ผลของสารเสริมชีวนะ (*Bacillus subtilis* และ *Lactobacillus acidophilus*) ต่อประสิทธิภาพการ ใช้อาหาร สมรรถภาพการเจริญเติบโต และประชากรจุลินทรีย์ในทางเดินอาหารส่วนปลายของกระต่ายหย่านม

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญา วิทยาศาสตรมหาบัณฑิต
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THE EFFECTS OF PROBIOTIC SUPPLEMENT *(Bacillus subtilis* AND *Lactobacillus acidophilus)* ON FEED EFFICIENCY, GROWTH PERFORMANCE AND MICROBIAL POPULATION IN DISTAL GASTROINTESTINAL TRACT OF WEANING RABBITS

Mr. Lam Phuoc Thanh

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Animal Nutrition
Department of Animal Husbandry
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Thesis Title	THE EFFECTS OF PROBIOTIC SUPPLEMENT (*Bacillus subtilis* AND *Lactobacillus acidophilus*) ON FEED EFFICIENCY, GROWTH PERFORMANCE AND MICROBIAL POPULATION IN DISTAL GASTROINTESTINAL TRACT OF WEANING RABBITS
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กระต่ายพันธุ์นิวซีแลนด์ ไวท์ อายุ 28-29 วัน จำนวน 64 ตัว ถูกใช้ในการศึกษาผลของสารเสริมชีวนะต่อ ประสิทธิภาพการใช้อาหาร สมรรถภาพการเจริญเติบโต คะแนนลักษณะมูล สัมประสิทธิ์การย่อยได้ที่ปรากฏที่จุดสิ้นสุดของทางเดินอาหาร การคงอยู่ของไนโตรเจน การหมักที่ไส้ตัน และประชากรจุลินทรีย์ในทางเดินอาหารส่วนปลายของกระต่ายหย่านม แบ่งกระต่ายออกเป็น 4 กลุ่ม แต่ละกลุ่มได้รับอาหารทดสอบเพียงอย่างเดียวอย่างต่อเนื่องนาน 6 สัปดาห์ อาหารทดสอบประกอบด้วย อาหารพื้นฐานได้แก่ อาหารสำเร็จรูปที่ปราศจากสารเสริมชีวนะ (กลุ่มควบคุม) อาหารพื้นฐานผสม 1) *B. subtilis* (BS) ที่ $1x10^6$ cfu/g อาหาร 2) *L. acidophilus* (LA) ที่ $1x10^7$ cfu/g อาหาร และ 3) สารผสม *B. subtilis* ที่ $0.5x10^6$ และ *L. acidophilus* ที่ $0.5x10^7$ cfu/g อาหาร (BL) ผลการศึกษาแสดงว่า กระต่ายหย่านมกลุ่มที่ได้รับอาหารพื้นฐานเสริม LA และ BL มีอัตราการเพิ่มขึ้นของน้ำหนักตัวมากกว่า มีอัตราการแลกเนื้อต่ำกว่า มีสัมประสิทธิ์การย่อยได้ที่ปรากฏที่จุดสิ้นสุดของทางเดินอาหารสูงกว่า และคะแนนลักษณะมูลต่ำกว่ากระต่ายหย่านมกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) กระต่ายที่ได้รับอาหารพื้นฐานเสริม LA มีการคงอยู่ของไนโตรเจนมากกว่า มีปริมาณกรดไขมันระเหยได้ในไส้ตันมากกว่า มีประชากรแลคโตแบซิลไลในลำไส้มากกว่า และมีประชากรโคไลฟอร์มน้อยกว่ากระต่ายกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ไม่พบความแตกต่างใดระหว่างกระต่ายกลุ่มที่ได้รับอาหารพื้นฐานเสริม BS เมื่อเปรียบเทียบกับกระต่ายกลุ่มควบคุม สรุปได้ว่า จากการศึกษาครั้งนี้ *L. acidophilus* มีศักยภาพที่เป็นประโยชน์เมื่อนำมาใช้ในรูปของสารเสริมชีวนะในกระต่ายหย่านม.

ภาควิชา : สัตวบาล ลายมือชื่อนิสิต .....

สาขาวิชา : อาหารสัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: GROWTH PERFORMANCE / GUT BACTERIA / PROBIOTIC / RABBIT

LAM PHUOC THANH: THE EFFECTS OF PROBIOTIC SUPPLEMENT (*Bacillus subtilis* AND *Lactobacillus acidophilus*) ON FEED EFFICIENCY, GROWTH PERFORMANCE AND MICROBIAL POPULATION IN DISTAL GASTROINTESTINAL TRACT OF WEANING RABBITS. ADVISOR: ASST. PROF. UTTRA JAMIKORN, Ph.D., 49 pp.

Sixty-four healthy weaning New Zealand White rabbits (28-29 days of age) were used to investigate the effects of probiotic supplement on feed efficiency, growth performance, fecal score, coefficient of total tract apparent digestibility, nitrogen retention, cecal fermentation, and gut microbial populations of weaning rabbits. The animals were fed four diets for 6-week study. A commercial diet with no probiotic was used as a basal diet (control). Three probiotic diets composed of basal diet mixed with either: 1) *B. subtilis* (BS) spores at $1x10^6$ cfu/g feed, 2) *L. acidophilus* (LA) at $1x10^7$ cfu/g feed, or 3) a mixture of *B. subtilis* at $0.5x10^6$ cfu/g feed and *L. acidophilus* at $0.5x10^7$ cfu/g feed (BL). The results showed that the weaning rabbits supplemented with LA and BL had greater average daily gain, lower feed conversion ratio, greater coefficient of total tract apparent digestibility, and lower fecal score than the rabbits fed the control diet ($P<0.05$). The animals fed LA had greater nitrogen retention, cecal volatile fatty acids concentration and intestinal lactobacilli population, and lower intestinal coliform population as compared to the control group ($P<0.05$). No significant differences were found on almost all parameters in the animals supplemented with BS as compared to the control group. In conclusion, the present results suggest that *L. acidophilus* has the potential benefits in terms of probiotic effects in weaning rabbits.

Department : Animal Husbandry Student's Signature .....

Field of Study : Animal Nutrition Advisor's Signature .....

