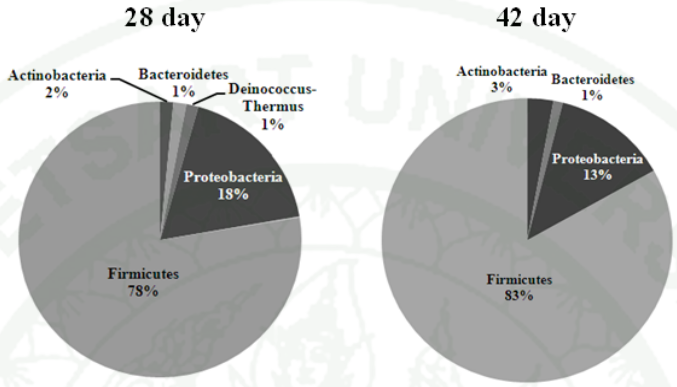

Figure 6 Pie charts presenting the phylum composition of the ileum bacterial communities at 28 d and 42 d. Phylum compositions were determined through 16S rDNA amplicon pyrosequencing. These data represent the average of twelve chickens.

1.2.2 Genus level analysis

The genus level was further analyzed from pyrosequence data. The absolute population size of each genus was calculated by multiplying the total bacterial amount determined by q-PCR by the relative ratio of each group determined by amplicon pyrosequencing. At 28 d, 126 genus were generated from 11,592 sequences (Table 7). The population of lactic acid bacteria including *Weissella, Leuconostoc*, *Lactobacillus* and *Lactococcus* were dominant at around 4 log 16s rDNA gene copy. This result was similar with the report of Gong *et al.* (2002, 2007). *Lactobacillus* was dominant species mostly found in chicken ileum. In addition, genus *Lachnospiraceae Incertae Sedis* belongs to order *Clostridiales* was also detected as dominant one. At 42 d, the 102 bacterial genus were generated from 10,195 sequences

(Table 7). Similar to 28 d, the dominant genus of *Weissella, Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Lachnospiraceae Incertae Sedis* were observed for 3 log 16s rDNA gene copy. Some pathogens of genus *Acinetobacter, Citrobacter, Escherichia, Campylobacter, Chryseobacterium* and *Raoultella* were detected for 3 log 16s rDNA gene copy at 28 d. However, they all decreased at the age of 42 d. In addition, two important pathogens causing strong effect to consumer, *Shigella* and *Klebsiella* were detected at 2 log 16s rDNA gene copy. However their amounts at 42 d had no effect on chicken health during chicken growth. These findings showed that the dominant lactic acid bacteria and *Lachnospiraceae Incertae Sedis* could suppress the growth of some pathogens allowing healthy in chicken.

Table 7 The bacteria genera found in the chicken ileum at 28 d and 42 d.

Genus	Ileum 28d [gene copy]		Ileum 42d [gene copy]	
	Average*	SD*	Average	SD
Weissella	2.50E+04	3.34E+04	8.25E+03	1.08E+04
Leuconostoc	2.26E+04	2.81E+04	8.33E+03	1.18E+04
Lachnospiraceae Incertae Sedis	2.05E+04	3.38E+04	5.46E+03	5.56E+03
Lactobacillus	1.20E+04	1.70E+04	7.24E+03	1.22E+04
Lactococcus	1.11E+04	1.47E+04	3.73E+03	6.13E+03
Acinetobacter	7.53E+03	9.92E+03	2.06E+03	3.25E+03
Faecalibacterium	5.86E+03	9.43E+03	6.97E+02	7.02E+02
Citrobacter	5.83E+03	7.15E+03	1.40E+03	2.44E+03
Subdoligranulum	4.95E+03	8.24E+03	5.20E+02	8.75E+02
Pseudomonas	4.25E+03	6.13E+03	6.32E+01	1.02E+02
Escherichia	3.84E+03	4.76E+03	8.56E+01	8.46E+01
Streptococcus	2.96E+03	3.71E+03	2.90E+03	6.27E+03
Thermus	2.40E+03	3.97E+03	0.00E+00	0.00E+00
Enterococcus	2.02E+03	2.84E+03	7.90E+02	1.17E+03
Enterobacter	1.81E+03	2.42E+03	2.78E+02	3.33E+02
Bifidobacterium	1.78E+03	2.47E+03	1.16E+03	1.51E+03
Campylobacter	1.67E+03	2.10E+03	1.21E+01	2.31E+01
Dorea	1.29E+03	2.45E+03	6.78E+01	8.41E+01

Table 7 (Continued)

Genus	Ileum 28d [gene copies]		Ileum 42d [gene copies]	
	Average*	**SD***	**Average**	**SD**
Ruminococcus	1.22E+03	1.58E+03	6.76E+02	8.35E+02
Veillonella	1.22E+03	1.64E+03	4.80E+02	7.74E+02
Chryseobacterium	1.18E+03	1.77E+03	1.40E+02	1.95E+02
Raoultella	1.17E+03	1.67E+03	4.15E+02	7.63E+02
Papillibacter	9.96E+02	1.73E+03	1.85E+02	2.35E+02
Anaerofilum	9.56E+02	1.74E+03	2.15E+02	2.89E+02
Arcobacter	9.00E+02	1.06E+03	3.65E+02	5.97E+02
Ruminococcaceae Incertae Sedis	8.56E+02	1.13E+03	3.16E+02	4.89E+02
Erysipelotrichaceae Incertae Sedis	8.45E+02	1.57E+03	3.11E+02	3.10E+02
Kluyvera	8.20E+02	1.07E+03	2.59E+02	4.43E+02
Mesorhizobium	7.73E+02	1.15E+03	1.57E+01	3.99E+01
Allobaculum	7.09E+02	1.05E+03	9.87E+01	2.61E+02
Shigella	6.67E+02	1.24E+03	2.83E+00	9.81E+00
Microvirgula	6.33E+02	8.10E+02	1.34E+02	1.71E+02
Achromobacter	5.54E+02	6.81E+02	1.11E+02	1.14E+02
Chryseomonas	5.41E+02	1.02E+03	0.00E+00	0.00E+00
Enhydrobacter	5.27E+02	7.66E+02	1.16E+02	1.16E+02
Agrobacterium	4.65E+02	6.71E+02	1.23E+01	2.39E+01
Aeromonas	4.54E+02	5.28E+02	1.75E+02	3.29E+02
Bacteroides	4.46E+02	5.94E+02	2.48E+02	2.07E+02
Roseomonas	3.66E+02	5.33E+02	0.00E+00	0.00E+00
Clostridium	3.21E+02	4.60E+02	4.50E+01	8.14E+01
Comamonas	3.18E+02	5.46E+02	6.52E+01	7.41E+01
Caldimonas	3.12E+02	5.82E+02	0.00E+00	0.00E+00
Acidovorax	3.10E+02	4.42E+02	9.78E+01	1.44E+02
Stenotrophomonas	2.90E+02	4.75E+02	1.70E+01	3.52E+01
Alistipes	2.63E+02	3.30E+02	1.64E+01	4.07E+01
Delftia	2.60E+02	3.54E+02	0.00E+00	0.00E+00

Table 7 (Continued)

Genus	Ileum 28d [gene copies]		Ileum 42d [gene copies]	
	Average*	SD*	Average	SD
Klebsiella	2.55E+02	3.95E+02	1.59E+02	2.78E+02
Erysipelothrix	2.32E+02	4.39E+02	0.00E+00	0.00E+00
Phyllobacterium	2.22E+02	3.37E+02	0.00E+00	0.00E+00
Methylobacterium	2.13E+02	3.97E+02	0.00E+00	0.00E+00
Prevotella	2.10E+02	3.15E+02	2.51E+01	4.21E+01
Dietzia	2.09E+02	2.66E+02	4.82E+01	7.97E+01
Eggerthella	1.98E+02	2.66E+02	5.46E+01	7.00E+01
Morganella	1.92E+02	2.36E+02	2.03E+01	4.14E+01
Hespellia	1.73E+02	2.92E+02	2.47E+01	5.37E+01
Bacillus g	1.71E+02	2.94E+02	0.00E+00	0.00E+00
Propionibacterium	1.69E+02	2.12E+02	3.38E+01	6.13E+01
Vitreoscilla	1.65E+02	2.71E+02	8.29E+00	2.87E+01
Actinomyces	1.65E+02	2.06E+02	2.83E+00	9.81E+00
Anaerostipes	1.57E+02	2.22E+02	4.43E+01	8.83E+01
Bacillus c	1.55E+02	2.93E+02	1.51E+01	3.92E+01
Xanthomonas	1.55E+02	2.93E+02	0.00E+00	0.00E+00
Staphylococcus	1.48E+02	2.01E+02	2.51E+01	5.43E+01
Coprobacillus	1.39E+02	1.75E+02	9.34E+00	2.23E+01
Streptophyta	1.29E+02	1.69E+02	4.48E+01	6.50E+01
Serratia	1.24E+02	1.89E+02	9.68E+00	2.34E+01
Collinsella	1.19E+02	1.89E+02	9.37E+01	1.81E+02
Parabacteroides	1.17E+02	1.42E+02	2.87E+01	4.57E+01
Luteococcus	1.10E+02	1.65E+02	0.00E+00	0.00E+00
Paenibacillus	1.09E+02	1.60E+02	0.00E+00	0.00E+00
Flavimonas	1.07E+02	1.59E+02	0.00E+00	0.00E+00
Sulfurospirillum	1.06E+02	1.65E+02	5.98E+01	1.11E+02
Anaerotruncus	1.02E+02	1.24E+02	4.28E+01	8.09E+01
Aquitalea	9.93E+01	1.42E+02	2.39E+01	4.54E+01
Peptostreptococcaceae Incertae Sedis	9.74E+01	1.84E+02	2.09E+02	5.82E+02

Table 7 (Continued)

Genus	Ileum 28d [gene copies]		Ileum 42d [gene copies]	
	Average*	SD*	Average	SD
Anaerovorax	9.71E+01	1.49E+02	1.68E+01	5.83E+01
Leminorella	9.37E+01	1.60E+02	1.09E+01	3.78E+01
Flavobacterium	9.09E+01	1.28E+02	5.91E+01	9.13E+01
Nesterenkonia	8.48E+01	1.06E+02	1.05E+01	2.49E+01
Aeromicrobium	8.43E+01	1.59E+02	2.84E+01	7.52E+01
Erwinia	8.12E+01	1.14E+02	4.57E+01	6.45E+01
Anaerofustis	7.87E+01	1.49E+02	0.00E+00	0.00E+00
Prosthecobacter	7.74E+01	1.46E+02	0.00E+00	0.00E+00
Halomonas	7.08E+01	1.34E+02	0.00E+00	0.00E+00
Roseburia	6.96E+01	9.93E+01	1.22E+01	3.10E+01
Corynebacterium	6.66E+01	9.48E+01	8.62E+01	2.49E+02
Brevundimonas	6.15E+01	9.14E+01	3.72E+01	8.89E+01
Uruburuella	5.98E+01	8.77E+01	5.47E+00	1.89E+01
Vagococcus	4.72E+01	8.92E+01	4.37E+01	1.51E+02
Angulomicrobium	4.22E+01	7.96E+01	0.00E+00	0.00E+00
Bordetella	4.22E+01	7.96E+01	0.00E+00	0.00E+00
Novosphingobium	4.22E+01	7.96E+01	0.00E+00	0.00E+00
Sphingobium	4.22E+01	7.96E+01	0.00E+00	0.00E+00
Catenibacterium	3.27E+01	6.18E+01	3.70E+00	1.28E+01
Cedecea	3.27E+01	6.18E+01	0.00E+00	0.00E+00
Diaphorobacter	3.27E+01	6.18E+01	0.00E+00	0.00E+00
Megamonas	3.27E+01	6.18E+01	0.00E+00	0.00E+00
Xylanibacter	2.45E+01	3.62E+01	0.00E+00	0.00E+00
Vogesella	2.36E+01	4.46E+01	1.51E+01	3.92E+01
Desulfovibrio	2.36E+01	4.46E+01	3.70E+00	1.28E+01
Acidaminococcus	2.36E+01	4.46E+01	0.00E+00	0.00E+00
Bilophila	2.36E+01	4.46E+01	0.00E+00	0.00E+00
Mahella	2.36E+01	4.46E+01	0.00E+00	0.00E+00
Shinella	2.36E+01	4.46E+01	0.00E+00	0.00E+00
Williamsia	2.36E+01	4.46E+01	0.00E+00	0.00E+00

Table 7 (Continued)

Genus	Ileum 28d [gene copies]		Ileum 42d [gene copies]	
	Average*	SD*	Average	SD
Acetivibrio	1.76E+01	3.33E+01	6.56E+00	1.55E+01
Akkermansia	1.76E+01	3.33E+01	0.00E+00	0.00E+00
Ammoniphilus	1.76E+01	3.33E+01	0.00E+00	0.00E+00
Thauera	1.76E+01	3.33E+01	0.00E+00	0.00E+00
Alicycliphilus	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Brevibacillus	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Parascardovia	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Salmonella	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Silanimonas	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Sphingomonas	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Tatumella	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Trabulsiella	9.36E+00	1.77E+01	0.00E+00	0.00E+00
Hymenobacter	6.77E+00	1.28E+01	0.00E+00	0.00E+00
Rothia	6.47E+00	9.07E+00	1.24E+01	4.31E+01
Paracoccus	6.47E+00	9.07E+00	2.83E+00	9.81E+00
Dendrosporobacter	4.75E+00	8.98E+00	0.00E+00	0.00E+00
Tessaracoccus	4.75E+00	8.98E+00	0.00E+00	0.00E+00
Dysgonomonas	3.73E+00	7.05E+00	1.09E+01	3.78E+01
Isobaculum	3.73E+00	7.05E+00	0.00E+00	0.00E+00
Bacillus a	3.38E+00	6.39E+00	8.29E+00	2.87E+01
Leptothrix	3.38E+00	6.39E+00	0.00E+00	0.00E+00
Holdemania	0.00E+00	0.00E+00	3.37E+01	1.17E+02
Pelomonas	0.00E+00	0.00E+00	2.18E+01	7.57E+01
Ochrobactrum	0.00E+00	0.00E+00	1.57E+01	2.89E+01
Haemophilus	0.00E+00	0.00E+00	1.51E+01	5.22E+01
Thermoactinomyces	0.00E+00	0.00E+00	1.24E+01	4.31E+01
Alkanindiges	0.00E+00	0.00E+00	1.09E+01	3.78E+01
Cloacibacterium	0.00E+00	0.00E+00	1.09E+01	3.78E+01
Dermacoccus	0.00E+00	0.00E+00	1.09E+01	3.78E+01
TM7_genera_incertae_sedis	0.00E+00	0.00E+00	9.11E+00	1.67E+01

Table 7 (Continued)

Genus	Ileum 28d [gene copies]		Ileum 42d [gene copies]	
	Average*	**SD***	**Average**	**SD**
Branhamella	0.00E+00	0.00E+00	6.31E+00	1.50E+01
Caloranaerobacter	0.00E+00	0.00E+00	5.99E+00	2.07E+01
Acetanaerobacterium	0.00E+00	0.00E+00	5.61E+00	1.94E+01
Aerococcus	0.00E+00	0.00E+00	4.78E+00	1.66E+01
Bacillus d	0.00E+00	0.00E+00	3.73E+00	1.29E+01
Moryella	0.00E+00	0.00E+00	3.73E+00	1.29E+01
Dialister	0.00E+00	0.00E+00	3.70E+00	1.28E+01
Duganella	0.00E+00	0.00E+00	3.70E+00	1.28E+01
Ethanoligenens	0.00E+00	0.00E+00	3.70E+00	1.28E+01
Oribacterium	0.00E+00	0.00E+00	3.70E+00	1.28E+01
Actinobaculum	0.00E+00	0.00E+00	2.83E+00	9.81E+00
Eubacterium	0.00E+00	0.00E+00	2.83E+00	9.81E+00

* Average and SD values were calculated from 12 broiler chicken.

1.2.3 Species level analysis

Microbial composition data in species level were further analyzed from sequence generated. According to seqmatch analysis, a total number of microbial species in each chicken was different. At 28 d, there was 43 to 209 species observed from 12 chickens. The prevalent species found more than 80% of total samples were composed of 19 species (Table 8). *Leuconostoc citreum*, *Weissella cibaria* and *Weissella confuse* were the first, second and third dominant species which alredy occupies for 25% of total amount of bacteria. Moreover, one hazard species *Shigella boydii* was found from 80% of all ilea samples. This bacteria was not harmful to chicken but it could contaminate in the food causing diarrhea and bacillary dysentery in human (Senchenkova *et al.*, 2006).

Table 8 The dominant bacteria species found in chicken ileum at 28 d.

Dominant species	Incidence (n = 12)	Average* (gene copy)	Max	Min	SD*
Leuconostoc citreum	12	2.57E+04	8.23E+04	2.98E+02	2.84E+04
Weissella cibaria	12	1.78E+04	7.50E+04	3.53E+02	2.18E+04
Weissella confusa	12	1.33E+04	5.30E+04	9.84E+01	1.60E+04
Faecalibacterium prausnitzii	12	9.43E+03	7.22E+04	2.46E+01	1.97E+04
Candidatus Arthromitus	11	5.68E+03	2.69E+04	0	7.53E+03
Lactococcus plantarum	11	5.34E+03	2.37E+04	0	7.44E+03
Citrobacter freundii	12	4.98E+03	1.86E+04	4.92E+01	5.97E+03
Lactococcus lactis	12	3.74E+03	1.41E+04	2.46E+01	4.36E+03
Lactobacillus crispatus	10	3.37E+03	1.41E+04	0	4.47E+03
Pseudomonas rhodesiae	11	2.94E+03	1.54E+04	0	4.47E+03
Acinetobacter venetianus	11	2.92E+03	1.30E+04	0	3.88E+03
Escherichia coli	11	2.80E+03	1.75E+04	0	4.68E+03
Shigella boydii	10	2.31E+03	1.24E+04	0	3.49E+03
Ruminococcus obeum	12	2.24E+03	1.07E+04	5.43E+01	2.96E+03
Blautia wexlerae	12	1.96E+03	7.33E+03	4.92E+01	2.21E+03
Acinetobacter johnsonii	10	1.78E+03	7.90E+03	0	2.41E+03
Streptococcus bovis	10	1.46E+03	5.22E+03	0	1.63E+03
Veillonella caviae	10	9.82E+02	3.38E+03	0	1.12E+03
Enterobacter hormaechei	10	5.10E+02	1.73E+03	0	5.83E+02

* The average and standard variation (SD) values were calculated from 12 chickens.

By 42 d, the range of 42 to 147 species was detected in ileal samples. The 27 prevalent species were observed (Table 9). Comparing to 21 d, the increasing of prevalent species were mainly from the group of strictly anaerobe and anaerobe bacteria in the class clostridia such as *Ruminococcus obeum, Blautia producta*, *Clostridium fusiformis*. However, the first, second and third dominant species was similar contained *Leuconostoc citreum*, *Weissella cibaria* and *Weissella confuse,* respectively. We could not observe *Shigella boydii* as prevalent species at 42

d whereas three pathogens were observed instead. *Klebsiella oxytoca* and *Klebsiella pneumonia* were detected at about 4 log of 16s rDNA gene copy. These strains were opportunistic pathogen frequently causing urinary tract infections (UTI), septicaemia or pneumonia in immunocompromised individuals (Struve and Krogfelt, 2003). In addition, *Clostridium amygdalinum* causing osteitis in human was found at 80% of total sample. However the pathogenic bacteria had no effect in chicken health. These bacteria colonized in chicken intestine as normal microflora. However it may concern their contamination during meat production process.

Table 9 The dominant bacteria species found in chicken ileum at 42 d.

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Leuconostoc citreum	12	8.12E+03	4.31E+04	1.70E+03	8.04E+00
Weissella cibaria	12	4.68E+03	2.32E+04	7.41E+02	4.40E+00
Weissella confusa	12	3.75E+03	2.08E+04	4.38E+02	3.23E+00
Lactobacillus salivarius	11	3.39E+03	2.74E+04	0	1.24E+01
Citrobacter freundii	12	1.52E+03	9.98E+03	5.48E+01	1.70E+00
Lactobacillus crispatus	11	1.49E+03	1.07E+04	0	4.79E+00
Lactococcus plantarum	12	1.49E+03	8.14E+03	1.10E+02	1.53E+00
Faecalibacterium prausnitzii	11	1.28E+03	3.84E+03	0	2.26E+00
Lactococcus lactis	12	1.23E+03	8.54E+03	1.49E+02	1.44E+00
Lactococcus raffinolactis	12	8.73E+02	5.12E+03	1.81E+02	8.75E-01
Clostridium amygdalinum	10	8.14E+02	4.51E+03	0	2.12E+00
Acinetobacter venetianus	11	7.48E+02	4.86E+03	0	7.52E-01
Ruminococcus obeum	10	7.06E+02	2.53E+03	0	3.05E+00
Acinetobacter johnsonii	12	6.57E+02	3.81E+03	6.73E+01	6.27E-01
Blautia producta	11	5.92E+02	2.76E+03	0	1.10E+00
Blautia hydrogenotrophica	10	4.79E+02	1.55E+03	0	8.48E-01
Klebsiella oxytoca	12	4.74E+02	3.15E+03	4.98E+01	4.35E-01
Streptococcus bovis	11	4.21E+02	2.63E+03	0	4.96E-01
Ruminococcus bromii	10	4.17E+02	1.89E+03	0	9.01E-01

Table 9 (Continued)

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Clostridium fusiformis	10	4.07E+02	2.36E+03	0	9.70E-01
Veillonella caviae	12	3.97E+02	2.23E+03	4.98E+01	4.36E-01
Arcobacter butzleri	11	2.99E+02	1.71E+03	0	4.05E-01
Acinetobacter calcoaceticus	11	2.80E+02	1.84E+03	0	3.96E-01
Clostridium nexile	11	2.45E+02	5.03E+02	0	4.56E-01
Blautia luti	10	2.26E+02	7.90E+02	0	5.56E-01
Klebsiella pneumoniae	10	2.08E+02	9.19E+02	0	9.24E-01
Enterobacter aerogenes	10	1.97E+02	6.57E+02	0	4.20E-01

* The average and standard variation (SD) values were calculated from 12 chickens.

1.3 Microbial communities found in chicken's cecum at 28 d and 42 d

1.3.1 Phylum level

Cecum contained the largest microbial in gastrointestinal tract in chicken. Extensive bacterial fermentation had occurred and resulted in further nutrient absorption and detoxification of harmful substances and prevention of pathogen colonization (Gong *et al.*, 2007). The 14,059 and 14,676 sequences generated from 28 d and 42 d in chicken cecum indicated that more than 90% of total microbial communities belonged to phylum *Firmicutes* (Figure 7). The amount of phylum *Firmicutes* was detected at 99% at 21 d and their level decreased to 97% at 42 d. Consequently, the increasing 2% of phylum *Bacteriodetes* at 42 d was observed. In addition, phylum *Actinobacteria* was observed both 28 d and 42 d at 1% of total microbial. The results suggested that healthy chicken contained abundant of phylum *Firmicutes* and low level of phylum *Actinobacteria* and *Bacteroidetes* in cecum.

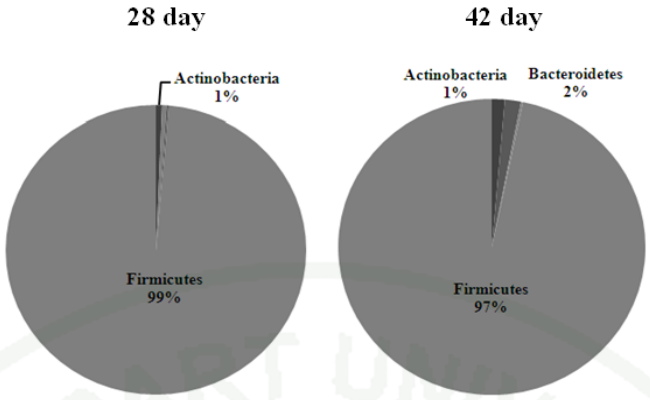

Figure 7 Pie charts presenting the phylum composition of the cecum bacterial communities at 28 d and 42 d. Phylum compositions were determined through 16S rDNA amplicon pyrosequencing. These data represent the average of twelve chickens.

1.3.2 Genus level

The 14,059 and 14,676 sequences analysed by pyrosequencing technique generated 48 and 45 genus at 28 d and 42 d, respectively (Table 10). Most of bacteria in cecum belonged to obligate anaerobe or strictly anaerobe bacteria such as genus *Lachnospiraceae Incertae Sedis, Subdoligranulum*, *Faecalibacterium*, *Ruminococcus*, *Anaerofilum* and *Dorea* etc. In addition, the group of lactic acid producing bacteria were smaller when compared to ileum region. At 28 d, the first, second and third dominant genus were *Lachnospiraceae Incertae Sedis, Subdoligranulum* and *Faecalibacterium* at around 4 log 16s rDNA gene copy. The genus order was changed in caeca sample at 42 d. By 42 d, the first, second and third dominant genus was *Lachnospiraceae Incertae Sedis, Lactobacillus* and *Subdoligranulum,* respectively. Unlike ileum, the pathogenic bacteria in family *Enterobacteriaceae, Shigella* and *Klebsiella* were detected at very low population around 10^1 copies of 16s rDNA gene at both 28 d and 42 d.

Table 10 The bacteria genera found in the chicken cecum at 28 d and 42 d.

Genus	Cecum 28 day[gene copy]		Cecum 42 day[gene copy]	
	Average	**SD**	**Average**	**SD**
Lachnospiraceae Incertae Sedis	8.85E+04	4.87E+04	1.05E+05	3.47E+04
Subdoligranulum	2.66E+04	1.48E+04	1.95E+04	1.15E+04
Faecalibacterium	1.78E+04	1.69E+04	1.63E+04	1.22E+04
Ruminococcus	1.46E+04	8.68E+03	1.40E+04	1.03E+04
Lactobacillus	1.42E+04	1.14E+04	4.84E+04	4.75E+04
Anaerofilum	4.91E+03	6.29E+03	2.70E+03	1.58E+03
Papillibacter	4.02E+03	4.50E+03	6.60E+03	8.82E+03
Ruminococcaceae Incertae Sedis	3.82E+03	3.14E+03	6.61E+03	3.28E+03
Dorea	2.44E+03	2.72E+03	1.85E+03	2.03E+03
Streptococcus	1.59E+03	2.46E+03	2.05E+03	3.58E+03
Eggerthella	1.47E+03	1.32E+03	1.58E+03	9.32E+02
Anaerotruncus	1.21E+03	1.21E+03	1.18E+03	1.29E+03
Hespellia	1.08E+03	1.66E+03	2.65E+02	5.24E+02
Erysipelotrichaceae Incertae Sedis	8.49E+02	8.95E+02	1.03E+03	1.47E+03
Acetivibrio	7.07E+02	1.64E+03	7.76E+01	2.69E+02
Enterococcus	6.88E+02	1.45E+03	6.98E+02	7.78E+02
Alistipes	5.49E+02	7.94E+02	1.84E+03	2.16E+03
Bacteroides	4.04E+02	5.84E+02	2.68E+03	2.67E+03
Bifidobacterium	2.52E+02	4.75E+02	2.79E+03	5.76E+03
Escherichia	2.03E+02	3.30E+02	7.86E+01	1.66E+02
Coprobacillus	1.97E+02	3.08E+02	2.75E+02	2.53E+02
Anaerovorax	1.82E+02	2.42E+02	1.03E+02	2.42E+02
Parabacteroides	1.73E+02	2.51E+02	9.36E+02	1.20E+03
Mahella	1.14E+02	3.45E+02	0.00E+00	0.00E+00
Syntrophothermus	8.80E+01	1.82E+02	4.52E+01	8.81E+01
Anaerostipes	8.77E+01	1.73E+02	8.81E+01	2.66E+02
Leuconostoc	7.20E+01	1.37E+02	6.50E+01	1.56E+02
Catenibacterium	6.41E+01	1.54E+02	6.68E+01	1.66E+02

Table 10 (Continued)

Genus	Cecum 28 day[gene copy]		Cecum 42 day[gene copy]	
	Average	**SD**	**Average**	**SD**
Bilophila	5.34E+01	1.25E+02	1.88E+02	3.78E+02
Peptostreptococcaceae Incertae				
Sedis	3.33E+01	1.15E+02	5.04E+01	1.19E+02
Anaerofustis	3.15E+01	1.09E+02	1.18E+01	4.07E+01
Eubacterium	3.15E+01	1.09E+02	0.00E+00	0.00E+00
Parabacteroides	1.73E+02	2.51E+02	9.36E+02	1.20E+03
Prevotella	3.15E+01	1.09E+02	0.00E+00	0.00E+00
Holdemania	3.11E+01	7.35E+01	9.15E+01	2.14E+02
Allobaculum	2.94E+01	7.05E+01	9.51E+01	1.94E+02
Thermus	2.88E+01	6.74E+01	0.00E+00	0.00E+00
Roseburia	2.67E+01	6.25E+01	0.00E+00	0.00E+00
Veillonella	2.66E+01	9.21E+01	0.00E+00	0.00E+00
Raoultella	2.10E+01	7.26E+01	8.87E+01	3.07E+02
Enterobacter	2.10E+01	7.26E+01	0.00E+00	0.00E+00
Shigella	2.10E+01	7.26E+01	0.00E+00	0.00E+00
Oribacterium	1.44E+01	4.98E+01	7.82E+01	2.19E+02
Bacillus c	1.44E+01	4.98E+01	0.00E+00	0.00E+00
Caloranaerobacter	1.44E+01	4.98E+01	0.00E+00	0.00E+00
Turicibacter	1.44E+01	4.98E+01	0.00E+00	0.00E+00
Klebsiella	1.33E+01	4.61E+01	4.43E+01	1.54E+02
Citrobacter	1.33E+01	4.61E+01	0.00E+00	0.00E+00
Clostridium	1.33E+01	4.61E+01	0.00E+00	0.00E+00
Rikenella	0.00E+00	0.00E+00	1.18E+02	4.09E+02
Slackia	0.00E+00	0.00E+00	8.11E+01	1.49E+02
Corynebacterium	0.00E+00	0.00E+00	6.73E+01	2.33E+02
Bacillus d	0.00E+00	0.00E+00	6.13E+01	1.59E+02
Aerococcus	0.00E+00	0.00E+00	4.43E+01	1.54E+02
Acetanaerobacterium	0.00E+00	0.00E+00	2.80E+01	9.69E+01
Arcobacter	0.00E+00	0.00E+00	2.20E+01	7.61E+01

Table 10 (Continued)

Genus	Cecum 28 day[gene copy]		Cecum 42 day[gene copy]	
	Average	SD	Average	SD
Propionibacterium	0.00E+00	0.00E+00	1.70E+01	5.88E+01
Collinsella	0.00E+00	0.00E+00	1.55E+01	5.38E+01
Weissella	0.00E+00	0.00E+00	1.10E+01	3.81E+01

1.3.3 Species level

At 28 d, the range of 64 to 145 species was generated from sequence data of 12 chickens. The 39 species were detected at more than 80% of sampling collection (Table 11). Most of bacteria belonged to class *Clostridia* such as *Faecalibacterium prausnitzii*, *Clostridium glycyrrhizinilyticum, Ruminococcus lactaris* and *Blautia coccoides*. The first, second and third species were *Faecalibacterium prausnitzii*, *Clostridium glycyrrhizinilyticum* and *Ruminococcus lactaris*, respespectively which were found in all caeca samples. In addition, no virulent pathogenic was observed as prevalent species.

Table 11 The dominant bacteria species found in chicken cecum at 28 d.

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Faecalibacterium prausnitzii	12	4.44E+04	1.05E+05	6.06E+03	2.69E+04
Clostridium glycyrrhizinilyticum	12	1.90E+04	3.50E+04	1.25E+04	6.68E+03
Ruminococcus lactaris	12	1.65E+04	4.69E+04	1.89E+03	1.33E+04
Blautia producta	11	1.30E+04	1.07E+05	0	3.01E+04
Blautia coccoides	12	1.15E+04	3.61E+04	4.74E+02	1.19E+04
Ruminococcus bromii	12	1.08E+04	2.79E+04	2.97E+03	7.66E+03
Clostridium fusiformis	12	8.27E+03	4.59E+04	2.12E+02	1.44E+04

Table 11 (Continued)

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Clostridium nexile	12	8.04E+03	1.85E+04	1.51E+03	5.45E+03
Clostridium leptum	12	7.95E+03	1.80E+04	1.04E+03	5.52E+03
Blautia hydrogenotrophica	12	7.22E+03	1.90E+04	3.45E+02	6.33E+03
Clostridium orbiscindens	12	7.14E+03	2.23E+04	4.01E+02	8.14E+03
Lactobacillus salivarius	11	6.29E+03	2.82E+04	0	7.71E+03
Butyricicoccus pullicaecorum	12	5.10E+03	1.60E+04	4.25E+02	5.00E+03
Elbe River	12	5.02E+03	2.29E+04	4.74E+02	6.26E+03
Ruminococcus callidus	12	4.50E+03	9.00E+03	6.90E+02	2.63E+03
Clostridium fimetarium	12	4.41E+03	1.54E+04	2.14E+02	5.55E+03
Robinsoniella peoriensis	12	3.99E+03	7.04E+03	5.18E+02	2.61E+03
Clostridium citroniae	12	3.82E+03	9.56E+03	1.73E+02	2.98E+03
Bacteroides capillosus	12	3.40E+03	9.00E+03	5.18E+02	2.60E+03
Clostridium amygdalinum	12	3.30E+03	1.24E+04	9.28E+02	2.99E+03
Blautia luti	10	3.25E+03	2.34E+04	0	6.44E+03
Ruminococcus obeum	12	3.14E+03	1.28E+04	3.78E+02	3.76E+03
Blautia schinkii	12	2.99E+03	9.45E+03	5.57E+02	2.45E+03
Ruminococcus albus	12	2.66E+03	6.20E+03	1.04E+03	1.67E+03
Ruminococcus torques	12	2.35E+03	5.69E+03	2.51E+02	1.72E+03
Coprobacillus cateniformis	12	2.29E+03	3.71E+03	7.55E+02	9.91E+02
Clostridium sporosphaeroides	10	2.23E+03	1.18E+04	0	3.62E+03
Clostridium aldrichii	10	2.20E+03	6.42E+03	0	2.31E+03
Clostridium viride	12	2.14E+03	4.41E+03	4.17E+02	1.02E+03

Table 11 (Continued)

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Ruminococcus flavefaciens	11	1.72E+03	9.58E+03	0	2.57E+03
Clostridium aldenense	12	1.49E+03	3.78E+03	5.57E+02	9.46E+02
Bacteroides xylanolyticus	12	1.39E+03	3.61E+03	2.12E+02	9.40E+02
Eggerthella hongkongensis	11	1.33E+03	4.53E+03	0	1.32E+03
Ruminococcus gauvreauii	10	1.17E+03	5.20E+03	0	1.47E+03
Clostridium lactatifermentans	10	1.15E+03	4.91E+03	0	1.34E+03
Anaerotruncus colihominis	11	8.20E+02	3.19E+03	0	8.53E+02
Eubacterium cylindroides	10	7.54E+02	3.02E+03	0	8.86E+02
Butyrivibrio fibrisolvens	10	6.65E+02	2.00E+03	0	6.62E+02
Hespellia porcina	11	6.39E+02	4.41E+03	0	1.21E+03

* The average and standard variation (SD) values were calculated from 12 chickens.

At 42 d, fifty three species were observed at more than 80% sampling collection (Table 12). Most of prevalent species were similar to 28 d samples which belong to gram positive anaerobic bacteria in the class *Clostridia*. The increasing of prevalent species at 42 d was mainly from higher diversity in species level. The first, second and third species were *Faecalibacterium prausnitzii*, *Lactobacillus crispatus* and *Clostridium glycyrrhizinilyticum*, respectively. *Faecalibacterium prausnitzii* and *Clostridium glycyrrhizinilyticum* were observed in

all samples except *Lactobacillus crispatus* was observed at 91.67% of total sample collection.

Table 12 The dominant bacteria species found in chicken cecum at 42 d.