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LIST OF ABBREVIATIONS

ADF	=	Acid detergent fiber
ADFI	=	Average daily feed intake
ADG	=	Average daily gain
AOAC	=	Association of official analytical chemists
BCFA	=	Branch chain fatty acids
BD	=	Basal diet
BL	=	*Bacillus subtilis* plus *Lactobacillus acidophilus*
BS	=	*Bacillus subtilis*
BW	=	Body weight
CF	=	Crude fiber
CFU	=	Colony forming units
CP	=	Crude protein
CTTAD	=	Coefficient of total tract apparent digestibility
d	=	Day
DCF	=	Digestible crude fiber
DCP	=	Digestible crude protein
DE	=	Digestible energy
DEE	=	Digestible ether extract
DM	=	Dry matter
DNFE	=	Digestible nitrogen-free extract
EE	=	Ether extract
FCR	=	Feed conversion ratio
GE	=	Gross energy
GIT	=	Gastrointestinal tract
GLM	=	General linear model
ISO	=	International organization for standardization
LA	=	*Lactobacillus acidophilus*

LAB	=	Lactic acid bacteria
LTB	=	Lauryl triptose broth
ME	=	Metabolizable energy
MPN	=	Most probable number
MRS	=	DeMan, Rogosa, Sharpe
MYP	=	Mannitol Egg York Polymyxin-B
N	=	Nitrogen
NDF	=	Neutral detergent fiber
NFC	=	Non-fiber carbohydrate
NFE	=	Nitrogen-free extract
OM	=	Organic matter
PI	=	Performance index
RH	=	Relative humidity
SCAN	=	Scientific committee for animal nutrition
SD	=	Standard deviation
SE	=	Standard error
SEM	=	Standard error of means
spp.	=	Subspecies
t	=	Temperature (^{o}C)
Td	=	Total days
TDN	=	Total digestible nutrients
THI	=	Temperature-humidity index
VFA	=	Volatile fatty acids

CHAPTER I
INTRODUCTION

Rabbit raising is common for pets, laboratory animals, meat and fur. During the past few years, the countries like China, Indonesia, Vietnam and Thailand were seriously affected by Bird flu. Rabbits can be a potential replacement source of poultry meat regarding to this situation. Cholesterol content of rabbit meat was lower than common red meats and poultry meat (Lukefahr et al., 1989). Rabbit husbandry shall be more environmentally friendly regarding to negligible methane production and nitrous oxide emissions when compare to other domestic species (Yang et al., 2003). Increase economic value has caused rabbit industry searching for new technologies to improve rabbit health and welfare.

Rabbit production in intensive system can cause many physiological and environmental stresses, especially during weaning period. These stresses result in spreading of enteric diseases such as *E. coli*, coccidiosis and epizootic rabbit enteropathy which have negative effect on animal health status and growth performance (Licois et al., 2000). Incorporation of antibiotics in feedstuff can reduce digestive disorders and improve growth performance of farm animals (Barton, 2000). However, bacterial resistance to antibiotics has urged scientists to find new alternatives to the use of antibiotics in animal production (Smith et al., 2002). In addition, on the base of consumer demand, European legislation has been banning the antibiotic use as growth promoters for all livestock production in order to avoid crossed resistance in humans.

To improve food safety, modulate intestinal microbial balance, improve animal health, and growth performance, probiotics appear to be possible alternative feed additives for the antibiotic products. Probiotics composing of live bacteria, yeast, or bacterial spores have been reported to prevent enteric diseases of weaning rabbits (Fortun-Lamothe and Drouet-Viard, 2002). Probiotics could promote gut colonization

and keep the balance of gut microflora by competing with the growth of harmful microorganisms, reducing the gut pH by producing organic acids, and improving digestibility of feed by producing some enzymes and vitamins (Falcão-e-Cunha et al., 2007). These functions are known to enhance the health status, feed efficiency, and growth performance of the weaning animals. However, there are few studies of probiotic supplement in rabbits than that in other monogastric farm species. Moreover, studies showing the effects of probiotic supplement on feed efficiency, growth performance, and gut microbial populations of weaning rabbits are still limited.

Objectives of this study were to investigate the effects of single and double strains of probiotic supplement on feed efficiency, growth performance, fecal score, coefficient of total tract apparent digestibility, nitrogen retention, cecal fermentation, and gut microbial populations of weaning rabbits. Hypothesis of this study was that probiotic supplement of weaning rabbits can increase number of gut beneficial microflora populations that results in reduction of harmful intestinal bacteria, increase nutrient digestibility, gut-microbial fermentation, and growth performance.

CHAPTER II
LITERATURE REVIEW

2.1 The digestive physiology of the rabbit

2.1.1 The digestive system of the rabbit

The digestive system of the rabbit (figure 2.1) is characterized by relative importance of the cecum and colon. As a result, the activity of cecal microflora is important not only for the processes of digestion and nutrient utilization, but also in control of digestive pathology. Furthermore, caecotrophy, the behavior of ingestion of soft feces, makes digestion of cecal microbes more important for the overall utilization of nutrients by the rabbit (Portsmouth, 1977). Mouth is the first digestive organ of the rabbit to ingest the feed into the stomach by transport through the esophagus. Rabbit stomach has a very weak muscular layer and is always partially filled. The rabbit stomach capacity is about 34 % of the total capacity of digestive system (Portsmouth, 1977). The pH of adult rabbit stomach is always very acid (1-5). The stomach pH usually depends on the site of determination, presence or absence of soft feces, time after feed intake and stages of the rabbit (de Blas and Wiseman, 2010). Small intestine is usually approximate 12 % of the gastrointestinal volume in the rabbit (O'Malley, 2005). This organ is the segment where a large part of digestion and absorption take place by either passive or active transport through the intestinal mucosa. In the small intestine, the digesta contents are liquid with 8-10 % DM, especially in the upper part (Fortun Lamothe and Gidenne. 2006). The pH is slightly basic in the upper part (7.2-7.5) and more acid than that in the end of ileum (6.2-6.5). So the small intestine pH is around 7 (Nicodemus et al., 2002). The jejunum is the longest segment of the small intestine. Large intestine is predominant by a big cecum which is characterized by a weak muscular layer and contents. The rabbit cecum is characterized by presence of a rich microbial community, which plays an important role in feed digestion through fermentation of non-absorbed nutrients and maintains the intestinal health by

preventing colonization of pathogenic bacteria strains (Carabaño et al., 2006). Digesta contents in the rabbit cecum are about 20-22% DM (Lebas et al., 1997). The rabbit cecum capacity is approximately 49 % of total digestive tract capacity (Portsmouth, 1977). The pH value in cecal contents is slightly acid from 5.4 to 6.8 (García et al., 2002). Yeasts, protozoa, *E. coli*, and clostridia species were also observed in the cecal flora (O'Malley, 2005).