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Faecalibacterium prausnitzii	12	3.65E+04	5.88E+04	1.42E+04	1.65E+04
Lactobacillus crispatus	11	2.78E+04	1.46E+05	0	4.14E+04
Clostridium glycyrrhizinilyticum	12	1.98E+04	4.63E+04	6.46E+03	1.36E+04
Blautia producta	12	1.86E+04	5.95E+04	7.46E+02	1.65E+04
Blautia coccoides	12	1.52E+04	2.67E+04	2.69E+02	7.19E+03
Clostridium orbiscindens	12	1.25E+04	6.55E+04	1.05E+03	1.83E+04
Blautia hydrogenotrophica	12	1.21E+04	2.27E+04	4.32E+03	7.20E+03
Blautia wexlerae	10	1.07E+04	5.27E+04	0	1.91E+04
Clostridium fusiformis	11	9.00E+03	5.15E+04	0	1.46E+04
Clostridium amygdalinum	12	8.92E+03	1.91E+04	1.59E+03	4.56E+03
Ruminococcus bromii	12	8.33E+03	2.07E+04	1.06E+03	6.06E+03
Lactobacillus johnsonii	11	8.10E+03	6.84E+04	0	1.92E+04
Clostridium leptum	12	7.79E+03	2.65E+04	6.36E+02	7.22E+03
Lactobacillus salivarius	12	7.42E+03	1.67E+04	1.13E+03	4.52E+03
Clostridium nexile	12	7.13E+03	1.82E+04	2.13E+03	5.55E+03
Butyricicoccus pullicaecorum	12	6.09E+03	1.77E+04	8.08E+02	5.65E+03
Bacteroides capillosus	12	6.06E+03	1.05E+04	1.06E+03	3.56E+03
Blautia schinkii	12	5.60E+03	3.23E+04	9.44E+02	8.56E+03
Ruminococcus callidus	12	5.45E+03	1.46E+04	2.04E+02	4.76E+03
Blautia luti	12	4.77E+03	1.42E+04	2.69E+02	4.97E+03
Blautia luti	12	4.77E+03	1.42E+04	2.69E+02	4.97E+03
Ruminococcus obeum	12	4.43E+03	2.13E+04	2.69E+02	5.91E+03

Table 12 (Continued)

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Ruminococcus albus	11	4.11E+03	1.33E+04	0	3.89E+03
Clostridium viride	12	4.03E+03	1.50E+04	5.32E+02	4.23E+03
Clostridium aldenense	11	3.77E+03	1.40E+04	0	3.95E+03
Clostridium citroniae	12	3.39E+03	7.12E+03	6.17E+02	2.46E+03
Elbe River	11	2.93E+03	5.39E+03	0	1.72E+03
Eubacterium hallii	12	2.65E+03	7.65E+03	1.49E+03	1.70E+03
Robinsoniella peoriensis	12	2.62E+03	8.44E+03	3.18E+02	2.26E+03
Oscillibacter valericigenes	12	2.47E+03	1.35E+04	2.04E+02	3.68E+03
Clostridium aldrichii	10	2.22E+03	9.44E+03	0	2.51E+03
Clostridium lactatifermentans	11	2.21E+03	4.26E+03	0	1.73E+03
Streptococcus alactolyticus	11	2.13E+03	1.23E+04	0	3.71E+03
Eubacterium plautii	12	1.99E+03	7.27E+03	4.08E+02	1.79E+03
Acetivibrio cellulolyticus	12	1.98E+03	6.71E+03	5.27E+02	1.71E+03
Clostridium fimetarium	12	1.89E+03	5.37E+03	5.39E+02	1.70E+03
Clostridium sporosphaeroides	11	1.86E+03	5.67E+03	0	1.65E+03
Eubacterium cylindroides	10	1.60E+03	5.19E+03	0	1.87E+03
Ruminococcus gauvreauii	11	1.51E+03	5.27E+03	0	1.38E+03
Ruminococcus gnavus	10	1.41E+03	7.56E+03	0	2.05E+03
Catabacter hongkongensis	10	1.40E+03	3.25E+03	0	1.13E+03

Table 12 (Continued)

Microbial species	Incidence (n = 12)	Average (gene copy)	Max	Min	SD
Clostridium lavalense	11	1.39E+03	6.02E+03	0	1.59E+03
Ruminococcus torques	11	1.33E+03	2.61E+03	0	8.74E+02
Clostridium alkalicellum	10	1.33E+03	2.36E+03	0	8.73E+02
Lactobacillus reuteri	10	1.29E+03	7.98E+03	0	2.19E+03
Clostridium clostridioforme	12	1.26E+03	3.36E+03	2.69E+02	1.08E+03
Eggerthella hongkongensis	10	1.19E+03	3.69E+03	0	1.03E+03
Bacteroides xylanolyticus	10	1.16E+03	4.70E+03	0	1.54E+03
Clostridium spiroforme	11	1.15E+03	3.36E+03	0	9.64E+02
Coprobacillus cateniformis	12	1.14E+03	2.68E+03	3.08E+02	6.84E+02
Ruminococcus flavefaciens	12	1.13E+03	3.76E+03	2.04E+02	1.04E+03
Anaerotruncus colihominis	12	1.09E+03	4.25E+03	2.04E+02	1.20E+03
Eubacterium tortuosum	10	9.15E+02	3.78E+03	0	1.05E+03
Butyrivibrio fibrisolvens	10	6.95E+02	2.24E+03	0	5.70E+02

***** The average and standard variation (SD) values were calculated from 12 chickens.

Similar to other studies on microbiota using molecular technique, *Lactobacillus* was abundant in chicken ileum. Whereas in this study other lactic acid bacteria including *Weisella, Leuconostoc* and *Lactococcus* were more abundant. Especially *Leuconostoc* and *Lactococcus* were not detected in the previous studies reported by Le *et al.* (2003), Gong *et al.* (2002b) and Gong *et al.* (2007). In cecum, the bacterial communities were highly diversity. The bacteria in genus *Clostridium*, *Faecalibacterium* and *Ruminococcus* were the largest group (Gong *et al.*, 2002a; Le *et al.*, 2003) while *Lachnospiraceae Incertae Sedis*, *Subdologranulum*, *Faecalibacterium*, *Ruminococcus* and *Lactobacillus* were observed in the biggest

group for more than 50% of total bacteria in this studies. However microbial abundant in cecum of all researches are similar in order *Clostridiales* which is anaerobic and strictly anaerobic bacteria. The composition of chicken intestinal microbiota can be significant influenced by diet, growth condition and other factor such as age of birds or strain of birds (Gong *et al.*, 2007). In addition, microbial identification methods were also critical factor in microbiota studies. Therefore the microbial composition difference between this study and other previous researches were observed.

According to biodata analysis from healthy chicken using pyrosequencing technique, it could be concluded that good quality meat products may come from the healthy chicken with high abundance of *Firmicute* in both ileum and cecum during growing stage. Dominant bacterial species in ileum belonged to lactic producing bacteria. Moreover, in cecum, obligate or strictly anaerobic bacteria were a domain group in the class of *Clostridia*. Microbial communities at 28 d and 42 d were stable both in ileum and cecum and low amount of pathogen group were detected. Base on the results, high standard variation in each genus or species were observed. In addition, bacteria composition pattern in each chicken were different. It was possible that these chickens were not come from same mother even they are the same genetic strain. Microbial composition in intestine was depended on their mother feed since they born (Lorenzo, 2008). These factors would create some individual error causing such huge variation.

2. Optimization of HRM technique to determine microorganisms found in chicken intestine

The HRM is a PCR based technique which is able to identify microorganism according to melting temperature behavior of genomic DNA (Jeffery *et al.*, 2007). Comparing to other PCR base technique, this technique is faster, sensitive and convenient to apply in the field. Therefore, HRM technique was optimized by monitoring dominant species found in chicken intestine. According to intestinal microbiota identification in healthy chicken experiment, *L. crispatus, L. salivarius, Leuconostoc citreum, W. cibaria and W. confusa* were the dominant species found in

ileum. Therefore genomic DNA mixtures of these bacteria were applied for HRM study. Five primer pairs specific for each species were applied in amplification and then melting analysis was performed.

2.1 Primer specificity

At beginning, the primers were tested for capability and specificity to amplify DNA target bacteria. The PCR products amplified from the different of targets and primers generated size and melting temperature difference (Table 13). The difference plot of HRM data showed that melting peak of five target bacteria could well separate into five peaks (Figure 8). In addition, each primer was tested again in the reaction mixture of genomic DNA template from five species. The melting temperature analysis results were similar to pure template DNA (data not shown). It supposed that the HRM technique with five primer pairs could identify targets bacteria in both pure and DNA template mixture. Although the different melting temperature occurred only 0.6 °C and 0.8 °C of *W. cibaria* and *Leu. citreum* , *Leu. citreum* and *L. salivarius*, respectively. This finding showed that HRM technique was able to generate significant melting peak difference in each product. This result related with Reed and Wittwer (2004) who applied HRM technique for mutation screening in genomic DNA. They claimed that HRM technique can identify heterozygote single base change in PCR product.

Table 13 Melting temperature and size of amplified PCR products from 5 target bacteria using HRM technique.

Target	Melting temperature (°C)	Size (bp)
L. crispatus	85	154
L. salivarius	83	140
Leu. citreum	83.8	119
W. confusa	86.2	126
W. cibaria	84.4	122

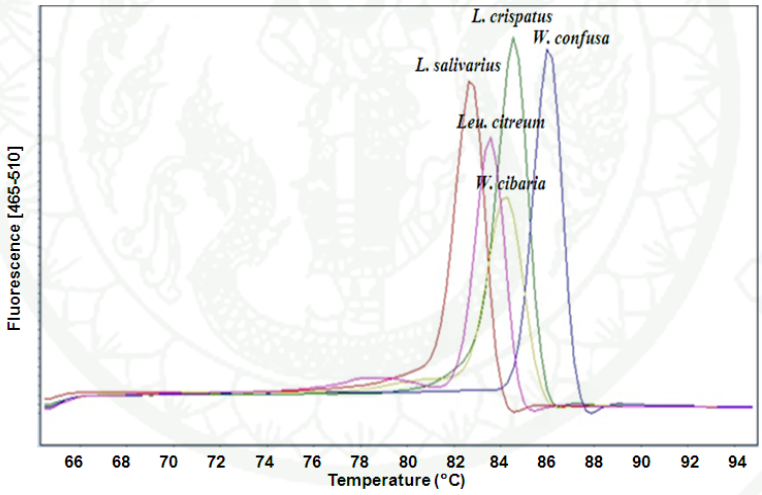

Figure 8 The difference plot of HRM data from PCR products of five targets bacteria. The target names were labeled on the top of peaks.

2.2 Multiplex PCR in mixture of targets bacteria

In chicken intestine, various dominant species were colonized. The identification technique was necessary to detect more than one species in the same

tube and time in the field. Therefore multiple primers were optimizated for more than one target amplification.

Duplex PCR amplification in one PCR reaction was firstly optimized. Two target bacteria were chosen from different genus. Primer specific for *L. crispatus* and *W. confusa* were mixed together at final concentration 0.4 pmol/µl in one PCR reaction. Each primer pair to perform PCR amplification was used as the positive control. All PCR reactions were done using DNA template mixture. After amplification step, melting temperature analysis was performed. The melting peak of three PCR products was represented in differentiated plot (Figure 9). The melting temperature of PCR products from *L. crispatus* and *W. confusa* was 85 °C and 86.2 °C, respectively. Expectedly, melting temperature of PCR products from duplex primers had two peaks at 85 °C and 86.2 °C similar to positive control.

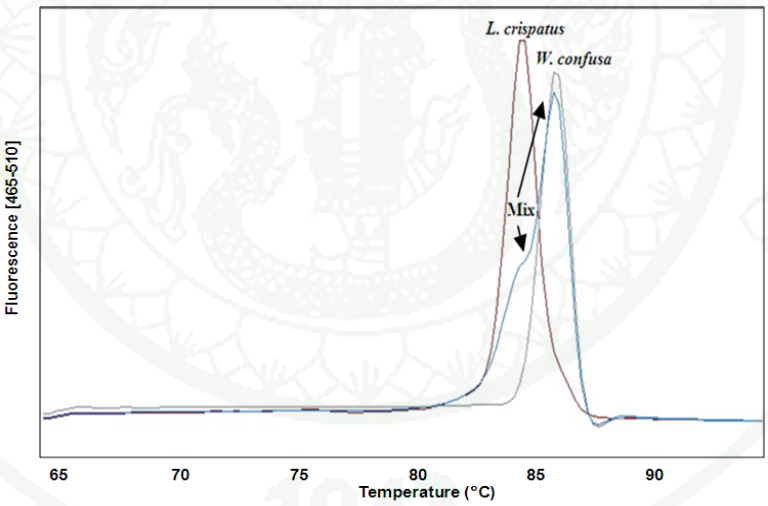

Figure 9 The difference plot of HRM data from PCR product of *L. crispatus* primer, *W. confusa* primer and duplex PCR amplification *(L. crispatus* and *W. confusa*).

The duplex PCR amplification specific for *L. salivarius* and *W. cibaria* were further optimized at the same way. In the result, positive control of *L. salivarius*

and *W. cibaria* template showed one melting peak at 83 °C and 84.4 °C, respectively. Moreover, duplex primers generated two peaks at 83 °C and 84.4 °C as well (Figure 10). This finding suggested that duplex primer pairs could be applied to two bacterial identification performed in one PCR reaction by HRM analysis.

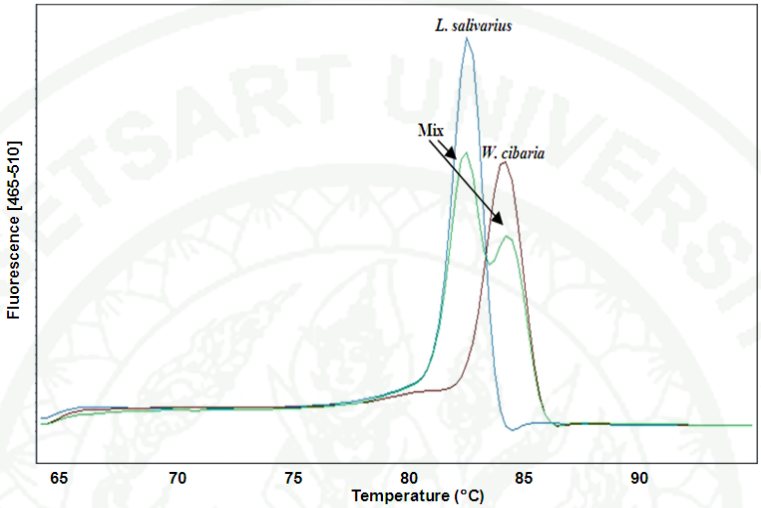

Figure 10 The difference plot of HRM data from PCR product of *L. salivarius* primer, *W. cibaria* primer and duplex PCR amplification *(L. salivarius* and *W. cibaria*).

Besides, triplex PCR amplifications were further performed by adding one specific primer of *Leuconostoc citreum* to duplex PCR amplifications. The final concentrations of all primers were 0.4 pmol/µl. The positive controls were done using PCR products of dulplex PCR amplification and *Leuconostoc citreum.* According to difference plot of HRM results, the melting temperature of PCR product from *L. salivarius*, *W. cibaria* and *Leuconostoc citreum* were generated only two peaks at 83 °C and 84.4 °C (Figure 11). The results were similar to PCR products of *L. crispatus*, *W. confusa* and *Leuconostoc citreum.* Only two peaks at 85 °C and 86.2 °C were observed. Althought melting peaks of triplex PCR amplification were a few difference from dulplex PCR amplification. This melting peak represented only two peaks that not indicated come from three target bacteria.

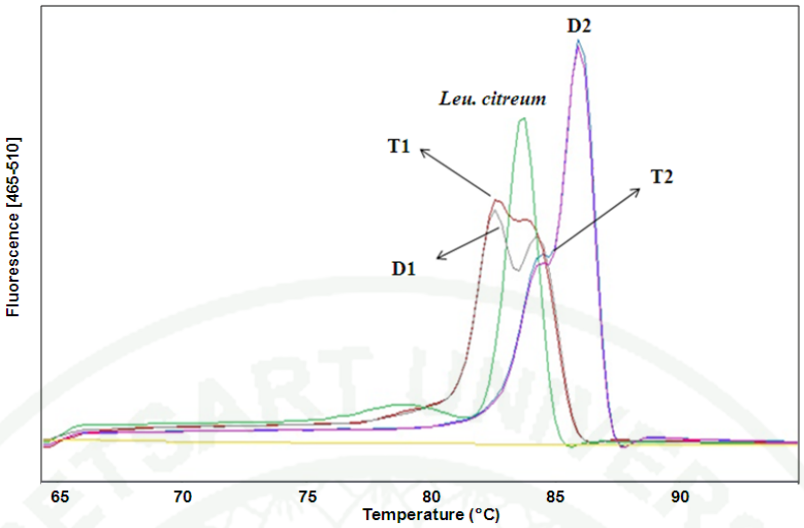

Figure 11 The difference plot of HRM data from PCR product of *Leuconostoc citreum* primer, duplex mix primer of *L. salivarius* and *W. cibaria* (D1), duplex mix primer of *L. crispatus* and *W. confuse* (D2), triplex mix primer of *L. salivarius*, *W. cibaria* and *Leuconostoc citreum* (T1) and triplex mix primer of *L. crispatus, W. confusa* and *Leuconostoc citreum* (T2).

Multiplex PCR amplification of five target bacteria contained *L. crispatus, L. salivarius, Leuconostoc citreum, W. cibaria and W. confuse*. Only three melting peak at 82.5 °C, 84.5 °C and 86.2 °C were observed (Figure 12). It inferred that HRM technique disabled to identify five target PCR products at the same tube and time.

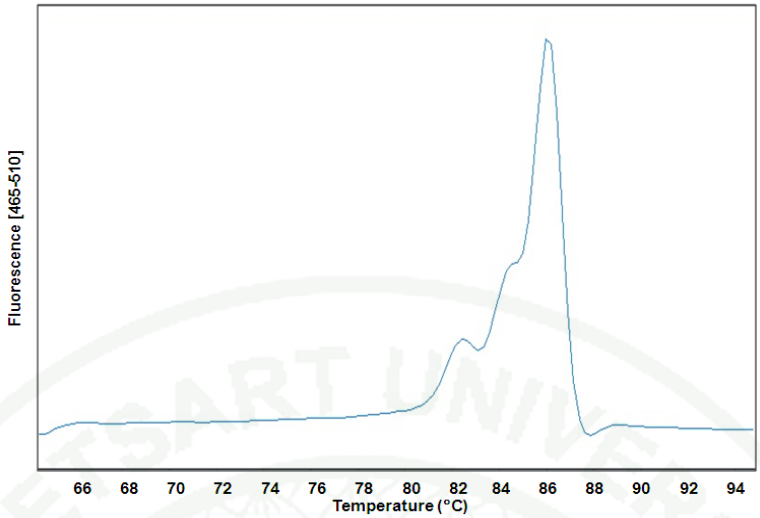

Figure 12 The difference plot of HRM data from multiplex PCR product of *L. crispatus, L. salivarius, Leuconostoc citreum, W. cibaria and W. confusa.*

In the results suggested that HRM technique did not appropriate to monitor microbial change in chicken intestine. Due to chicken intestine contained a lot of dominant bacteria. Its limitation was number of primer used in reaction.

3. Monitoring the effect of including *Lactobacillus reuteri* KUB-AC5 during posthatch feeding on the growth and ileum microbiota of broiler chickens

Probiotic strain, *Lactobacillus reuteri* KUB-AC5, was isolated from chicken intestine. The isolate displayed the hallmark features of good survival at low pH values and tolerance to high bile concentrations. Moreover, it showed antimicrobial activity against Gram-negative bacteria such as *Escherichia coli* and *Salmonella* sp. (Nitisinprasert *et al.*, 2000). In this context, we evaluate the role of *L. reuteri* KUB-AC5 as a feed supplement for chicken growth and microflora modulation in the chicken intestine. The present study aimed to evaluate the effect of a single application of *L. reuteri* KUB-AC5 over 7 days at a relatively low dosage of 10^5 cfu/g feed on broiler chicken performance and gut microbiota by combinatorial approach of 16S rDNA pyrosequencing and real-time PCR quantification.

3.1 Broiler production response to inclusion of probiotic *Lactobacillus reuteri* KUB-AC5

Broilers consumed a diet supplemented with a single probiotic and remained healthy throughout the experiment with high survival rate of 94-99% during 6 weeks as shown in Table 14. It was noticed that 8 dead chickens were from two pens of probiotic treatment at the last week causing significant lower survival rate of only 94% than the control. This would be due to the strong sunlight at this pen position, the high temperature up to 38-40°C during the day time and individual chicken error. While their other performances were similar to those of broilers fed a control diet. The weekly weight gain, feed intake and feed conversion ratio of broilers from day 1 to 42 are presented in Table 14. Only body weight gain in the first week was statistically different between the probiotic and control groups ($p = 0.03$). The body weight in the probiotic group increased by approximately 5% more than the body weight gain in the control group. However feed consumption and feed conversion ratio at first week were not significant difference ($p > 0.05$). During 42 d growth, both weight gain and feed conversion ratio of control treatment tended higher and lower than the one of probiotic treatment, respectively. However, there were no more significant differences ($p > 0.05$) observed in weight gain, feed consumption and feed conversion ratio during the 42 d experimental period. The variation of both weight gain and feed consumption noted as standard deviation were high up to 84 and 87, respectively. Since the animal trial was performed as an open system. Variation of temperature and humidity had occurred during the experiment. In addition, the chicks used in this experiment were not from the same mother even they all were Ross. These factors would create some individual error causing such huge variation in this experiment. Therefore, it could be concluded that single dose of 6 day at cell concentration of 10^5 CFU/g feed had no effect on chicken growth.

Table 14 Body weight, feed consumption and feed conversion ratio observed in broiler chickens fed a diet without (control) and with the probiotic. The parameter values were calculated from the mean value of each treatment. There were 250 chickens per treatment.

Parameter	**Week**	**Control treatment**	**Probiotic treatment**	**Level of significance**
Body weight gain	1	112.95±2.97[b]	117.36±2.28[a]	0.03
(g/week)	2	292.34±10.88	293.29±8.50	0.88
	3	446.84±15.60	454.90±38.78	0.68
	4	444.46±42.77	440.46±66.13	0.91
	5	524.68±38.65	450.64±83.70	0.13
	6	524.43±38.83	480.65±72.11	0.27
	Overall	2345.69±89.54	2281.21±181	0.28
Feed consumption	1	146.83±2.82	148.67±1.78	0.25
(g/week)	2	373.72±10.25	378.72±8.42	0.42
	3	608.32±13.71	610.35±17.99	0.84
	4	828.66±26.26	837.05±32.53	0.67
	5	1033.90±25.59	985.67±71.88	0.21
	6	1259.02±38.84	1228.55±87.11	0.50
	Overall	4250.46±92.58	4189.02±192.71	0.55
Feed conversion ratio	1	1.30±0.04	1.27±0.01	0.17
(F/G)	2	1.28±0.06	1.29±0.04	0.71
	3	1.36±0.04	1.35±0.11	0.81
	4	1.88±0.15	1.93±0.29	0.70
	5	1.98±0.13	2.23±0.28	0.13
	6	2.41±0.12	2.58±0.24	0.19
	Overall	1.81±0.03	1.87±0.08	0.09

Table 14 (Continute)

Parameter	Week	Control treatment	Probiotic treatment	Level of significance
Survival rate (%)	1	98.08±0.00	97.31±1.05	0.18
	2	99.61±0.88	100.00±0.00	0.37
	3	98.82±1.75	99.20±1.10	0.69
	4	99.21±1.08	98.80±1.09	0.58
	5	98.67±2.98	98.65±2.00	0.99
	6	99.09±2.03[a]	94.11±3.74[b]	0.04

Data were mean ± standard deviations. F/G, grams of feed per gram of body weight gain. [a-b] Means within a row that are not identified with a common superscript were found to differ significantly ($P<0.05$).

3.2 Effect of KUB-AC5 feeding on total bacterial amount in ileum

Single dose of KUB-AC5 supplementation was performed at the age of day 1–7. Lu *et al.* (2003) found that microbial communities in chicken ileum at days 7 and 21 were stable. In addition, the effect of probiotic treatment to microbial population by PCR-DGGE performed in our laboratory resulted in stable microbial abundance of day 1 and day 7. While at growing stage of day 21 to 42, development of microbial diversity could be detected. This might be well developed intestine during growing stage. Therefore, we decided to look for the effect of single dose of KUB-AC5 to bacterial changes during growing stage of 21 d - 42 d. Total bacterial amounts in the ileum samples were quantified by real-time PCR targeting total bacterial 16S rDNA (Figure 13). At day 21, rDNA amounts in the probiotic group tended to be higher than in the control group, although the difference was not statistically significant. The amount of 16S rDNA in ileum tended to increase from day 21 to day 42 in probiotic-treated chicks, while that in control group tended to slightly decrease. At day 42, the amount was significantly higher in the probiotic group than in the control group ($p = 0.03$).

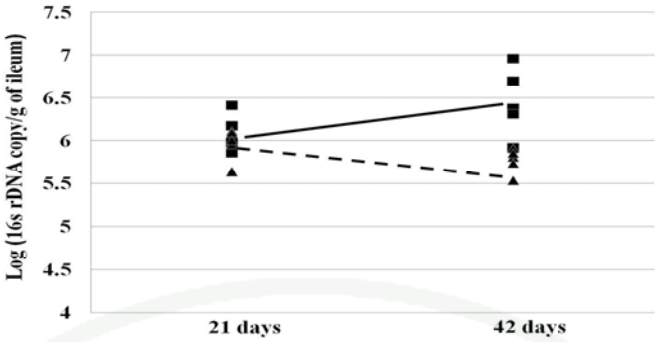

Figure 13 Total bacterial amounts (16S rDNA copy number) in the ileum of each chick from day 21 to day 42. Probiotic and control treatments are shown by square and triangle legend, respectively. The trend line of probiotic and control treatments from day 21 to day 42 were represented by solid and dash lines, respectively.16S rDNA copy number was quantified by Q-PCR using the total DNA as a template.

3.3 Effect of KUB-AC5 feeding on ileum bacterial composition

3.3.1 Phylum level analysis

The bacterial composition of ileum samples was profiled by pyrosequencing of 16S rDNA amplicons. The composition data were averaged among five chicks in each group and represented by a pie chart of phylum composition (Figure 14). Five phyla, i.e., *Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Proteobacteria* and *Firmicutes*, were found from 21 and 42-day-old chickens. In both probiotic and control groups, *Proteobacteria* and *Firmicutes* were the first and second most dominant phyla, respectively, and comprised more than 95% of the total biota together. Although the difference was not statistically significant, the relative ratio of *Firmicutes* was higher in the probiotic group (32%) than in the control group (12%). At 42d, *Firmicutes* became the most abundant in both groups, while phylum *Proteobacteria* was decreased to 1 and 9% in the probiotic and control groups, respectively.

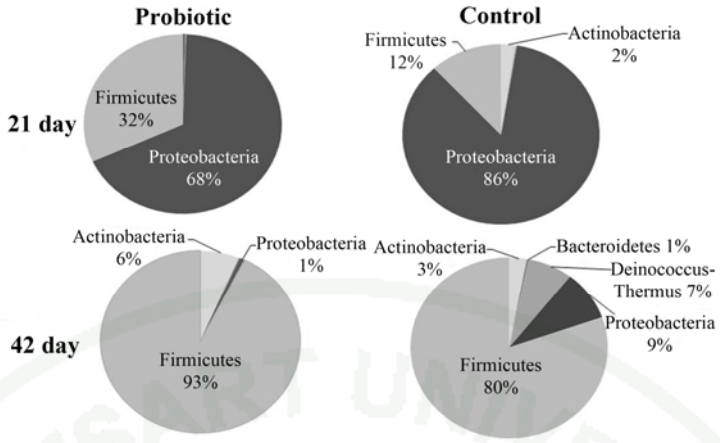

Figure 14 Pie charts presenting the phylum composition of the ileum bacterial communities. Phylum compositions were determined through 16S rDNA amplicon pyrosequencing. These data represent the average of five chickens.

The absolute population size of each phylum was calculated by multiplying the total bacterial amount determined by q-PCR by the relative ratio of each group determined by amplicon pyrosequencing (Figure 15). There was no difference in the population level of each phylum between the probiotic and control groups at day 21. However, a statistically significant difference ($p < 0.05$) was found at day 42 for phyla *Actinobacteria* and *Firmicutes.* The population level of these two phyla was higher in the probiotic group than in the control group. However, the level of phylum *Proteobacteria* was lower in the probiotic group than in the control group ($p = 0.055$) at day 42.

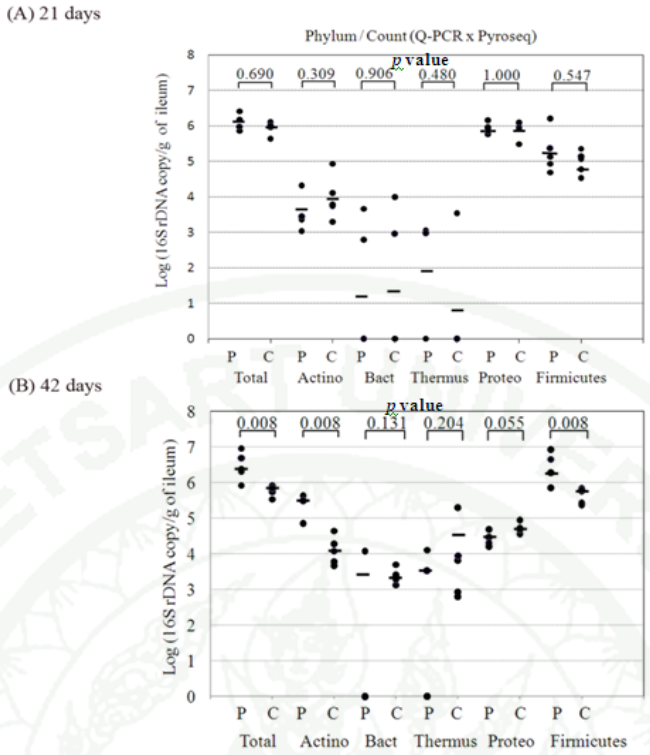

Figure 15 The population of each phylum in the chicken ileum at day 21 (A) and day 42 (B). 16S rDNA copy number of each phylum was calculated by total q-PCR count × pyrosequence relative rate for each chicken and plotted. Horizontal bars represent the average of five chickens in the probiotic group (P) or control group (C). Significant differences between the two groups were determined by a Mann-Whitney U-test, and the P-values are shown at the top of the graph.

3.3.2 Genus level analysis

Pyrosequencing data were further analyzed at the genus level. The population size of each genus was calculated in the same way as for the phyla, and the 30 most abundant genera are listed for day 21 and day 42 in Tables 15 and 16, respectively. At day 21, 31 and 38 genera were found in the probiotic and control groups, respectively. At day 21, *Erwinia* was the dominant genus, occupying more than 50% of the total population except for in one chick. The *Erwinia* belongs to the

family *Enterobacteriaceae*, is a well-known genus of plant pathogens (Lee and Yu, 2006; Mills *et al.*, 2006; Pirhonen *et al.*, 1988). This appears to originate from the feed composition components, such as soy bean meal, copra meal, corn gluten or peanut meal and grew dominantly in the early life stage. The population levels of *Erwinia* were similar in the control and probiotic groups, while the average population of the second most dominant genus, *Lactobacillus*, was one-order higher in the probiotic group than in the control. However, statistical analysis of the genuses showed no significant difference ($p > 0.05$) between the probiotic and control groups.

Table 15 The 30 most abundant genera found at day 21 in the chicken ileum.

Genus	Probiotic[gene copy][1]		Control[gene copy]		Level of significant
	Average[2]	SD[2]	Average	SD	
Erwinia	8.06E+05	3.19E+05	7.57E+05	3.19E+05	0.69
Lactobacillus	3.51E+05	5.96E+05	5.34E+04	3.90E+04	0.42
Pantoea	1.57E+04	1.27E+04	1.11E+04	4.41E+03	0.69
Brevundimonas	1.30E+04	6.03E+03	1.86E+04	8.05E+03	0.31
Achromobacter	1.17E+04	4.33E+03	1.51E+04	1.06E+04	1.00
Weissella	7.25E+03	3.44E+03	1.11E+04	7.84E+03	0.69
Leuconostoc	4.67E+03	3.29E+03	6.26E+03	5.02E+03	0.84
Enterococcus	4.19E+03	4.48E+03	8.85E+03	1.56E+04	0.84
Corynebacterium	3.35E+03	5.89E+03	1.58E+04	2.56E+04	0.29
Streptococcus	2.31E+03	3.89E+03	6.82E+02	8.23E+02	0.67
Staphylococcus	2.18E+03	2.77E+03	1.41E+04	2.72E+04	0.75
Lactococcus	2.16E+03	7.76E+02	2.38E+03	2.03E+03	0.84
Citrobacter	1.34E+03	9.51E+02	1.15E+03	1.14E+03	1.00
Bacteroides	9.19E+02	2.06E+03	1.85E+02	4.14E+02	1.00
Acinetobacter	8.27E+02	1.14E+03	2.16E+03	2.26E+03	0.59
Raoultella	6.49E+02	1.01E+03	0	0	0.18
Thermus	6.36E+02	5.85E+02	6.87E+02	1.54E+03	0.48
Bifidobacterium	5.78E+02	9.84E+02	7.32E+02	1.01E+03	1.00
Enterobacter	4.76E+02	6.53E+02	4.82E+02	8.04E+02	1.00
Nocardiopsis	4.60E+02	1.03E+03	0	0	0.42
Faecalibacterium	4.47E+02	6.12E+02	0	0	0.18

Table 15 (Continue)

Genus	Probiotic [gene copies][1]		Control [gene copies]		Level of significant
	Average[2]	SD[2]	Average	SD	
Ruminococcus	3.78E+02	8.46E+02	0	0	0.42
Lachnospiraceae Incertae Sedis	3.13E+02	4.44E+02	0	0	0.18
Escherichia	2.27E+02	5.08E+02	0	0	0.42
Brevibacterium	1.89E+02	4.23E+02	0	0	0.42
Nesterenkonia	1.89E+02	4.23E+02	0	0	0.42
Aerococcus	1.89E+02	4.23E+02	3.91E+03	8.74E+03	1.00
Bacillus f	1.89E+02	4.23E+02	0	0	0.42
Brevibacillus	1.89E+02	4.23E+02	3.43E+02	7.68E+02	1.00
Chryseobacterium	1.24E+02	2.78E+02	2.00E+03	4.47E+03	1.00

[1] calculated by total q-PCR count × pyrosequence relative rate.

[2] calculated from five chickens in each group.

At day 42, 59 and 83 genera were observed in the probiotic and control groups, respectively. Genus *Erwinia* was dramatically reduced from day 21 to 42. At this point, genus *Lactobacillus* became dominant in both the probiotic and control groups, and its level was significantly higher in the probiotic group than in the control ($p < 0.05$). This result suggested that probiotic treatment indirectly introduced the conditions for enrichment of a number of *Lactobacillus* species. This trend was more evident in the growing stage of day 42. In the control group, the two most abundant genera were *Weisella* and *Leuconostoc*, which belong to the *Lactobacillus*–containing lactic acid bacteria. However, these two genera were significantly reduced in the probiotic group compared to the control group ($p < 0.05$). Instead, *Corynebacterium* became the second most abundant genus. Altogether, 15 genera were significantly reduced in the probiotic group, while only 3 genera were enriched. However, the molecular mechanism of enrichment for these groups of bacteria in the probiotic-treated ileum remains unclear at the moment. Notably, 5 genera belonging to phylum *Proteobacteria*, e.g., *Acinetobacter*, *Aeromonas*, *Chryseobacterium*, *Citrobacter* and *Klebsiella* were reduced in the probiotic group.

Table 16 The 30 most abundant genera found at day 42 in the chicken ileum.

Genus	Probiotic[gene copy][1]		Control[gene copy]		Level of significant
	Average[2]	SD[2]	Average	SD	
Lactobacillus[a]	3.26E+06	3.05E+06	3.09E+05	2.35E+05	0.01
Weissella[b]	1.57E+04	1.75E+04	6.11E+04	1.64E+04	0.01
Leuconostoc[b]	1.08E+04	5.23E+03	4.61E+04	1.19E+04	0.01
Thermus	3.23E+03	5.51E+03	4.38E+04	8.85E+04	0.20
Lactococcus[b]	7.31E+03	5.85E+03	1.65E+04	6.42E+03	0.10
Acinetobacter[b]	2.10E+03	1.57E+03	1.29E+04	5.90E+03	0.01
Staphylococcus	5.47E+03	6.01E+03	1.07E+04	5.39E+03	0.15
Corynebacterium[a]	1.50E+05	1.31E+05	9.52E+03	1.13E+04	0.01
Citrobacter[b]	2.40E+02	5.37E+02	8.96E+03	3.27E+03	0.01
Enterococcus	9.35E+03	1.47E+04	6.47E+03	4.25E+03	0.69
Erwinia	3.09E+03	5.22E+03	3.31E+03	3.37E+03	0.59
Veillonella[b]	1.20E+02	2.68E+02	3.03E+03	1.90E+03	0.04
Caldimonas	5.66E+02	1.27E+03	2.73E+03	5.75E+03	0.61
Aerococcus[b]	1.20E+02	2.68E+02	2.66E+03	2.11E+03	0.02
Streptococcus	2.90E+03	2.36E+03	2.51E+03	1.02E+03	1.00
Achromobacter[b]	2.83E+02	6.33E+02	1.78E+03	6.19E+02	0.03
Brevundimonas	8.49E+02	1.90E+03	1.61E+03	1.50E+03	0.27
Kluyvera	1.07E+03	1.26E+03	1.56E+03	9.11E+02	0.40
Bacillus	7.48E+03	6.99E+03	1.50E+03	2.54E+03	0.14
Brevibacillusb	0	0	1.39E+03	2.59E+03	0.07
Propionibacterium[b]	0	0	1.24E+03	9.76E+02	0.03
Dietzia[a]	1.53E+04	5.99E+03	1.20E+03	1.02E+03	0.01
Raoultella	2.53E+03	5.33E+03	1.16E+03	3.38E+02	0.14
Helicobacter[b]	0	0	1.12E+03	1.19E+03	0.07
Arcobacter	3.87E+02	8.66E+02	1.12E+03	1.05E+03	0.18
Klebsiella[b]	0	0	1.06E+03	6.44E+02	0.02
Microvirgula	3.87E+02	8.66E+02	9.28E+02	3.90E+02	0.13
Enhydrobacter[b]	0	0	9.24E+02	1.04E+03	0.07
Chryseobacterium[b]	0	0	9.18E+02	7.67E+02	0.02
Aeromonas[b]	0	0	8.99E+02	9.57E+02	0.02

Table 16 (Continute)

Genus	Probiotic[gene copy][1]		Control[gene copy]		Level of
	Average[2]	SD[2]	Average	SD	significant
Enhydrobacter[b]	0	0	9.24E+02	1.04E+03	0.07
Chryseobacterium[b]	0	0	9.18E+02	7.67E+02	0.02
Aeromonas[b]	0	0	8.99E+02	9.57E+02	0.02

[1] calculated by total q-PCR count × pyrosequence relative rate.