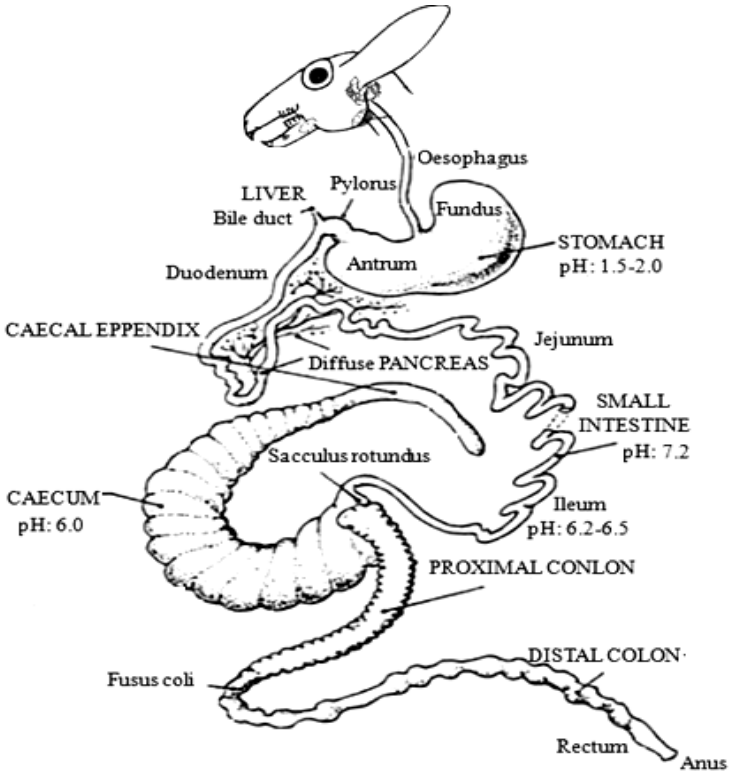

Figure 2.1 Digestive tract of a New Zealand White rabbit fed *ad libitum* a pelleted balanced diet (Lebas et al., 1997)

2.1.2 Age-related changes in function of the digestive system

Stomach glands are evident in late fetuses, while true villi and intestinal glands are observed at 29 days' gestation. Lactase activity is high value at newborn and remains constant until 25 days of age, while sucrase and maltase activity increase and

reach a peak at around 28-32 days (Gallois et al., 2008). Suckling rabbits begin to eat solid food at around 18 days of age. The cecum which develops faster than the rest of the digestive tract, is filled by digesta and microflora from 3 to 7 weeks of age, reaching a peak at 7-9 weeks of age (García et al., 2004). The pH of cecum is also affected by age which decreased from 6.8 at 15 days of age to 5.6 at 50 days of age (Padilha et al., 1995).

2.1.3 Role of intestinal microflora in digestion and absorption of nutrients

The rabbit is both a monogastric and an herbivore. Together with caecotrophy, the presence of cecal microbial population permits the rabbit to obtain additional amino acids, energy, and vitamins. During the first week of age, the digestive system of rabbit is colonized by strict anaerobes, predominantly *Bacteroides* spp. At 15 days of age, the numbers of amylolytic bacteria seem to be stabilized, whereas those of colon, *Colibacilli* spp. decreased as the numbers of cellulolytic bacteria increase (Padilha et al., 1995). The enzyme activities of intestinal microflora such as cellulase, pectinase, xylanase and urease determine ability of the rabbit to utilize fiber sources (de Blas and Wiseman, 2010). Some studies on the enzymatic activities of rabbit microflora reported that the main activities of intestinal bacteria were decreasing order, ammonia use, ureolytic, proteolytic, cellulolytic, xylanolytic, pectinolytic, and mucolytic (Emaldi et al., 1979; Forsythe and Parker, 1985). In young rabbits, the gut microflora is unstable until 30-40 days of age (Forthun-Lamothe and Boullier, 2007).

2.1.4 Cecal fermentation patterns

Cecal pH and volatile fatty acid (VFA) concentrations are classical variables characterising the extent and the pattern of cecal fermentation. The VFA concentration in the cecum has been used as an indirect method to estimate microbial activity in the rabbits. The VFA has been proposed as a protective factor against pathogenic (*E. coli*) infections *in vitro* (Wallace et al., 1989), but no significant effects have been observed in

rabbits *in vivo* (Gidenne and Licois, 2005). The cecal VFA concentrations averaged ranged 18.1-99.8 mmol/l (García et al., 2002). Result of the cecal microflora fermentative activity, VFA is produced with a predominance of acetate (77 % on average, range 65-87 %), follow by butyrate (17 % on average, range 6-28 %) and then propionate (6 % on average, range 3-11 %) (García et al., 2002). However, VFA proportions will change with the age of animal such as propionate/butyrate ratio becomes lower than 1 after 25-30 days of age (García et al., 2002). Cecal ammonia concentration slightly falls with age, while cecal VFA has reverse trend. Both increase in VFA and decrease in ammonia concentration cause a fall in cecal pH from 15 to 42 days of age (Gidenne et al., 2002). VFA produced by cecal microbial fermentation affects cecal pH. Cecal pH is alkaline in the morning and acid in mid afternoon (Brewer and Cruise, 1994). This may be due to the higher soft feces excretion and consumption (high bacterial protein) in the morning than that in the afternoon (Carabaño and Merino, 1996).

2.2 Colonization and microflora composition of gastrointestinal tract (GIT)

2.2.1 Colonization of GIT

The colonization of gastrointestinal microflora is essential to maintain intestinal health by preventing colonization of pathogenic bacteria, modulating the immune system and degrading unabsorbed substrates in the small intestine. The microflora develops according to the age and the gastrointestinal segments. In young rabbit, the implantation of microflora varies along the intestinal tract and its composition evolves with age and time after weaning (Padilha et al., 1995). According to Gouet and Fonty (1979), microflora in adult rabbit stomach remains at low level after weaning. This may be due to the very acidic environment in the stomach contents (pH<2). The microflora is present as soon as the first week of age and regularly increases until the 7^{th} day after the birth. The microbial population in the small intestine is 10-100 times higher than that in the stomach. The proportion of facultative anaerobic bacteria in small intestine of rabbits is sometimes a little higher before weaning. In small intestine, facultative anaerobic bacteria declines after weaning (28-30 days) and stabilizes at

10^6-10^8 bacteria/g of contents (Gouet and Fonty, 1979). After the first week of life, the cecum contains an abundant microflora at 10^7-10^9 bacteria/g of contents, while this is 10^9-10^{10} bacteria/g of contents after the second week. The number of facultative anaerobic bacteria is sometimes equivalent to that of strictly anaerobes during 1^{st}-2^{rd} week period. From the third week, the numbers of facultative anaerobic bacteria fall dawn to 10^2-10^4 bacteria/g of contents. This microflora is absent after weaning, whereas the strictly anaerobic microflora remains stable to 10^9-10^{11} bacteria/g of contents (Zomborszky-Kovács et al., 2000). In the colon, the microflora follows an identical evolution to that of the cecum, however, the total number of bacteria there is systematically lower (Gouet and Fonty, 1979). The number of bacteria present in the feces does not exceed more than 30 % of what appears in cecum. This may be related to the existence of a "lytic factor" secreted by the colon (Carabaño et al., 2006).

2.2.2 Microflora compositions in GIT

The first important characteristic of cecal microflora in healthy rabbit is weak prevalence of fungi and an absence of protozoa, contrary to the ruminants (Bennegadi et al., 2003). The second characteristic of the digestive microflora in the healthy young rabbit is that the facultative anaerobic microflora has a simple composition dominated by *Streptococcus* spp. until the 14^{th} day of age, whereas intestinal pathogens are detected only occasionally (Gouet and Fonty, 1979). After weaning, facultative anaerobic bacteria isolated from the intestinal tract mainly belong to the Gram-positive genera such as *Bacillus* spp., *Enterococcus* spp. and *Staphylococcus* spp., and Gram-negative bacteria as Enterobacter or *Escherichia coli* (Canganella et al., 1992).

2.3 Challenges for weaning period

Weaning is critically stressful period for young animals due to separation from their mother, changes in their physical environment, differences in housing, and even mixing with other social groups. In addition, there is a transition from milk to solid feed

that decreases the intake of milk immunoglobulins and several bactericide nutrients such as short-chain fatty acids or peptides (Skrivanova et al., 2005). The first two weeks of post-weaning can be considered as one of the most critical periods of adaptation with potential stress (McCracken and Kelly, 1993). Post-weaning GIT disorders in young animals result in not only many changes in the GIT physiology, but also in enteric microbiology and immune responses (Lallès et al., 2007). Consumption of fiber and other nutrients after weaning can result in an impairment of villi morphology. This change is either in the mucosal structure and translocation of GIT bacteria. The weaning rabbits excrete more pathogenic *Colibacilli* spp. in the cecum than suckling animals, because milk intake seems to contain a transitory protection against several important pathogens. Animal requirements for growth, intestinal development and immune capacity are not also met due to an insufficient feed intake after weaning. In addition, substitution of solid feed for doe milk in young animals also leads to decrease nutrient digestibility, and higher flow of undigested substrates at the distal ileum. The development of the cecum and large intestine seems to improve this status in small intestine by weaning animals, so that bacterial colonization might favor to development and diversification of immune system (Gallois et al., 2005).

2.4 Nutrient requirements of growing rabbits

In the growing rabbits, the maximum average daily gain is achieved when the dietary DE concentration is about 2,627-2,770 kcal/kg DM. Crude protein requirements are shown in relation to dietary energy by DCP/DE ratio. The dietary DCP/DE ratios below and above this optimum impair growth performance and feed efficiency. The recommended dietary crude protein levels for growing rabbits range between 165-176 g/kg DM and 112 to 121 g DCP/kg DM. These values correspond to a DCP/DE ratio of 0.043-0.044 g/kcal. Rabbits are able to achieve a good growth performance with high fiber diets as a result of their cecal microbial digestion, maximal growth gains are reached a peak at diets containing around 198-231 g ADF/kg DM. The beneficial

effects of dietary fat are less pronounced for growing rabbits (de Blas and Wiseman, 2010). The nutrient requirements of growing rabbits are shown in the table 2.1.

Table 2.1 Nutrient requirements of growing rabbits (kg DM basis)

Nutrients	
Digestible energy (kcal)	2,675
Metabolizable energy (kcal)	2,579
Crude protein (g)	165 (156-176)
Digestible crude protein (g)	114 (110-121)
Neutral detergent fiber (g)	374 (363-385)
Acid detergent fiber (g)	209 (198-220)
Crude fiber (g)	171 (165-176)
Ether extract[1] (g)	32 (10-71)

Source: de Blas and Wiseman, 2010, [1]: Villamide et al., 2009

2.5 Probiotics

2.5.1 Definition

Probiotics are generally defined as live microbial supplements which improve intestinal microbial balance, resulting in beneficial effects on the host (Fuller, 1989). A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract (Naidu et al., 1999). Live microorganisms when administered in adequate amounts confer a healthy benefit on the host (Reid et al., 2003). Probiotic microorganisms may or may not be normal residents in the GIT. Microorganisms such as *Lactobacillus* spp., *Bacillus* spp., *Enterococcus* spp., *Pediococcus* spp., *Bifidobacterium* spp., and *Streptococcus* spp. are commonly used as probiotic supplement in animal feed. Other microorganisms are yeasts including *Saccharomyces*

spp. Vanbelle et al. (1990) indicated that potential microorganism strains used as probiotics must have some characteristics as follow: (1) acid tolerance to ensure their survival during passage through the stomach, (2) bile tolerance to ensure their survival during passage through the upper small intestine as duodenum, (3) resistance against the lytic enzymes of saliva (lysozyme) and digestive enzymes, (4) acid production as an efficient "acid barrier" in the upper gut, (5) production of antimicrobial substances, (6) attachment or adhesion by the fimbriation to the brush-border cells, (7) immunological modulation, (8) heat tolerance in order to survive during processing of feeds, and (9) tolerance of feed antimicrobials in order to combine with medicated feed.

2.5.2 Modes of action

The beneficial modes of action of probiotics include: (i) reduction of toxic substances, (ii) increase absorption and enzyme activities, (iii) increase production of some antimicrobial substances, (iv) competition for adhesion to epithelial cells and increase resistance to colonization, and (v) stimulation of the host immune system (Falcão-e-Cunha et al., 2007).

Reduction of toxic substances: intestinal microflora produces ammonia and amines may have deleterious effects on the host animal. For example, amines produced after weaning have been shown to associate with diarrhea in animals (Porter and Kenworthy, 1969). It has been reported that the level of amines produced within the gut can be reduced by *L. acidophilus* (Hill et al., 1970). It is also claimed that probiotic can detoxify pathogenic toxins, for example *L. bulgaricus* was shown to neutralize *E. coli* enterotoxin (Mitchell and Kenworthy, 1976).

Increase absorption and enzyme activities: probiotic bacteria can break down the compounds produced within the GIT which are not hydrolyzed by enzymes in the stomach and small intestine such as urea, bacterial mucoproteins, mucosal residues,

mucus, and uric acid. This action alters urea circulation which may improve the production of non-essential amino acids in animals (Mason, 1980). Probiotic supplement significantly increased regarding α- and β-galactosidase and microbial glycolytic activities. Bacterial glycolytic enzymes play an important role in the fermentation of undigested carbohydrates, leading to enhance animal performance and health. Glycolytic enzymes are active in the following ways: α-galactosidase contributes to the hydrolysis of dietary α-galactosides such as stachyose, rafinose, and other oligosaccharide components of feedstuffs. The β-galactosidase contributes to the hydrolysis of β-galactosides as some lactose and prebiotics. The α-glucosidase contributes to starch fermentation, while β-glucosidase contributes to the hydrolysis of glucose monomers from non-starch polysaccharides. The *Lactobacillus* spp. supplement has been shown to increase the concentration of these enzymes in the small intestine (Mountzouris et al., 2007).