[2] calculated from five chickens in each group.

[a,b] significantly higher and lower level ($p \leq 0.05$) in probiotic groups, respectively.

3.3.3 Species level analysis.

The pyrosequence data were further analyzed for the species level, and the data are depicted in a pie chart for each chicken (Figure 16). The data show that the abundance of *Lactobacillus* was remarkably increased from day 21 to day 42. Except for in one chicken, some predominant *Lactobacillus* species were found in all tested chicken samples from both the probiotic and control groups at day 42.

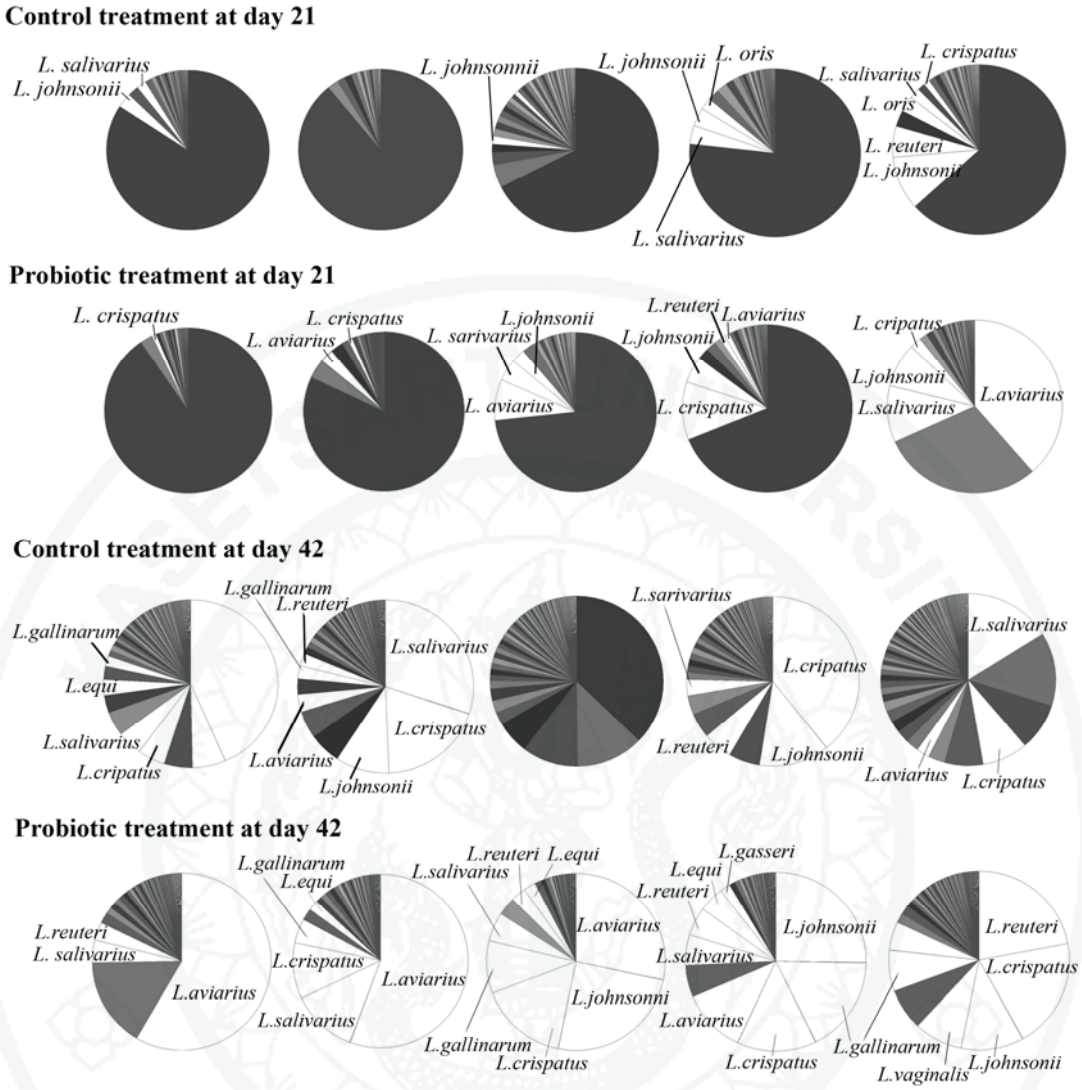

Figure 16 Pie charts representing the bacterial species composition in each chicken ileum sample at day 21 and day 42. *Lactobacillus* species are colored white and labeled by species name.

Population changes of each *Lactobacillus* species from day 21 to day 42 are shown in Figure 17. Most *Lactobacillus* species increased from day 21 to day 42, and their levels were mostly higher in the probiotic group than in the control. In particular, *L. aviarius* was markedly enriched in the probiotic group (p = 0.016).

It is also interesting that the administration of KUB-AC5 could increase the diversity of *Lactobacillus* species in the ileum. High microbial diversity is beneficial because high diversity of microorganisms indicates a healathy balance. In contrast, an imbalance promotes overgrowth of pathogenic or nonbeneficial microbes, and a disease may emerge (Karlsson *et al.*, 2010). These findings suggested that supplementation of *L. reuteri* KUB-AC5 recruited a gut environment favorable for the colonization and growth of certain groups of lactobacilli.

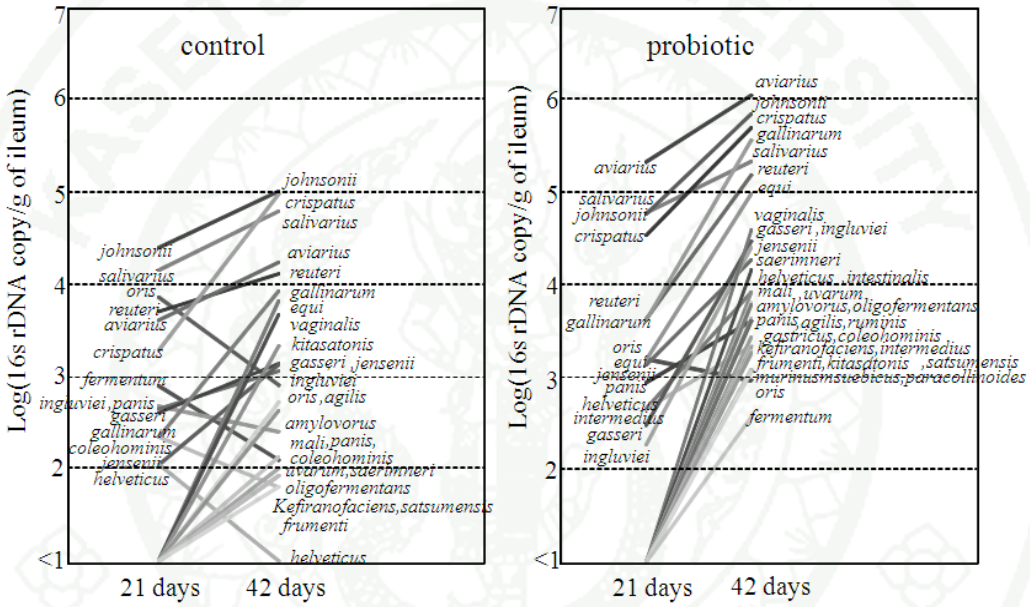

Figure 17 The population change of each *Lactobacillus* species from day 21 to day 42. 16S rDNA copy number of each species was calculated by total q-PCR count × pyrosequence relative rate. The data from five chicken samples in each group were averaged.

The level of *L. reuteri* was also quantified by Taqman PCR targeting the *L. reuteri* 16S rDNA sequence. The amount of rDNA at day 21 was not different between the probiotic and control groups, which suggests that KUB-AC5 did not efficiently colonize the ileum. However, the population level of *L. reuteri* increased from day 21 to day 42 only in the probiotic group and was significantly

higher than that of the control group at day 42 (Figure 18). Although it is unclear whether the increased *L. reuteri* were strain KUB-AC5 or a different strain.

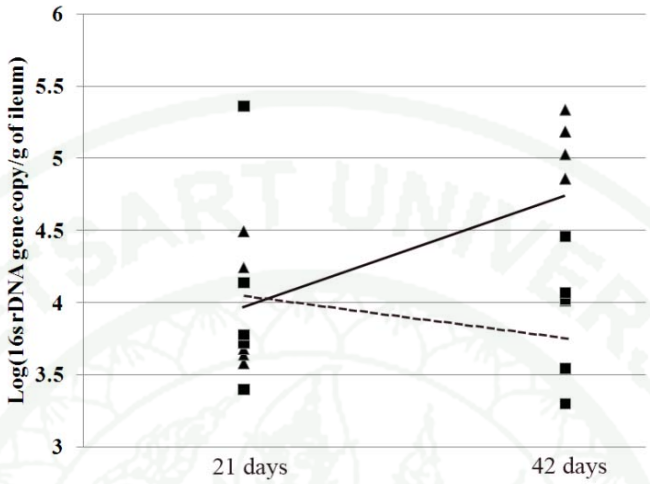

Figure 18 Population of *L. reuteri* from day 21 to day 42 in a probiotic treated-chicken [triangle] and the control [square]. The trend line of probiotic and control treatments from day 21 to day 42 were represented by solid and dash lines, respectively. *L. reuteri* 16S rDNA in each ileum sample was quantified by TaqMan PCR.

3.3.4 Antagonism of *Lactobacillus* against potential pathogenic bacteria existing in GI tract

As mentioned above, the effect of KUB-AC5 supplementation during feeding in the first week of life appeared at the growing stage of day 42 to enhance both the level and diversity of lactobacilli in the ileum. The correlation between the number of *Lactobacillus* and those pathogenic bacteria were analyzed (Figure 19). This enrichment effect of *Lactobacillus* was observed as a suppression of some non-beneficial bacterial groups including genus *Klebsiella*, genus *Chryseobacterium*, genus *Citrobacter*, genus *Aeromonas,* genus *Acinetobacter* and order *Campylobacterales*. Thus, *Lactobacillus* antagonism against five genera and one order in a later stage was indicated. It is well-known that genus *Aeromonas* and

order *Campylobacterales* are food-borne pathogens that are often found in chicken meat (Sachan and Agarwal, 2000; Sarimehmetoglu and Kuplulu, 2001; Willis and Reid, 2008). Notably, *Campylobacter* is the most common food-borne pathogen worldwide (Kilonzo-Nthenge *et al.*, 2008); it causes a gastroenteritis characterized by watery and bloody diarrhea. In the meat industry, poultry is an important source of human *Campylobacter* infection. It has been reported that chicken meat is frequently contaminated with *Campylobacter* (Bull *et al.*, 2006). The harmful *Campylobacter* colonizes the chicken intestine without any apparent harm to the birds. Its effect is only observed on human consumers. In addition, *Citrobacter* and *Chryseobacterium* occasionally cause food spoilage (Bayan M. and Abu-Ghazaleh, 2006; De Beer *et al.*, 2005) and *Klebsiella* and *Acinetobacter* have been found in chicken meat (Brisse and Duijkeren, 2005; Rathinavelu *et al.*, 2003b). KUB-AC5 antagonism against these non-beneficial groups of bacteria has been suggested for its potential value to the poultry industry.

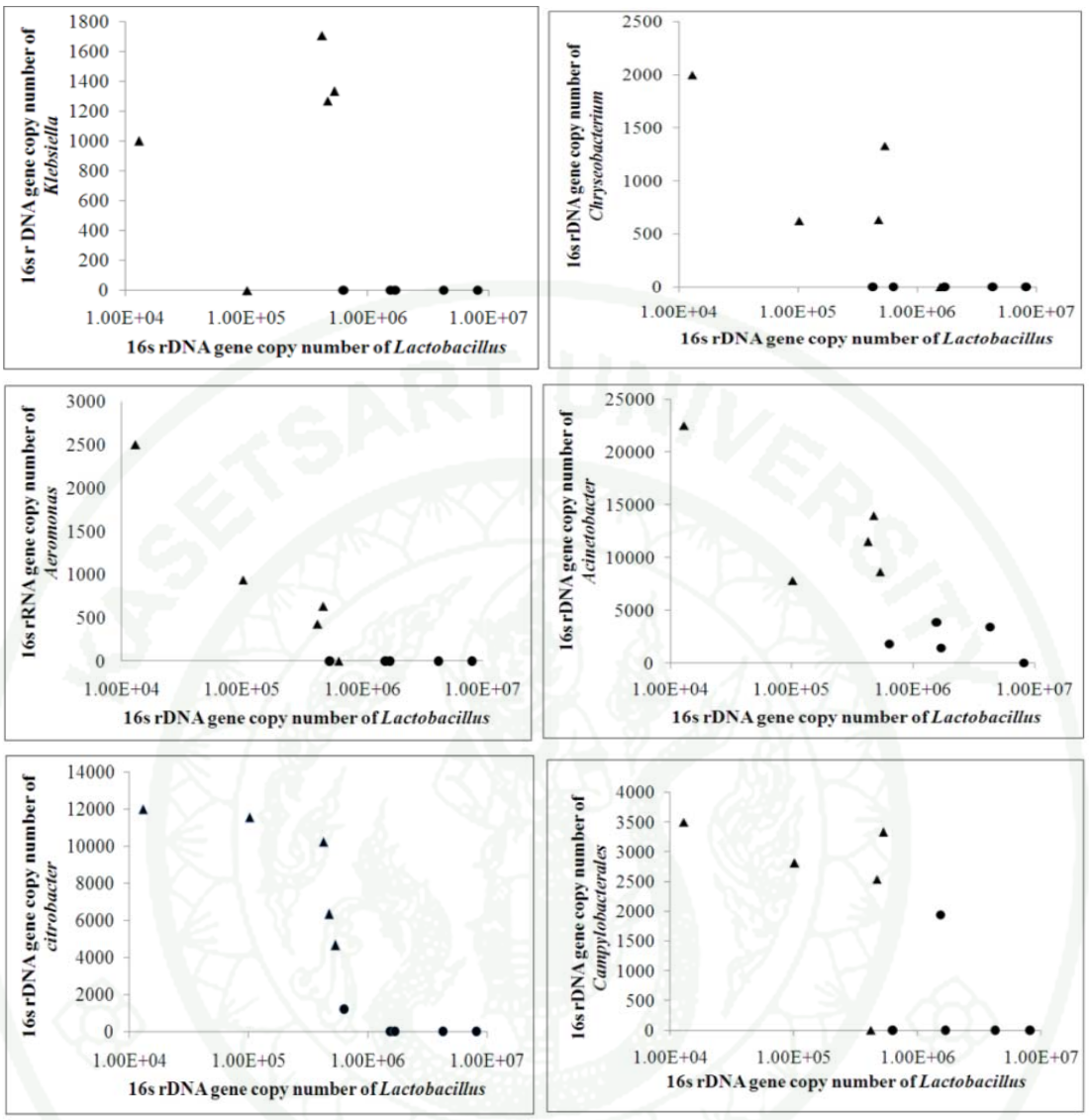

Figure 19 The antagonism of genus *Lactobacillus* to genus *Klebsiella*, genus *Chryseobacterium*, genus *Aeromonas,* genus *Acinetobacter,*genus *Citrobacter* and order *Campylobacterales*. The population size of each bacterial group was represented by 16S rDNA copy number calculated by total q-PCR count × pyrosequence relative rate. A pair of two different bacterial populations in each chicken sample is plotted in the two-dimensional graph. Circles and triangles represent probiotic-treated chicken and control chicken, respectively.

In conclusion, 5 log CFU/g feed of KUB-AC5 given as a single dose over the course of 7 days had a slight effect on chicken weight gain but greatly influenced the

ileum microbiota in growing stage of day 42. The mode of action of this KUB-AC5 probiotic is unknown. It was probably that KUB-AC5 played a role in low activities of adherence, antimicrobial activity or immune stimulation in broiler chicken proposed by Castillo *et al.* (2011). Further studies may be needed. In addition, provision of higher doses and/or longer-term feedings of KUB-AC5 should be also considered to improve growth performance.

4. Monitoring the effect of crude protein and essential amino acids on the growth and intestinal microflora of broiler chicken

Crude protein (CP) is an important component in feed ingredient and there is directly effect on chicken growth and health through intestinal microbiota (Rama Rao *et al.*, 1999; Sterling *et al.*, 2003). Therefore the monitoring of microbiota change according to the effect of CP might improve the feed formulars affecting on chicken growth and feeding efficiency in poultry production. In Thailand, soybean meal was a favorite CP source (Association of Animal Feed Mill of Thailand, 2000). The increasing of CP contents in feed formular leading to the chicken growth and microbial changes in GI tract is a hypothesis. In this study, three treatments of soybean meal based-diets containing 22% CP (the control), soybean meal based-diets containing 28% CP (high CP treatment) and soybean meal based-diets containing 22% CP as well as additional essential amino acid of methionine, lysine and threonine to obtain similar concentration as high CP diet (EAA treatment) were carried out. All diet formulars were formulated according to NRC recommendation (1994). These three diets were fed to chicken age of 1-21 d. Then, the diets were changed to the same feed formular containing 20% CP during the age of 21-35 d.

4.1 Broiler performance response to CP level

During 1 d – 35 d, the weekly weight gain, feed consumption and feed conversion ratio are represented in Table 17. At first week, feed consumption from the control and the EAA treatment were significant higher than the high CP treatment (p = 0.001). After the diets change to finisher stage at fourth week, the feed

consumption of high CP and EAA treatment were significantly higher than control. However, the body weight gain and feed conversion ratio from three feed formula were not significant different ($p > 0.05$).