Production of antimicrobial substances: probiotics can produce some antimicrobial substances such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants (surlactin and glycolipids) to aid their survival in the GIT. Price and Lee (1970) reported that under certain circumstances, some lactic acid bacteria (LAB) can produce detectable amounts of hydrogen peroxide. This compound will inhibit growth of many bacteria, especially pathogenic Gram-negative types. Homofermentative LAB use the glycolytic pathway to produce two pyruvates, which are further converted to lactate. Lactic acid has been shown to permeabilise the outer membrane of Gram-negative bacteria by liberation of its lipopolysaccharides that leads to loss of pathogenic bacteria viability (Alakomi et al., 2000). When there is a lack of sugar or oxygen, heterofermentative LAB such as some lactobacilli strains use the pentose phosphate pathway to produce 1 mol each of lactic acid, acetic acid/ethanol, CO_2, and ATP per mole of glucose consumed (Lee and Salminen, 2009). The CO_2 can directly create an anaerobic environment that will be toxic to some aerobic food microorganisms (Daniels et al., 1985). Probiotics can also produce bacteriocins such as nisin that has been

shown to bind the membrane-bound cell wall precursor lipid II, results in inhibition of cell wall synthesis and rapid efflux of small cytoplasmic compounds such as amino acids, inorganic phosphate, potassium, and ATP (Bauer and Dicks, 2005).

Competition for adhesion to epithelial cells and increase resistance to colonization: the normal flora fight against pathogenic bacteria through several ways: blocking pathogenic bacteria effects by producing bactericidal substances and competing with pathogens and toxins for adherence to the intestinal epithelium; regulating the immune responses by enhancing the innate immunity and modulating pathogen-induced inflammation via toll-like receptor-regulated signal pathways; regulating intestinal epithelial homeostasis by promoting intestinal epithelial cell survival, enhancing barrier function, and stimulating protective responses (Corcionivoschi et al., 2010). The strategy for inhibition of pathogenic bacteria bases on the ability of probiotic bacteria to bind pathogens in intestinal epithelial tissue. Probiotic *Lactobacillus* spp. had an inhibitor effect on some types of pathogenic bacteria that included *E. coli*, *Klebsiella pneumoniae, Shigella flexneri, Salmonella typhimurium, Enterobacter cloacae, Pseudomonas aeruginosa, Enterococcus faecalis* and *Clostridium difficile* (Forestier et al., 2001).

Stimulation of the host immune system: intestinal lymphoid tissues make contact with food components, antigens, beneficial bacteria, and pathogen. Antigens and substances can trigger an immune response and enter the body through the intestinal mucosa that is essential in controlling host immunity to invasion of pathogenic bacteria. Probiotics are directly taken up through transcytosis by micro-fold epithelial cells and engulfed by macrophages or dendritic cells, which eventually triggers immune responses of the host. Cytokines modulate the immune functions of dendritic cells (as antigen-presenting cells), T and B cells (Shida and Nanno, 2008). *L. casei* strains could directly act with the intestinal epithelial cells and maintain integrity of the epithelial barrier. These mechanisms include suppression of Nuclear Factor-Kappa B (NF-kB) activation, stimulating the activation of the anti-apoptotic protein Akt to prevent

apoptosis, and enhancing mucin secretion by epithelial cells (Yan and Polk, 2002). The main cytokines produced by macrophages and dendritic cells are IL-12 and IL-10, respectively. *Lactobacillus* strains activate macrophages to produce more IL-12 in murine models which may also promote differentiation of naive $CD4^+$ T cells (nThs) into Th1 cells, leading to natural killer (NK) cell activity (Ichikawa et al., 2007). Epithelial cells can differentiate the difference between probiotic and pathogenic microorganisms. Pathogenic bacteria induce pro-inflammatory responses in epithelial cells by activating transcription NF-kB, while non-pathogenic species as probiotic may alleviate to the side of pro-inflammatory response by blocking this factor (Neish et al., 2000).

2.5.3 *Bacillus* spp.

The *Bacillus* strains commonly used in animal feed are *B. subtilis, B. licheniformis, B. cereus* var. *toyoi, B. clausii,* and *B. coagulans* (Cutting, 2011). The *Bacillus* strains are able to produce spores that afford protection from enzymatic degradation, heat, and the acidic condition of the stomach. Spores are able to survive on the low pH of gastric barrier. The optimal pH values for growing of the *Bacillus* spores are ranged from 5.5 to 5.8. These organisms have some nutrient requirements such as nucleotide bases, amino acids, peptides, vitamins, minerals, fatty acids, and carbohydrates. The optimal levels of *B. subtilis* spores use for growing animals ranged from $0.48x10^6$ to $1.28x10^6$ cfu/g feed (SCAN, 2000). The *B. subtilis* and its related species have long been established as industrial bacteria for producing of various enzyme secretions such as amylase, protease, lipase, xylanase, pullulanase, and chitinase (Westers et al., 2004). The *B. subtilis* is capable of producing a variety of antibacterial agents, such as surfactin, potent biosurfactants as an antibiotic (Branda et al., 2001). The *B. subtilis* is able to quickly mature to produce spores against multiple stress conditions which include alkaline, acidic or oxidative stress, osmotic, heat, and ethanol (Hecker and Volker, 2001).

2.5.4 *Lactobacillus* spp.

The *Lactobacillus* spp. is a group of LAB, Gram-positive, non-spore-forming that ferment carbohydrates to produce lactic acid as a major end-product. Reduction of pH and fermentation of large amounts of carbohydrates are the primary actions by LAB to inhibit food-borne pathogens. The *Lactobacillus* strains mainly used in animal feed are *L. casei, L. plantarum, L. acidophilus, L. farciminis,* and *L. rhamnosus* (Anadón et al., 2006). The Scientific Committee for Animal Nutrition (SCAN) in Europe reported that *Lactobacillus* spp. can use as probiotic for animals at the rate of 10^6-10^8 cfu/g feed (SCAN, 2003). The previous studies reported that due to the limited resistance to gastric juice and lack of adhesive capacity to the intestinal tract, the use of lactobacilli as probiotic in growing rabbits may be limited. However, the optimal pH for growing of *L. acidophilus* ranged 5.5- 6.0 (Gomes and Malcata, 1999). The *L. acidophilus* is capable of surviving in acidic environment with pH from 4 to 5 or below. It is able to pass harsh environments of the stomach and move through to the small intestine. The *L. acidophilus* had good survival rate as faced with pH 2.0 or mixed into the gastric juice/product at pH 1.4, and 45 % survived in a medium that contains 0.3 % bile for 3 h (Deraz et al., 2007).

2.6 The effects of probiotics in livestock

2.6.1 Feed intakes and growth rate

The supplement of *B. cereus* var. *toyoi* at $2x10^5$ cfu/g diet to d37-d79 growing rabbits increased average daily gain (ADG) from 38.2 g/day in the control group to 40 g/day in the treatment group ($P<0.05$), while no effect was observed on average daily feed intake (ADFI) (Trocino et al., 2005). Kritas et al. (2008) found that supplementation to d38-d93 rabbit diet with probiotic containing *B. licheniformis* and *B. subtilis* at $1.28x10^6$ cfu/g feed did not affect the ADFI, while ADG was increased from 30 g/day in the control to 33 g/day in the treatment group ($P<0.05$). Giang et al. (2010) showed that LAB supplement at $1.3x10^7$-$3x10^8$ cfu/g feed to weaning piglets increased ADG to 298 g/day after 5 week feeding ($P<0.05$) as compared to the control group (271 g/day).

Giang et al. (2012) reported that weaning piglets supplemented with LAB complex alone at $4.4x10^7$ cfu/g feed or combination of this LAB complex with $1.2x10^9$ cfu/g feed *B. subtilis* H4 improved ADFI, ADG, and reduced FCR as compared to the control after 5 week feeding ($P<0.001$).

2.6.2 Fecal score and health risk index

Giang et al. (2010) reported that LAB diets containing $1.3x10^7$-$3x10^8$ cfu/g feed to weaning piglets decreased fecal scores from 0.22 in the control group to 0.11-0.14 in the treatment groups ($P<0.05$) after 2 weeks feeding. Weaning piglets fed the diet supplemented with LAB complex alone at $4.4x10^7$ cfu/g feed or combination of this LAB complex with $1.2x10^9$ cfu/g feed *B. subtilis* H4 decreased the fecal scores to 0.12 as compared to 0.21 in the animals fed the control diet after two weeks feeding ($P<0.001$) (Giang et al., 2012). In these studies, the fecal scores were assigned from 0 to 3. The Matusevicius et al. (2006) found that supplementation to d35-d77 rabbit diet with *Bacillus strains* at $1.28x10^6$ cfu/g feed reduced morbidity, mortality, and health risk index by 2, 17 and 20 %, respectively as compared to the control group ($P<0.05$). The *B. cereus* var. *toyoi* supplement at $2x10^5$ cfu/g diet to d37-d79 growing rabbits decreased morbidity rate to 7.6 % in the treatment group when compared to 18.2 % in the control group (Trocino et al., 2005).

2.6.3 Nutrient digestibility and retention

Sen et al. (2011) reported that d22-d35 broiler diet supplemented with *B. subtilis* at $1x10^5$ and $1x10^6$ cfu/g feed increased crude protein retention to 62.1 % and 62 % as compared to 59.8 % in the control ($P<0.05$). No significant difference was observed when the chickens were fed at $1x10^4$ cfu/g feed. Onbasilar and Yalçin (2008) found that supplementation of 42 day weaning rabbits with probiotic complex at $4.5x10^8$ cfu/g feed was not significant effect on the digestibility of DM, OM, CP, and ADF after 6 week feeding. The LAB supplement at $2.5x10^6$ cfu/g feed to d1-d42 chicken increased α-

galactosidase and β-glucosidase ($P<0.05$) when compared to the control and avilamycin groups (Mountzouris et al., 2007). Giang et al. (2010) reported that LAB complex supplement at $1.3x10^7$-$3x10^8$ cfu/g feed to weaning piglets increased total digestibility of crude protein and crude fiber by 4 % and 8 % after 2 week feeding when compared to the control group ($P<0.05$). Weaning piglets supplemented with LAB complex alone at $4.4x10^7$ cfu/g feed or combination of this LAB complex with $1.2x10^9$ cfu/g feed *B. subtilis* H4 enhanced the total tract apparent digestibility of OM (by 3 and 5 %) and CP (by 2 and 4 %) as compared to the control group after 5 week feeding ($P<0.001$) (Giang et al., 2012).

2.6.4 Fermentative patterns

Giang et al. (2010) reported that LAB complex supplement at $1.3x10^7$-$3x10^8$ cfu/g feed to weaning piglets increased intestinal VFA concentration to 342 mmol/kg DM after 5 week feeding when compared to the control group (284 mmol/kg DM) ($P<0.05$). *Lactobacillus* spp. mixture supplement at $5x10^{10}$-$1x10^{11}$ cfu/day to rat diet increased VFA concentration in the portal blood from 942 mmol/l (control) to 1555 mmol/l in the probiotic group after 12 day feeding ($P<0.05$) (Branning et al., 2009). Maertens et al. (1994) reported that supplementation of *Bacillus* CIP 5832 (a *Bacillus* strain with microbiological characteristics similar to *B. subtilis*) at $1x10^6$ cfu/g feed in the d28-d70 weaning rabbit diet was not significant effect on cecal pH, NH_3 production, and total VFA concentration. Pascual et al. (2008) found that d28-d54 weaning rabbit diet supplemented with *Bacillus cereus* var. *toyoi* at $1x10^6$ cfu/g feed decreased cecal branch chain fatty acids (BCFA) concentration from 0.16 % in the control group to 0.03 % in the treatment group ($P<0.05$). However, there was not significant difference was observed in the cecal VFA concentration between probiotic and control groups.

2.6.5 Balance of gut microflora

Kritas et al. (2008) reported that supplementation to d38-d93 rabbit diet with probiotic containing *B. licheniformis* and *B. subtilis* at $1.28x10^6$ cfu/g feed reduced fecal *E. coli* (>10^7 cfu/g feces) from 8.3 % in the control group to 5.1 % in the probiotic treated-group ($P<0.05$). Vilà et al. (2009) reported that *B. cereus* var. *toyoi* supplement at $1x10^6$ cfu/g feed for 42 days feeding reduced *Salmonella* spp. enteritidis of broiler chicks infected with *Salmonella* spp. to 25 % when compared to 58 % in the control group ($P<0.05$). Doe rabbit and kit diets supplemented with *B. cereus* at $2x10^5$ cfu/g feed decreased cecal coliform counts of 3 week suckling rabbits to 4.3 $\log_{10}$ cfu/g of contents when compared to 5.9 $\log_{10}$ cfu/g of contents in the control group ($P<0.05$) (Bónai et al., 2008). Increasing levels of *B. subtilis* supplement from $1x10^4$ to $1x10^6$ cfu/g feed to d22-d35 broiler diet showed linear decrease in cecal *Clostridium* and coliform populations as compared to the control ($P<0.05$), while no significant difference was found on cecal LAB population (Sen et al., 2011). Weaning piglets supplemented with LAB complex alone at $4.4x10^7$ cfu/g feed or combination of this LAB complex with $1.2x10^9$ cfu/g feed *B. subtilis* H4 increased intestinal LAB population means, while the intestinal *E. coli* population means decreased as compared to the control ($P<0.001$) (Giang et al., 2012).

CHAPTER III
MATERIALS AND METHODS

3.1 Animal management and experimental design

Animal used and experimental protocols were approved by Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University, Thailand. Figure 3.1 shows the experimental schedule for this study.