Increasing of villi height caused the capability of greater absorption of available nutrients in intestine effecting to high growth performance (Samanya and Yamauchi, 2002). Villi size of jejunum and ileum were therefore determined at week 3 and 5 (Table 17). In jejuna samples, the villi of control treatment were the highest comparing to other treatments for both weeks 3 and 5 ($p < 0.05$). Whereas villi of ilea sample were similar in all treatment ($p \geq 0.05$). Laudadio *et al.* (2012) observed that villi height of bird fed with hyponutrient increased at duodenum and ileum resulting in improving absorbtion area in intestine. Therefore, in these studies the increasing of villi height in hypernutrient of high CP and EAA treatment might not necessary. However there was no significant interaction between jejunal villi height and growth performance in chicken.

There were many investigators reported that dietary protein level induced better growth performance and improved feed efficiency (Cahaner *et al.,* 1995; Jackson *et al.,* 1982; Rosebrough *et al.,* 1999). However, in this study, increasing CP and amino acid did not effect on the growth of chicken which corresponded to other results carried out by Smith *et al.* (1998); Summers *et al.* (1992); Parsons and Baker (1982). The growth performance of poultry did not only depend on protein concentration but it was also other factors such as energy content and balance of amino acid in feed (Dahiya *et al.,* 2005, 2007; Van Tuan, 2005). This was probably the reason that chicken performances of three treatments studied were similar.

Table 17 Body weight, feed consumption, feed conversion ratio and villi height observed in broiler chickens fed with control, high CP and EAA treatment. The parameter values were calculated from the mean value of each treatment. There were 300 chickens per treatment.

Parameter	**Week**	**Control**	**High CP**	**EAA**	***P* value**
Body weight gain	1	126.11 ± 7.01	118.97 ± 8.09	121.17 ± 12.19	0.236
(g/week)	2	327.67 ± 14.60	318.50 ± 10.23	321.00 ± 16.85	0.341
	3	529.07 ± 24.14	531.46 ± 24.66	540.69 ± 18.77	0.491
	4	741.00 ± 10.58	749.26 ± 28.88	729.28 ± 21.18	0.591
	5	742.23 ± 42.33	750.43 ± 42.58	736.64 ± 45.44	0.777
	Overall	2466.1 ± 53.8	2458.9 ± 57.9	2468.8 ± 60.2	0.920
Feed consumption	1	169.41^{a} ± 4.82	158.40^{b} ± 7.05	167.58^{a} ± 6.34	0.001
(g/week)	2	447.43 ± 12.83	461.29 ± 14.98	462.28 ± 24.29	0.140
	3	817.17 ± 61.60	804.28 ± 60.63	827.64 ± 73.87	0.729
	4	1177.49^{b} ±	1212.98^{a} ±	1203.58^{ab} ±	0.026
	5	16.19	29.15	36.04	0.467
	Overall	1359.03 ±	1385.95 ±	1363.59 ±59.67	0.680
		39.09	53.42	4023.7 ± 144.0	
		3985.4 ± 97.1	4022.9 ± 82.5		
Feed conversion	1	1.35 ± 0.06	1.33 ± 0.05	1.39 ± 0.12	0.260
ratio (F/G)	2	1.41 ± 0.07	1.40 ± 0.04	1.44 ± 0.04	0.370
	3	1.54 ± 0.08	1.52 ± 0.13	1.53 ± 0.10	0.835
	4	1.59 ± 0.03	1.62 ± 0.06	1.61 ± 0.04	0.348
	5	1.83 ± 0.07	1.85 ± 0.10	1.85 ± 0.09	0.856
	Overall	1.62 ± 0.03	1.64 ± 0.04	1.63 ± 0.04	0.460
Villi(mm)Jejunum	3	34.46^{a} ± 5.61	24.56^{b} ± 5.23	26.52^{b} ± 4.60	0.000
Ileum		28.70 ± 2.96	25.67 ± 4.58	27.42 ± 5.07	0.302
Jejunum	5	46.04^{a} ± 7.56	45.80^{ab} ± 3.80	40.07^{b} ± 7.68	0.091
Ileum		31.69 ± 4.32	32.06 ± 4.14	30.97 ± 3.94	0.837

^{a,b}Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

4.2 Development of standard curve by real time PCR assays

The real time PCR assays were successfully established for quantification of total bacteria and other interesting bacterial groups of *Lactobacillus* group, *Campylobacter*, *Acinetobacter*, *Psedomonus*, *Bifidobacterium*, *Bacteriod*es – *Prevotell* - *Porphyromonas* group, *C. coccoides–E. rectal* group, *C. perfringens* group and *Enterobacteriaceae* found in chicken gastrointestinal tract. The serial dilute standard DNA of 10 bacterial groups were presented in Figure 20 to Figure 29. The amplification efficiencies individual assays were nearly perfect amplification at 1.8 - 2.1. In addition, the error values which measure accuracy of quantification were below 99%. Base on the results, they were considered as acceptable for accurate quantification. The melting temperature (Tm) of desired PCR amplicon varied according to the complexity of intestinal flora population consisting of numorous variety of bacterial species or strain within the same group. The established assay could detect as low as 10^2 – 10^4 copy of specific bacterial 16s rDNA per one reaction, corresponding to 10^3 – 10^5 copy per gram wet weight of chicken intestine.

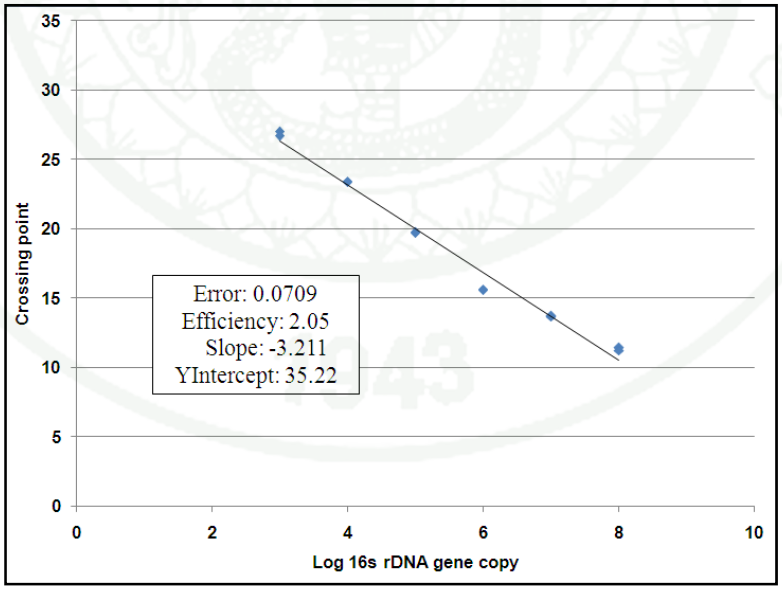

Figure 20 Standard curve of total bacteria using HAD peimer.

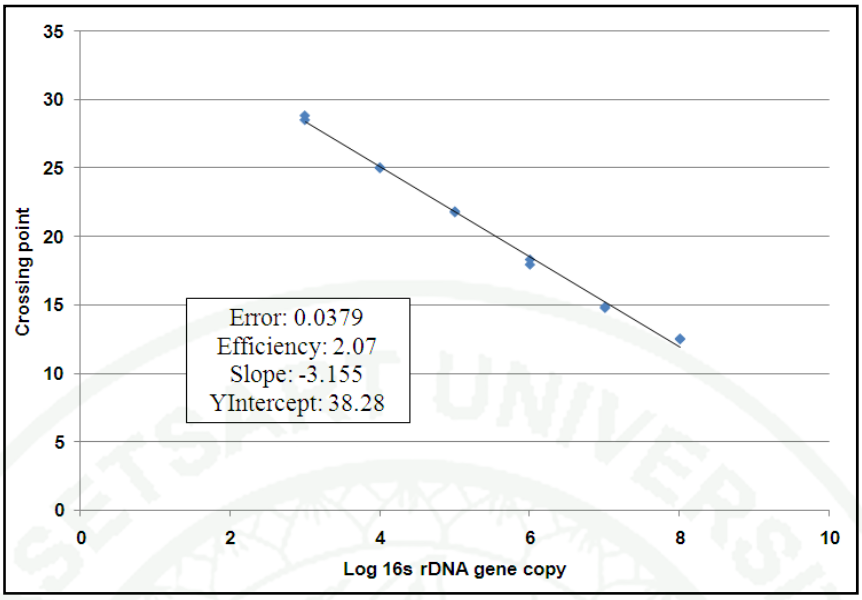

Figure 21 Standard curve of *Lactobacillus* group using LbF and LbR primers.

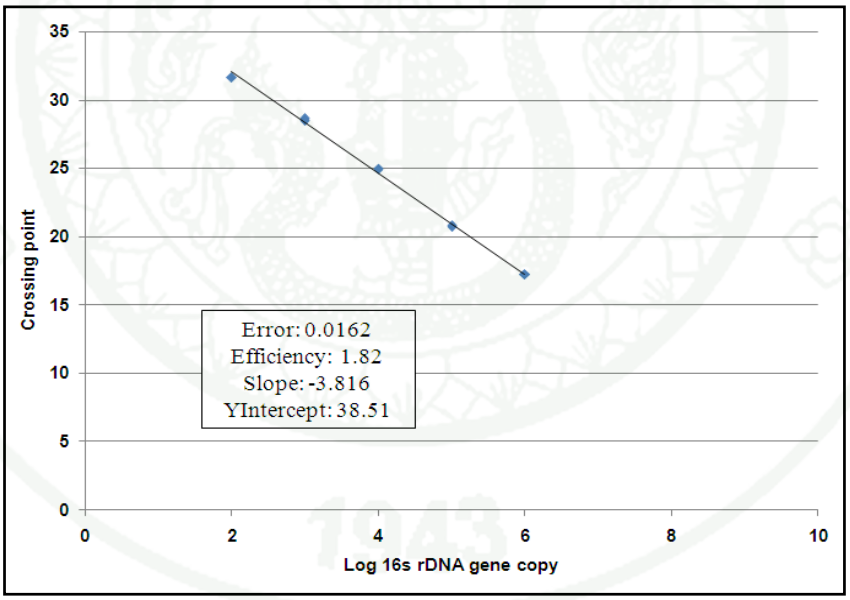

Figure 22 Standard curve of *Campylobacter* spp group using CamF and CamR primer.

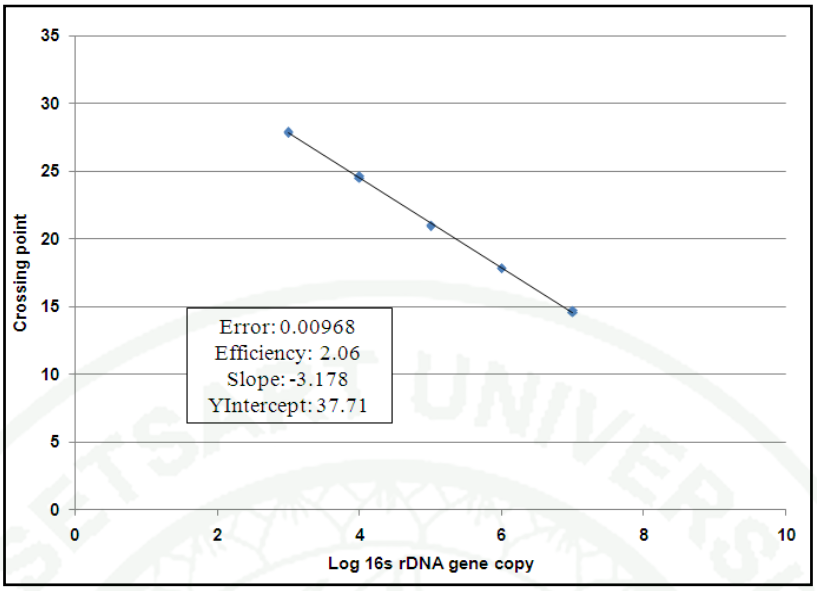

Figure 23 Standard curve of *Acinetobacter* group using AcF and AcR primer.

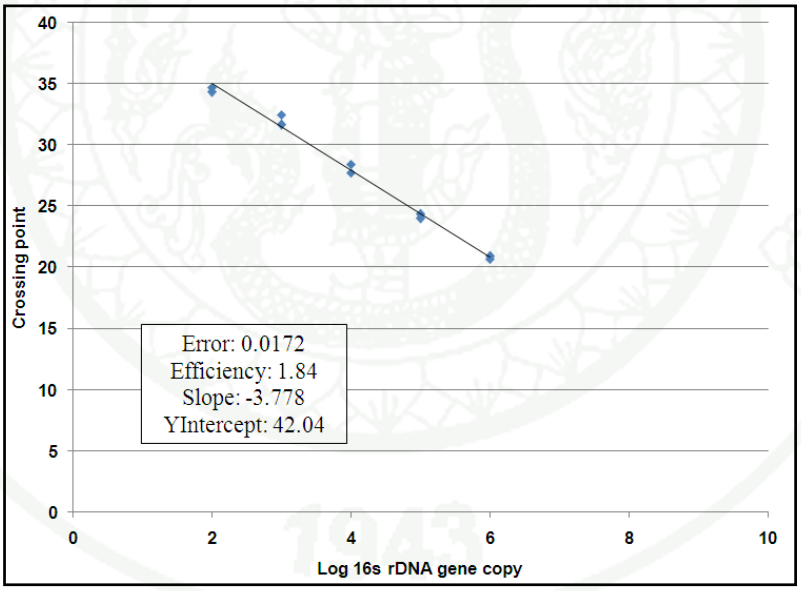

Figure 24 Standard curve of *Pseudomonas* group using PseF and PseR primer.

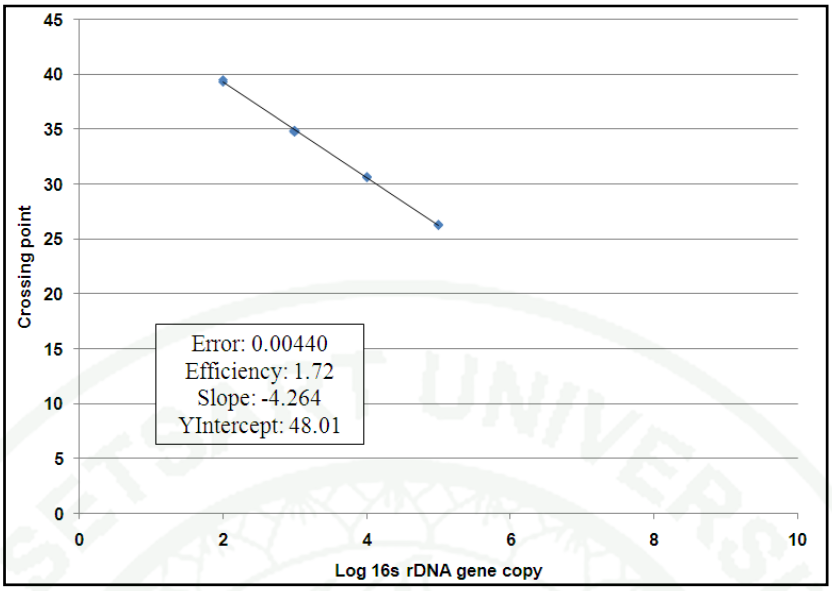

Figure 25 Standard curve of *Bifidobacterium* spp group using BifF and BifR primer.

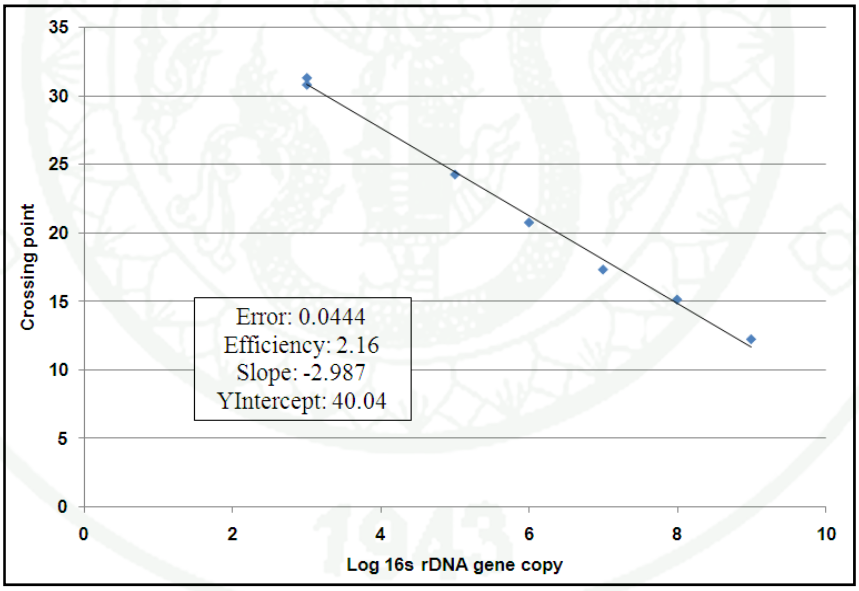

Figure 26 Standard curve of *Bacteriodes – Prevotell- Porphyromonas* group using BacPF and BacPR primer.

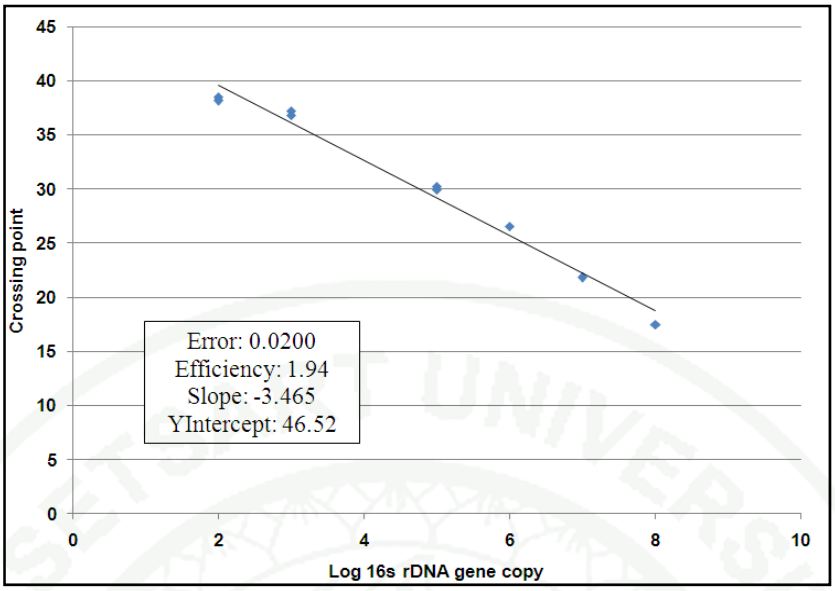

Figure 27 Standard curve of *C. coccoides – E. rectal* group using ClosF and ClosR primer.

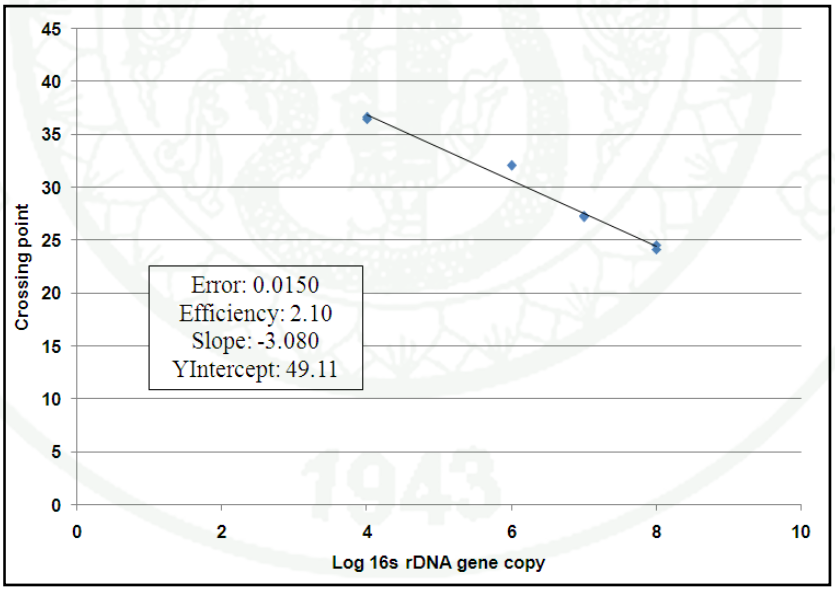

Figure 28 Standard curve of *C. perfringens* group using PerfF and PerfR primer.

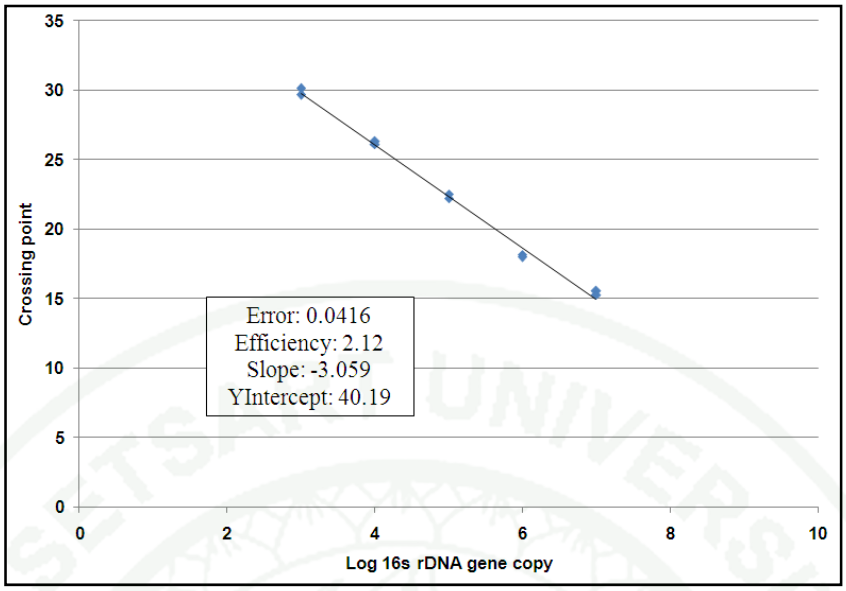

Figure 29 Standard curve of *Enterobacteriaceae* group using EnF and EnR primer.

4.3 Effect of CP and EAA on intestinal bacteria at starter stage

Total bacteria and 9 bacterial groups from jejunum, ileum and cecum were analysed by real time PCR. Total bacteria and specific bacterial group which mostly found in chicken intestine were quantified according to region of 16s rDNA gene (Gong *et al.*, 2007; Kiess *et al.*, 2010; Rathinavelu *et al.*, 2003a; Thitaram *et al.*, 2005; Zhu *et al.*, 2002). At chicken age of 21 d, twenty birds per treatment were randomly sampled. Considering total bacteria in jejunum, the total number of 16s rDNA genes between three treatments were not significant difference ($p > 0.05$) (Table 18). *Bacteroides-Prevotell* group, *Lactobacillus* group and *Pseudomonas* group were observed in this region as well. The *Pseudomonas* group was not found in control treatment but it could detect at 10 % of sampling in high CP and EAA treatment without significant difference among these three treatments. The populations of *Bacteroides-Prevotell* group and *Lactobacillus* group from high CP treatment significantly reduced ($p < 0.05$). The reduction of *Lactobacillus* group was significant occurred in EAA treatment as well ($p < 0.05$). However, a little percentage reducing at 10.28% and 4.51% were observed in high CP and EAA treatment, respectively. No phatogenic bacteria were observed in three treatments.

In ileum, the total bacteria were significantly promoted in EAA treatment ($p < 0.05$) (Table 19). Comparing to jejunum, the microbial community had greater diversity which contained *Lactobacillus* group, *Bacteroides-Prevotell*, *C. coccoides–E. rectal* subgroup, *C. perfringens* group and *Enterobacteriaceae*. The *C. perfringens* and *Enterobacteriaceae* which compose of several pathogenic species were found in all treatments at the similar concentrations (Tillman *et al.*, 2011). The amimal CP source containing high glycine content could enhance the growth of *C. perfringens* causing subclinical necrotic enteritis in chicken (Dahiya *et. al.,* 2007; Wilkie *et al.,* 2005) while high CP from soy bean had no effect on its growth corresponding to this study (Drew *et al.*, 2004). Interestingly, the copy number of *C. coccoides–E. rectal* group in ileum sample of high CP and EAA treatment were significantly higher than the control for two times ($p = 0$). It was possible that high percentage of these amino acids in diets promote the growth of *C. coccoides–E. rectal* group. However some species of the following genus belonging to *C. coccoides–E. rectal* group, *Clostridium, Eubacterium, Ruminococcus, Bytyrivibrio* and *Lachnospira* were non – pathogenic group (Rinttilä *et al.,* 2004). Therefore increasing of their cell numbers had no effect on chicken health. In addition, the copy number of *Lactobacillus* group was dominant in all treatments, and its concentration level of high CP treatment was significantly higher than control and EAA treatment ($p = 0$). The *Lactobacillus* group consisted of microbes in genera *Lactobacillus Weissella, Leuconostoc* and *Pediococcus* that mostly benefit to gut host (Rinttilä *et al.*, 2004; Tillman *et al.*, 2011). These results suggested that enrichment of both CP and EAA in feed content were no effect on phatogen group and it could promote the growth of beneficial bacteria in ileum.

In caecal region, the copy number of total bacteria from three treatments were not significantly different ($p > 0.05$). However, its numbers were the highest among three intestinal regions of jejunum, ileum and cecum (Table 20). Unlike ileum, number of *C. coccoides–E. rectal* group in control treatment was significant greater than high CP treatment ($p \leq 0.05$) and similar to EAA treatment ($p > 0.05$). In addition, high CP treatment was significantly reduced pathogenic bacteria in *Enterobacteriaceae* group ($p \leq 0.05$). This effect was not detected in EAA treatmet. It

deduced that the enrichment of 28% CP could inhibit the growth of *Enterobacteriaceae* group in cecum. Notably, no *Campylobacter* group was detected in any sample from EAA treatment, but 60% and 30% of control and high CP sample, respectively were detected at concentration level of 7.08×10^4 to 1.07×10^6 16s rDNA copy per gram. Dahiya *et al.* (2007) reported that 0.8% methionine supplement reduced colifrom, *C. perfringer* and *Streptococcus* group D in ileum. Since both EAA diet and high CP diet contained additional essential amino acid of lysine, methionine and threonine. It was, therefore, possible that enrichment of essential amino acid possibly reduced the number of *Campylobacter* group. However, the inhibition activity from in EAA treatment had more efficiency than 28% CP treatment. The *Campylobacter* can colonize in chicken intestine without any apparent harmful to the birds. Its enteritis effect is only observed on human consumers (Bull *et al.,* 2006). For other bacterial groups of *Lactobacillus* group and *Bacteroides-Prevotell* group, no significant difference between three treatments was observed ($p > 0.05$).

Table 18 Microbial quantification of jejunal samples from 21 day of chicken.

Bacteria	Control (n = 10)					High CP (n = 10)					EAA (n = 10)					P-value
	Mean*	SD	Min	Max	Incidence#	Mean	SD	Min	Max	Incidence	Mean	SD	Min	Max	Incidence	
All Eubacteria	7.68	0.28	7.36	8.20	10	7.33	0.40	6.25	7.57	10	7.66	0.46	6.94	8.25	10	0.102
Lactobacillus group	7.98[a]	0.19	7.75	8.22	10	7.16[c]	0.31	6.57	7.60	10	7.62[b]	0.34	7.04	8.00	10	0.000
Campylobacter spp.					0					0					0	na
Pseudomonas					0	3.35		3.35	3.35	1	4.03		4.03	4.03	1	na
*Bacteriod*es – *Prevotell Group*	4.57[a]	0.25	4.34	4.96	6	4.27[b]	0.37	3.64	4.68	10	4.58[a]	0.17	4.25	4.92	10	0.035
C. coccoides– E. rectal group					0					0					0	na
C. perfringens group					0					0					0	na
Enterobacteriaceae					0					0					0	na

na, not applicable.

* log no. of 16s rDNA gene copy per gram.

Number of detected chicken.

[a,b]Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

Table 19 Microbial quantification of Ilea samples from 21 day of chicken.

Bacteria	Control (n = 10)					High CP (n = 10)					EAA (n = 10)					P-value
	Mean*	SD	Min	Max	Incidence#	Mean	SD	Min	Max	Incidence	Mean	SD	Min	Max	Incidence	
All Eubacteria	7.41[b]	0.48	6.43	7.98	10	7.33[b]	0.34	6.81	7.82	10	7.96[a]	0.21	7.72	8.34	10	0.001
Lactobacillus group	6.37[b]	0.53	5.10	6.87	10	7.58[a]	0.47	6.85	8.29	10	6.33[b]	0.28	6.29	7.14	10	0.000
Campylobacter spp.					0					0					0	na
Pseudomonas					0					0					0	na
*Bacteriode*s – *Prevotell Group*	4.00	0.49	3.62	5.12	9	4.47	1.11	3.40	6.76	8	3.82	0.22	3.56	4.12	7	0.215
C. coccoides– E. rectal group	3.84[b]	0.29	3.30	4.26	9	6.46[a]	0.65	5.21	7.42	10	6.81[a]	0.45	5.91	7.60	10	0.000
C. perfringens group	6.27	0.28	5.85	6.62	10	6.36	0.33	5.71	6.83	10	6.44	0.15	6.21	6.63	10	0.375
Enterobacteriaceae	5.69	0.70	5.14	7.16	10	5.96	0.93	4.01	6.90	10	5.42	0.87	4.01	6.78	10	0.374

na, not applicable.

* log no. of 16s rDNA gene copy per gram.

Number of detected chicken.

[a,b]Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

Table 20 Microbial quantification of caeca samples from 21 day of chicken.

Bacteria	Control (n = 10)					High CP (n = 10)					EAA (n = 10)					P-value
	Mean*	SD	Min	Max	Incidence#	Mean	SD	Min	Max	Incidence	Mean	SD	Min	Max	Incidence	
All Eubacteria	9.55	0.24	9.28	10.03	10	9.37	0.24	9.05	9.73	10	9.65	0.37	8.85	10.06	10	0.114
Lactobacillus group	8.44	0.52	7.53	9.11	10	8.30	0.44	7.26	8.78	10	8.74	0.67	7.95	10.27	10	0.202
Campylobacter spp.	5.51	0.41	4.89	6.03	6	5.23	0.34	4.85	5.52	3					0	0.356
Pseudomonas					0					0					0	na
*Bacteriode*s – *Prevotell Group*	9.02	0.63	8.05	9.91	10	9.00	0.41	8.32	8.45	10	9.21	0.55	8.28	9.85	10	0.626
C. coccoides– E. rectal group	9.34[a]	0.30	8.92	9.94	10	8.96[b]	0.34	8.41	9.33	10	9.28[ab]	0.51	8.46	9.87	10	0.084
C. perfringens group					0					0					0	na
Enterobacteriaceae	7.32[a]	0.39	6.92	7.68	10	6.57[b]	0.57	5.77	7.56	10	7.09[a]	0.66	6.10	8.12	10	0.016

na, not applicable.

* log no. of 16s rDNA gene copy per gram.

Number of detected chicken.

[a,b]Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

4.4 Effect of CP and EAA on short chain fatty acid at starter stage

SCFA in jejunum, ileum and cecum were analyzed using HPLC technique (Figure 30). These are end products of metabolic activities of gut bacteria depending on their diet composition (Belenguer *et al.*, 2007). In the present studies, lactic acid and propionic acid were detected in three regions whereas acetic acid was detected only in cecum. Bacterial fermentation mainly occurs in cecum and leads to produce many type of SCFA (Meimandipour *et al.*, 2010). In the current study, many type of SCFA was detected in cecum as well.

In jejunum, the concentration level of lactic acid and propionic acid were similar in three treatments. The concentration of lactic acid obtained from these 3 treatments were higher than the one of propionic acid for about two times. Relating to previous q-PCR results, the lactic acid producing bacteria belonging to *Lactobacillus* group was also a dominant bacteria in jejunal samples which supported high lactic acid concentration obtained.

The effect of additional CP and EAA on SCFA production could observe in both ileum and cecum. In ileum, high CP treatment showed a significant greater amount of lactic acid compared to the control ($p \leq 0.05$) but it was similar to the EAA treatment. These results suggested that enrichment of EAA in high CP and EAA treatment could promote high number of *Lactobacillus* group and *C. coccoides–E. rectal* group previously reported and produce lactic acid as a main product. Chiba (2009) reported that some genus belonging to *C. coccoides–E. rectal* group such as *Lachnospira* can also produce lactic acid in intestine which supported this study. Considering to another acid, propionic acid, only about a half concentration of 19 - 23 μmol/g wet weight comparing to lactic acid concentration was observed. However, the propionic acid concentration occurred in ileum treated by these three treatments showed no significant difference.

In cecum, SCFA production was more complicate than other regions because many types of microbial fermentation had occurred. Three SCFA of lactic

acid, propionic acid and lactic acid were detected at the concentration of 6.7 - 12, 13 - 14 and 35 – 42 μmol/g wet weight, respectively as shown in Figure 30. Interestingly, the concentration of acetic acid was higher than lactic acid and propionic acid for 4.5 and 3 times, respectively. The concentrations of acetic acid detected from these three treatments were not significantly different. When microbiota previously observed were taken into account, it was noticed that the high number of both *Bacteroides-Prevotell* group and *C. coccoides–E. rectal* group of more than 9 log copy number had occurred. *Bacteroides-Prevotell* group is able to metabolize different carbon source to produce acetic acid as a major product while some genus in *C. coccoides–E. rectal* group especially genus *Clostridium* and *Ruminococcus* can utilize lactic acid and produce acetic acid in fermentation process (Chiba, 2009). These findings indicated that high acetic acid might be mainly from the metabolism of these two bacterial groups.

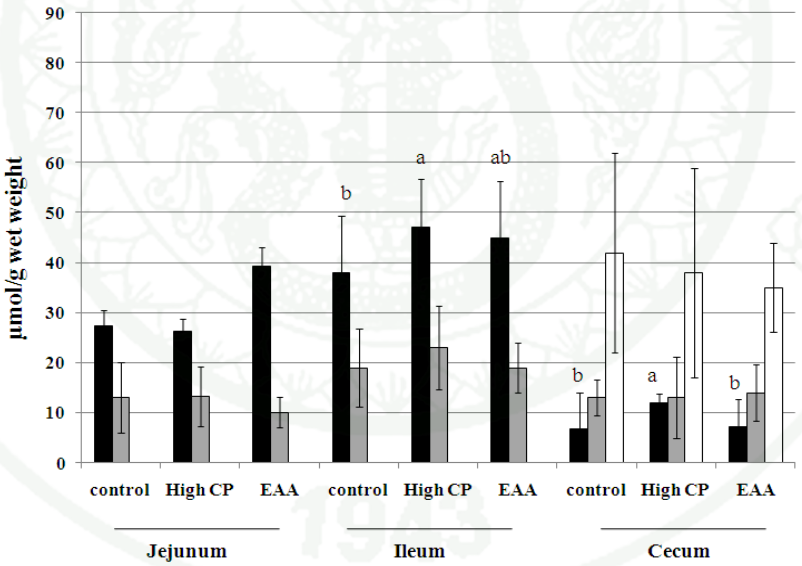

Figure 30 SCFA concentration from digesta of chicken intestine at 21 d. The black bars, gray bars and white bars are represented lactic acid, propionic acid and acetic acid, respectively. The error bars indicate standard derivation from each subject group (n=10).

[a,b]Different letter superscripts indicated a significant difference in each region ($p \leq 0.05$).

4.5 Effect of CP and EAA on intestinal bacteria at finisher stage

After 21 d, the same diet formular was fed to the chicken of three experimental treatments and microflora quantities were further analyzed at 35 d. In this stage, the CP contents were reduced to 20%. Although the diet ingredients were changed to the same in finisher stage, the intestinal microflora between three treatments were a bit different.

Considering all member of Eubacteria in jejunal samples, the total amount of bacteria in high CP and EAA treatment was significant higher than control (p = 0.026) (Table 21). In jejunum, four groups of bacteria including *Bacteriodes-Provotell*, *Campylobacter*, *C. coccoides–E. rectal* and *Lactobacillus* were observed at 35 d while *Pseudomonas, C. perfringens* group and *Enterobacteriaceae* were not detected. Similar to 21 d, *Lactobacillus* group was a dominant bacteria in this region. However, amount of them in high CP and EAA treatment became significant higher than the control (p = 0.031) and suppressed the growth of *Campylobacter* to 5.56 and 4.66 log copy number which were lower than the control with no significant difference (p > 0.266). These results suggested that jejunum condition of the high CP and EAA treatment at the finisher stage still supported the growth of *Lactobacillus* group and potentially suppressed the one of *Campylobacter*.