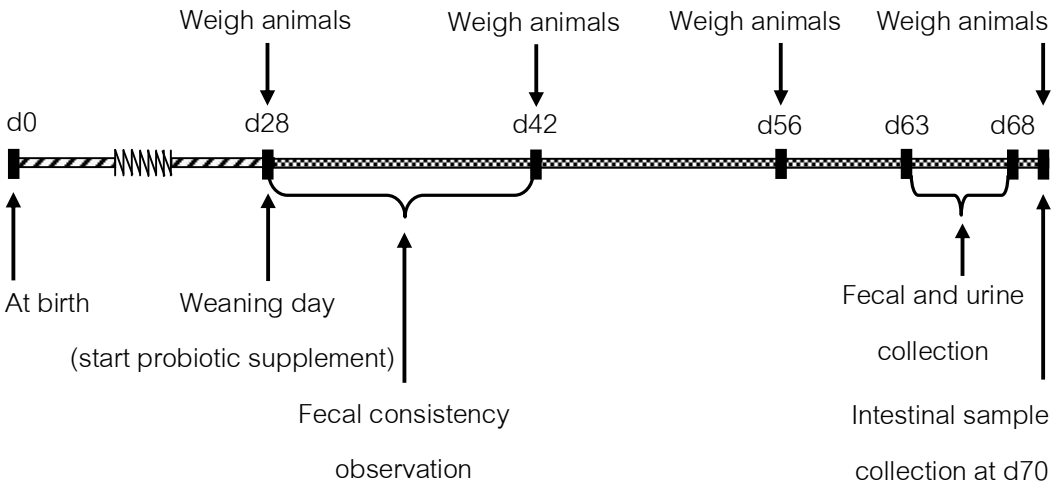

Figure 3.1 Experimental schedule

Sixty four healthy weaning New Zealand White rabbits (32 males and 32 females) age 28±1 day were used in this experiment. They were individually numbered at the ears for identification before starting the experiment. The animals were blocked by weaning weight with four levels (351, 457, 525, and 648 g) and sex (male and female). For each level of weaning weight, the weaning rabbits were randomly divided into 8 cages that included 4 cages of males and 4 cages of females (2 rabbits per each cage). One cage of male and one cage of female rabbits of each group were randomly assigned to one of the four diets for 6 weeks. The cages for fattening rabbits (60x50x45cm) were made of galvanized wire net, equipped with feeders and

automatic nipple drinkers. A natural photoperiod approximate 12 h light and 12 h dark was used in this experiment. During the 6-week feeding period, all rabbits were kept in open housing with the environmental temperature and relative humidity at about 30.7±1.25 ^{o}C and 66.7±7.77 %, respectively. A randomized complete block design was used with 4 treatments and 8 replicates per each treatment. The first block was weaning weight, while sex was for the second block.

3.2 Feed and feeding

A commercial diet for growing rabbits with no probiotic was used as a basal diet (control). Three probiotic diets included basal diet mixed with either: 1) *B. subtilis* (BS) spores at 1x10^{6} cfu/g feed, 2) *L. acidophilus* (LA) at 1x10^{7} cfu/g feed, or 3) a mixture of *B. subtilis* at 0.5x10^{6} cfu/g feed and *L. acidophilus* at 0.5x10^{7} cfu/g feed (BL). All treatments were applied to block 1 and block 2. The using doses of *B. subtilis* and *L. acidophilus* in this experiment were followed the indication of SCAN (2000, 2003).

Before weaning, both does and suckling rabbits had free access to the commercial feed. From 28 to 70 days of age, the weaning rabbits were offered either the basal diet (table 3.1) or the basal diet mixed with the probiotics. The basal diet was daily ground to pass through a 5-mm sieve. All treatment diets were daily prepared by mixing the basal diet with the probiotic powders (K.M.P. Biotech Co., Ltd., Thailand). The concentrations of *B. subtilis* and *L. acidophilus* products were approximately 1x10^{8} and 1x10^{9} cfu/g, respectively. Therefore, the BS and LA diets were prepared by mixing the basal diet with 1 % of *B. subtilis* product and 1% of *L. acidophilus* product, respectively, while the BL diet was prepared by mixing basal diet with 0.5 % of *B. subtilis* product and 0.5 % of *L. acidophilus* product. During the experiment, all probiotic products were kept in the refrigerator at about 4-6 ^{o}C. Feed and water were provided *ad libitum* to the animals for the entire study. Roughage was

not considered to offer to the animals in this experiment. The table 3.1 shows the nutrient compositions of the basal diet use in this study.

Table 3.1 Nutrient compositions (kg DM basis) of basal diet in the experiment

Chemical analysis	
Dry matter (g)	891
Organic matter (g)	924
Crude protein (g)	174
Neutral detergent fiber (g)	390
Acid detergent fiber (g)	203
Crude fiber (g)	155
Ether extract (g)	31.4
Ash (g)	75.6
Nitrogen free extract (g)	564
Non fiber carbohydrate (g)	329
Gross energy (kcal)	4,398
Digestible energy, DE (kcal)	2,747
Metabolizable energy (kcal)	2,580
Digestible crude protein, DCP (g)	118

3.3 Data collection

Feed samples were collected every day and mixed once a week throughout the experiment to determine nutrient compositions by proximate analysis (AOAC, 2005). Probiotic diet samples were also collected every day and mixed once a week to confirm the concentration of probiotic bacteria. Total feed offered and refused were daily weighed to calculate average daily feed intake. The rabbits were weighed at the d28 (weaning

day), d42, d56, and d70 (at the end of the experiment) to calculate average daily gain. The environmental temperature and humidity were also daily recorded at 9:00 am and 3:00 pm during the experiment. Fecal scores were daily evaluated every morning for 14 days after weaning. Fecal scores were assigned from 1 to 4, where score 1: normal, hard pellets; score 2: soft formed pellets; score 3: mixed soft pellets and moisture; and score 4: loose, softer, shapeless feces, completely liquid (Agin et al., 2005). Dead and sick animals were daily counted to calculate morbidity, mortality and health risk index. The feed intake was daily calculated, excluding the intake of dead animals.

At the fifth week of the experiment (63 day old), total fecal and urine output were collected everyday for 5-day period to investigate coefficient of total tract apparent digestibility and nitrogen retention (McDonald et al., 2002). On each day, approximately 40 g of fresh fecal samples were dried in oven at 55 ^{o}C for 48 h and stored in the fridge at -20 ^{0}C until further analysis for nutrient contents. The urine samples were kept in 10 % sulfuric acid (Young and Conway, 1942), and stored in the fridge at -20 ^{o}C for analysis of nitrogen concentration. At the end of the experiment (70-day old), 32 rabbits (one rabbit per each experimental unit) were sacrificed by an overdose injection of pentobarbital sodium at 60-70 mg/kg live weight to immediately obtain the intestine. The intestinal samples were kept in ice box and quickly transported to microbiological laboratory for enumeration of *Bacillus* spp., *Lactobacillus* spp., and coliform populations. The representative digesta contents from fresh cecum were used to measure pH and kept at -80 ^{o}C for VFA concentration analysis.