In ileum, all bacterial groups were detected from three treatments studied except *C. perfringens* group (Table 22). There was no significant of all eubacteria gene copies between three treatments was observed. *Pseudomonas* group was detected at only 20% in control while *Enterobacteriaceae* group was at 10% and 20% in control and high CP treatment, respectively. However, no significant different in all bacterial specific groups was observed at finisher stage (p > 0.05).

In ceeca samples, all bacterial groups found were similar to ilea samples (Table 23). All eubacteria of control and high CP treatment was the highest (p = 0.025). The population size of *C. coccoides–E. rectal* had become the major group for all treatments. Its number in the high CP treatment were significant greater than the

control and EAA treatment (p = 0.039). These results indicated that suppression of CP enrichment on *C. coccoides–E. rectal* group did not occurred when diet changing to finisher stage. However the other bacterial groups from three treatments were similar ($p > 0.05$).

It was noticed that both *Acinetobacter* and *Bifidobacterium* group were not observed at both 21 d and 35 d. According to healty chicken and probiotic experiment, these bacteria could detect at low concentration around 10^2 copy numbers. The limitation of real time PCR in this study was 10^2 - 10^3 copy numbers. Therefore, it was possible that their amount were lower than the limitation detection allowed by real time PCR analysis.

Table 21 Microbial quantification of jejunal samples from 35 day of chicken.

Bacteria	Control (n = 10)					High CP (n = 10)					EAA (n = 10)					P-value
	Mean*	SD	Min	Max	Incidence#	Mean	SD	Min	Max	Incidence	Mean	SD	Min	Max	Incidence	
All Eubacteria	7.89[b]	0.45	7.26	8.44	10	8.31[a]	0.33	7.61	8.88	10	8.32[a]	0.34	7.63	8.76	10	0.026
Lactobacillus group	8.30[b]	0.50	7.75	8.97	10	8.80[a]	0.40	8.08	9.53	10	8.81[a]	0.47	8.32	9.42	10	0.031
Campylobacter spp.	6.58		6.58	6.58	1	5.56	0.37	5.29	5.82	2	4.66		4.66	4.66	1	0.266
Pseudomonas					0					0					0	na
*Bacteriode*s – *Prevotell* Group	4.84	0.45	4.50	6.01	10	4.98	0.20	4.64	5.30	10	4.88	0.13	4.70	5.06	10	0.555
C. coccoides– E. rectal group	4.77	0.65	3.99	6.09	8	4.87	0.68	3.55	5.73	8	4.34	0.59	3.54	5.14	5	0.355
C. perfringens group					0					0					0	na
Enterobacteriaceae					0					0					0	na

na, not applicable.

* log no. of 16s rDNA gene copy per gram.

Number of detected chicken.

[a,b]Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

Table 22 Microbial quantification of Ilea samples from 35 day of chicken.

Bacteria	Control (n = 10)					High CP (n = 10)					EAA (n = 10)					P-value
	Mean*	SD	Min	Max	Incidence#	Mean	SD	Min	Max	Incidence	Mean	SD	Min	Max	Incidence	
All Eubacteria	7.74	0.43	6.82	8.25	10	7.97	0.51	6.71	8.54	10	8.04	0.28	7.60	8.70	10	0.313
Lactobacillus group	8.03	0.51	7.02	8.74	10	7.71	0.84	6.39	8.59	10	7.82	0.71	6.55	8.82	10	0.582
Campylobacter spp.	4.86	0.29	4.54	5.20	4	4.77	0.56	4.01	5.74	7	4.52	0.53	3.89	5.75	9	0.480
Pseudomonas	4.27	0.52	3.90	4.64	2					0					0	na
*Bacteriode*s – *Prevotell Group*	5.40	0.75	3.72	6.65	10	5.28	0.77	4.53	7.08	10	4.91	0.52	4.29	5.88	10	0.281
C. coccoides– E. rectal group	5.17	0.97	4.63	6.61	10	4.83	0.78	4.04	6.50	9	4.64	0.51	3.83	5.43	10	0.309
C. perfringens group					0					0					0	na
Enterobacteriaceae	5.37		5.37	5.37	1	5.71	0.55	5.32	6.10	2					0	0.700

na, not applicable.

* log no. of 16s rDNA gene copy per gram.

Number of detected chicken.

[a,b]Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

Table 23 Microbial quantification of caeca samples from 35 day of chickens.

Bacteria	Control (n = 10)					High CP (n = 10)					EAA (n = 10)					P-value
	Mean*	SD	Min	Max	Incidence#	Mean	SD	Min	Max	Incidence	Mean	SD	Min	Max	Incidence	
All Eubacteria	9.62[a]	0.30	9.28	10.03	10	9.66[a]	0.16	9.51	10.00	10	9.38[b]	0.24	8.91	9.58	10	0.025
Lactobacillus group	8.11	0.38	7.44	8.74	10	8.01	0.31	7.49	8.48	10	7.88	0.36	7.43	8.62	10	0.354
Campylobacter spp.	4.90	0.30	4.63	5.33	4	4.72	0.20	4.64	5.01	5	4.50	0.31	4.15	5.05	6	0.114
Pseudomonas	6.16	1.39	5.18	7.15	2	5.02		5.02	5.02	1	4.15	0.11	4.07	4.20	2	0.324
*Bacteriode*s – *Prevotell* Group	9.11	0.21	8.86	9.49	10	8.99	0.33	8.44	9.27	10	8.98	0.33	8.36	9.38	10	0.542
C. coccoides–E. rectal group	9.26[b]	0.40	8.67	9.80	10	9.40[a]	0.24	9.01	0.89	10	9.03[b]	0.27	8.86	9.46	10	0.039
C. perfringens group					0					0					0	na
Enterobacteriaceae	6.85	0.64	5.98	7.39	9	6.56	0.89	5.43	7.66	9	6.93	0.26	6.50	7.30	7	0.514

na, not applicable.

* log no. of 16s rDNA gene copies per gram.

Number of detected chicken.

[a,b]Different letter superscripts indicated a significant difference. ($p \leq 0.05$)

4.6 Effect of CP and EAA on short chain fatty acid at finisher stage

SCFA concentration of digesta from jejunum, ileum and cecum were further analyzed at 35 d (Figure 31). Both lactic acid and propionic acid were detected from jejunum and ileum of all three treatments while additional acetic acid was from cecum. The significant difference of only propionic acid concentration was observed in these three treatments. In jejunal sample, the level of propionic acid in the EAA treatment showed significant greater than control and high CP treatment ($p \leq 0.05$). Chiba (2009) reported that *Prevotella ruminocola* in *Bacteriodes-Provotell* group could produce propionic acid during their metabolism. However we could not observe the difference in number of these bacterial group from three treatment samples. Higher concentration of propionic acid found from the EAA treatment may be from the other bacterial groups which were not studied. Moreover lactic acid concentration of the high CP and EAA treatment tended to be higher than the control ($p = 0.137$). These finding supported the results of higher number of *Lactobacillus* group in the high CP and EAA than the control as previous study.

For ilea samples, lactic acid concentration at 35 d chicken digesta from all 3 treatments increased for 85% comparing to 21 d samples. However, there was no significant difference among these 3 tratments. The propionic concentration in EAA treatment was significantly higher than the control and the high CP treatment ($p = 0.041$) although microbial quantification previously observed showed no significant different.

Unlike two regions of jejunum and ileum, high concentration of acetic acid from caeca samples of 3 treatments were high up to 48 – 56 μmol/g wet weight which were higher than lactic acid and acetic acetic acid for 6.5 and 3.7 times, respectively. However, acetic acid concentration detected in these 3 treatments was not significantly different. These results supported by no significant difference of the number of *Bacteriodes-Provotella* producing acetic acid from three treatments previously observed. In addition, propionic acid concentration from the high CP treatment was significantly lower than the control and the EAA treatment ($p = 0.015$). However, the

propionic acid production mechanism by *Bacteriodes-Provotell*a group remains unclear at the moment.

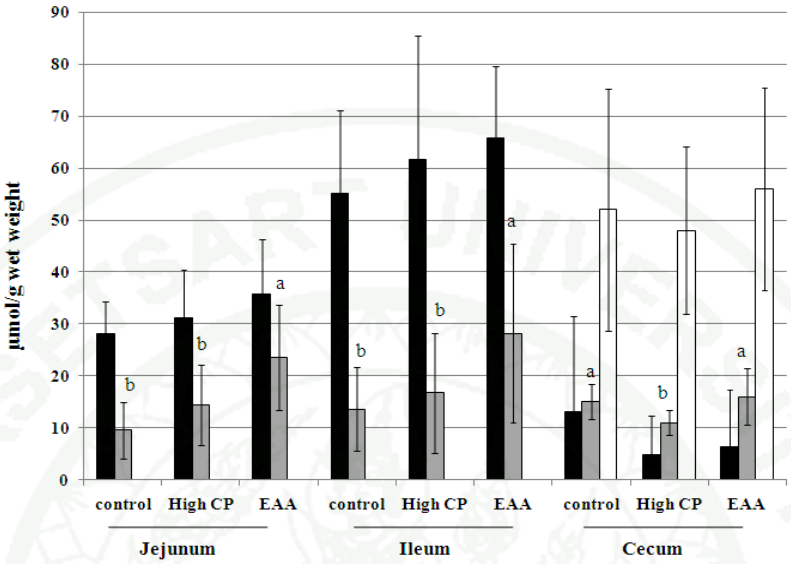

Figure 31 SCFA concentration from digesta of chicken intestine at 35 d. The black bars, gray bars and white bars are represented lactic acid, propionic acid and acetic acid, respectively. The error bars indicate standard derivation from each subject group (n=10).

[a,b]Different letter superscripts indicated a significant difference in each region ($p \leq 0.05$).

It was noticed that butyric acid was not observed in any samples at 21 d and 35 d. Whereas it was observed in cecum at around 7 – 25 µmol/g intestinal content proposed by Barnes et al. (1978) and van der Wielen *et al.* (2000). However, the most SCFA production in cecum was acetic acid that was similar to previous reports of Barnes *et al.* (1978) and van der Wielen *et al.* (2000). SCFA was an end product of intestinal microflora metabolism. Type and concentration of SCFA production depended on microbial metabolic pathway and microbial species in intestine which can survive by that diet treatments causing difference of butyric acid concentration observed.

In conclusion, additional crude protein or amino acid in feed formular had no effect to chicken performance. This might be that only increased CP or EAA are not enough for well growing in broiler chicken. Other components such as energy intake had to be considered. However the sensitive indicator as intestinal microbia between three treatments showed different.

The intestinal bacteria analyzed were based on molecular method of 16s rDNA gene. In the results, we observed that the major difference between three diets occurred in ilea and caeca region. The high CP concentration from soybean meal had no effect on the group of *C. perfringer* in ileum. This effect was different to the results of CP from animal source (Drew *et al.*, 2004). But it promoted the growth of *C. coccoides–E. rectal* group which was a normal flora and non pathogen bacteria in chicken. Moreover, the harmful bacteria *Campylobacter* group was not detected in caeca samples of EAA diet and detected only 3 birds from 10 birds in caeca samples of high CP diet. It was suggested that enrichment of EAA in these diets could suppress *Campylobacter* group which was more effective in in EAA diet contained 22% CP than high CP treatment. Nevertheless, the inhibition activity was not continued to finisher stage when amount of CP in diets were changed. These finding was the guideline to improve feed formula of chicken in the future.

DISCUSSION

It is generally accepted that the intestinal microbiota contributes to intestinal function and thus has significant impact on the growth and health of chickens (Gong *et al.,* 2007). In these studies, molecular techniques were applied to monitor intestinal microorganism in chicken intestine. Microbiota in ileum and cecum of healthy chickens were analyzed by pyrosequencing technique. The fundamental properties of pyrosequencing were both identification and diversity studies. The most abundance of bacteria belonged to phylum *Firmicutes* in two regions. In ileum, *Leuconostoc, Weissella, Lactobacillus* and *Lactococcus* which mainly produce lactic acid were the dominant group. Whereas the bacteria in class *Clostridia* including *Lachnospiraceae Incertae Sedis*, *Subdoligranulum, Faecalibacterium* and *Ruminococcus* were abundant in cecum. Althought the dominat bacteria in this study were similar to previous studies by Gong *et al.* (2007) and Lu *et al.* (2003). Most of bacteria composition in broiler chickens was different according to diets, growth condition or strain of birds etc. Therefore gut microflora was a sensitive indicator to monitor chicken health during stress treatment.

The new application in real time PCR, HRM was optimized for identification of intestinal microflora in chicken. HRM technique was able to identify bacteria based on their melting temperature behavior of DNA region (Cheng *et al.,* 2006). Compare to pyrosequencing technique, HRM is more convenient, faster and cost effective. Five dominant species found in ileum of healthy chicken including *L. crispatus, L. salivarius, Leuconostoc citreum, W. cibaria and W. confuse* were chosen for the target species. According to the results, HRM technique could identify bacteria in pure culture and duplex PCR amplication. However in chicken intestine, various dominant species were colonized. Only duplex PCR identification did not appropriate for intestinal microbiota identification in chicken.

Probiotic is the one important growth promoter of broiler chicken. It had impact on the host through intestinal microflora modification. The probiotic strain, *L. reuteri* KUB-AC5, administered to broiler chicken from 1 d – 7d at low single dose of

10^5 CFU/g feed. In the results, KUB-AC5 improved body weight gain at first week. Pyrosequencing technique was applied to monitor gut microflora change at 21 d and 42 d. Unlike healthy chicken experiment, the highest bacteria population of control and probiotic treatment at 21 d belonged to phylum *Proteobacteria* in genus *Erwinia* which is a well-known plant pathogens. The effect of probiotic and healthy chicken experiment were reared under environment and condition difference. Moreover the diets components between two treatments were different as well. Therefore the dominant bacteria of these experiments were not similar. However *Lactobacillus*, *Weissella* and *Leuconostoc* of probiotic experiment were detected at high population around 10^5 - 10^4 copy number similar to healthy chicken. At 42 d, *Lactobacillus* population became dominant in two treatments. Interestingly, KUB-AC5 promoted the number of *Lactobacillus* species and it could suppress some pathogenic bacteria.

Although pyrosequencing technique could apply to microbiodata studies in chicken. The analysis cost is very expensive for poutry industry when it is compared to other molecular techniques. Moreover, this technology was limited in quantification assay. Therefore, q-PCR was applied to study the effect of CP and EAA on intestinal microorganism in chicken. The high level of CP and EAA did not effect on chicken performance. Whereas jejuna villi height decreased in hypernutrient treatments. Ten bacterial groups including total bacteria of jejunum, ileum and cecum were chosen to be the target bacteria. According to the results, microbial communities in each CP treatment were similar whereas their population level were significant different. High CP and EAA from soy bean meal promoted the population of *C. coccoides-E. rectal* group in ileum which were different from CP of animal source (Dahiya et al., 2007). Notably, enrichment of EAA reduced the number of *Campylobacter* in cecum. In addition, CP and EAA level had effect on bacterial metabolism product. High CP and EAA level enhanced lactic acid production in ileum. The increasing of lactic acid production was observed in high CP treatment in cecum as well.

In these studies, no significant different of chicken performance in the term of weight gain, feed intake and feed conversion ratio was observed when the diet or growth condition were changed. On the other hand, the difference was detected on gut

microflora by molecular technique. Therefore the understanding of gut microflora could be an efficiency way to manipulate and improve poultry production in industry.

CONCLUSION

Sequence based technique, pyrosequencing was applied to detect bacteria in ileum and cecum of healthy chicken. In the results, more than 78% of total bacteria in ileum and cecum of healthy chicken belong to phylum *Firmicutes* and less than 18% of non beneficial bacteria in phylum *Proteobacteria* were observed. However in genus level between ileum and cecum was difference. In ileum, the dominant bacteria were in lactic acid producing group and some genus in order *Clostridiales.* There were *Weissella, Leuconostoc, Lactobacillus, Lactococcus*, *Lachnospiraceae Incertae Sedis* and etc. Whereas caeca samples contained most bacteria in obligate anaerobe or strictly anaerobe group such as *Lachnospiraceae Incertae Sedis, Subdoligranulum, Faecalibacterium* and *Ruminococcus* etc. In species level, the microbial diversity of caeca samples was higher than the group of ilea samples. Some pathogenic species were observed such as *Shigella buydii* and *Klebsiella pneumonia*. However a number of them were smaller when compared with all bacteria detection and no virulence effect was observed in chicken health. According to pyrosequncing data, we observed that bacteria in each chicken had individual pattern. Nevertheless, these bacterial compositions in all samples were stable at starter and finisher stage.

High resolution melting analysis (HRM) technique was optimized for microbial identification in intestinal chicken. According to melting temperature behavior of bacterial genomic DNA, HRM technique could indentify less than 3 target bacteria in the same reaction. Consequently, HRM technique was performed by one fluorescent dye for signal emission and these signals could not separate to different melting peak in the case of more than three targets bacteria. Therefore HRM technique did not appropriate to investigate the intestinal bacteria which contain abundance of microorganism.

Probiotic, *L. reuteri* KUB-AC5, effect on chicken performance and gut microflora were investigated using pyrosequencing technique. The results showed that probiotic concentration of 10^5 CFU/g feed had no effect on chicken growth. However,

the effect on ileum microorganism was observed. Probiotic strain introduced the condition for enrichment a number of beneficial bacteria, *Lactobacillus* species in ileum at 42 d. In addition, the enrichment of *Lactobacillus* could suppression of some pathogens group including genus *Klebsiella,* genus *Chryseobacterium,* genus *Citrobacter*, genus *Aeromonas,* genus *Acinetobacter* and order *Campylobacterales*.

The effect of CP and EAA level on chicken performance and intestinal microbiota were studies. Three diets were formulated according to CP and EAA concentration. The control and EAA diets contained 22% CP under NRA recommend (1994) whereas high CP diet contained high dietary protein from soy bean meal at 28%. In addition, EAA diet was formulated by supplementation of lysine, methionie and threonine for final concentration similar to high CP diet. According to the result, additional CP and EAA concentration to feed had no effect on chicken growth in the term of weight gain, feed intake and feed conversion ratio. However, gut microflora were changed. Major differences on diets treatment were in ileum and cecum. The enrichment of EAA in high CP treatment and EAA treatment promoted *C. coccoides–E. rectal* group at around 10^6 gene copies in ileum at 21 d. Moreover, their metabolite, lactic acid, increased in high CP and EAA treatment compared to the control. In cecum, *Campylobacter* group were not observed in EAA diet and found only 30 % of all sampling in high CP diet in cecum. Similar to ileum, lactic acid production was the highest in high CP diet. However, the circumstances were not continued to 35 d since the diets were changed.

In conclusion, gut microbiota analysis was a sensitive way to monitor chicken health according to various treatments. These data had direct impact on improvement chicken development and their health in commercial poultry industry.

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