3.4 Sample analyses

3.4.1 Temperature, humidity, and temperature-humidity index

The average daily temperature and humidity were calculated as an average of temperature and humidity at 9:00 am and 3:00 pm during 42 days of the experiment. The temperature-humidity index (THI) was calculated according to the following equation of Marai et al. (2001):

$$THI = t - \left[\left(0.31 - 0.31\left(\frac{RH}{100}\right)\right)(t - 14.4)\right]$$

Where t (^{0}C) = dry bulb temperature in degrees Celsius, and RH=RH percentage/100. THI values were classified as following: <27.8: absence of heat stress, 27.8-28.9: moderate heat stress, 29.0-30.0: severe heat stress, and >30.0: very severe heat stress (Marai et al., 2001).

3.4.2 Feed intakes and growth performance

$$\text{Average daily feed intake (ADFI, g DM/day)} = \frac{\text{total feed offered (g)} - \text{total feed refused (g)}}{\text{total days of the experiment}}$$

$$\text{Average daily gain (ADG, g/day)} = \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{total days of the experiment}}$$

Feed conversion ratio (FCR) = ADFI (g DM) / ADG (g)

$$\text{Performance index (PI, \%)} = \frac{\text{body weight (kg)}}{\text{FCR}} \times 100$$ (Amber et al., 2004)

3.4.3 Fecal score and health risk index

The average fecal score was calculated as the sum of the fecal score over the feeding period divided by the number of the experimental days. For calculation of morbidity, the sick rabbits had the diarrhea symptoms (fecal score of 4) were recorded only once. The suddenly dead animals without any signs of disease were not considered in the morbidity calculation. The health risk index was calculated as a sum of the morbidity and mortality (Bennegadi et al., 2000).

3.4.4 Chemical analyses

The feed and fecal samples were analyzed for dry matter (DM), organic matter (OM), crude protein (CP, N x 6.25), crude fiber (CF), ether extract (EE), and ash

according to the standard methods of AOAC (2005). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed following the method of Van Soest et al. (1991). Urine samples were analyzed for nitrogen content following the standard method of AOAC (2005). Gross energy (GE) in feed and fecal samples was determined by an automatic adiabatic oxygen bomb calorimeter. NFE and NFC were calculated by following equations:

NFE = OM – CP – EE – CF

NFC = OM – CP – EE – NDF

The ME content was estimated as following equation of Maertens et al. (2002):

ME (MJ/kg DM) = DE (0.995 – 0.048 DCP/DE)

Where: DE (MJ/kg DM) = 15.3 – 0.19 ADF (Fernández -Carmona et al., 2004),

DCP (g/kg DM) = – 34.67 + 0.876 CP (Villamide and Fraga, 1998).

The ME and DE after estimating were converted from MJ/kg DM to Kcal/kg DM.

3.4.5 Coefficient of total tract apparent digestibility (CTTAD) and nitrogen retention

$$\text{CTTAD (\%)} = \frac{\text{(nutrient in feed – nutrient in feces)}}{\text{nutrient in feed}} \times 100$$

Total digestible nutrients (TDN, %/DM) = DCP + DNFE + DCF + 2.25 DEE

Nitrogen retention (g/day) = nitrogen in feed – (nitrogen in feces + nitrogen in urine)

3.4.6 Cecal pH and fermentative products

The cecal pH value was determined after sacrifice by a manual automatic pH meter (pH meter PB 10, Goettingen, Germany). The cecal VFA concentration was determined by using a modified gas chromatographic method (Playne, 1985). In this analysis, approximately 7 g of cecal contents were centrifuged at 14,000xg for 15 minutes at 4 $^{\circ}$C in an Eppendorf centrifuge 5810R with A-4-81 Rotor (Eppendorf AG,

Hamburg, Germany). The supernatant was transferred to 2 ml eppendorf and centrifuged at 14,000xg for 15 minutes at 4 ^{o}C in an Eppendorf centrifuge 5810R with FA-45-30-11 Rotor. The 0.5 ml of supernatant was stored at -80 ^{o}C until injection into the gas chromatograph to determine for VFA concentration. All samples were warmed to about 10 ^{o}C at the time of injection into gas chromatograph. 1 µl of sample was injected into a gas chromatograph with an HP-FFAP 10-m x 0.53-mm x 1-mm capillary column packed with cross-linked polyethylene glycol TPA. An aflame ionization detector was used with an oven temperature of 200 °C, and a detector temperature of 250 °C for determination of acetate, propionate, and butyrate concentrations. The standard solutions (acetic, propionic, and butyric acids mixing) were prepared with 3 various concentrations. The first concentration included 26.7, 1.07, and 2.67 mmol/l acetic, propionic and butyric acids, respectively. The second concentration comprised 37.3, 4.27, and 5.33 mmol/l acetic, propionic and butyric acids, respectively. The last concentration was mixed by 48.0, 8.53, and 10.7 mmol/l acetic, propionic and butyric acids, respectively. The total VFA concentration was calculated as a sum of acetic, propionic and butyric acids.

3.4.7 Gut microbial populations

Probiotic powder products and feed samples were determined for *B. subtilis* and *L. acidophilus* bacteria populations. The intestinal samples including ileum, cecum, and colon were weighed approximately 10 g of content and intestinal wall and dissolved in a sterile peptone saline fluid (0.9%) by a 1:9 dilution. The further dilutions of the intestinal samples were from 10^{-2} to 10^{-4} to estimate coliform population, while these were from 10^{-3} to 10^{-5} to count the bacilli and lactobacilli populations. The bacilli were cultured on MYP (Mannitol Egg York Polymyxin-B) agar, under aerobic conditions for 24 h at 37 ^{o}C (ISO 7932, 2004), while the lactobacilli were cultured in MRS (DeMan, Rogosa, Sharpe) agar, under anaerobic conditions for 48 h at 37 ^{o}C (ISO 15214, 1998). The total intestinal coliform was cultured in Lauryl triptose broth (LTB), under aerobic

conditions for 24 h at 37 ^{o}C (ISO 4831, 2006). The bacilli and lactobacilli enumerations were expressed as $\log_{10}$ colony forming units (cfu) in 1 g of fresh intestinal sample, while the intestinal coliform enumeration was expressed as $\log_{10}$ most probable number (MPN) in 1 g of fresh intestine.

3.5 Statistical analysis

Statistical analysis of the data was carried out as randomized complete block design. The effect of treatment was analyzed by General Linear Model (GLM). The significant differences between the treatment means were tested by Tukey method at α=0.05.

CHAPTER IV
RESULTS

4.1 Temperature, humidity, and temperature-humidity index of the experiment

In this study, the average daily temperature and relative humidity were between 26.6 to 33.8 °C (30.7 ±1.25 °C on average) and 50 to 90 % (66.7 ±7.77 % on average), respectively. As the result, the THI was between 26.2 to 30.8. The average THI value of the experiment was about 29.0 ± 0.79 (figure 4.1).

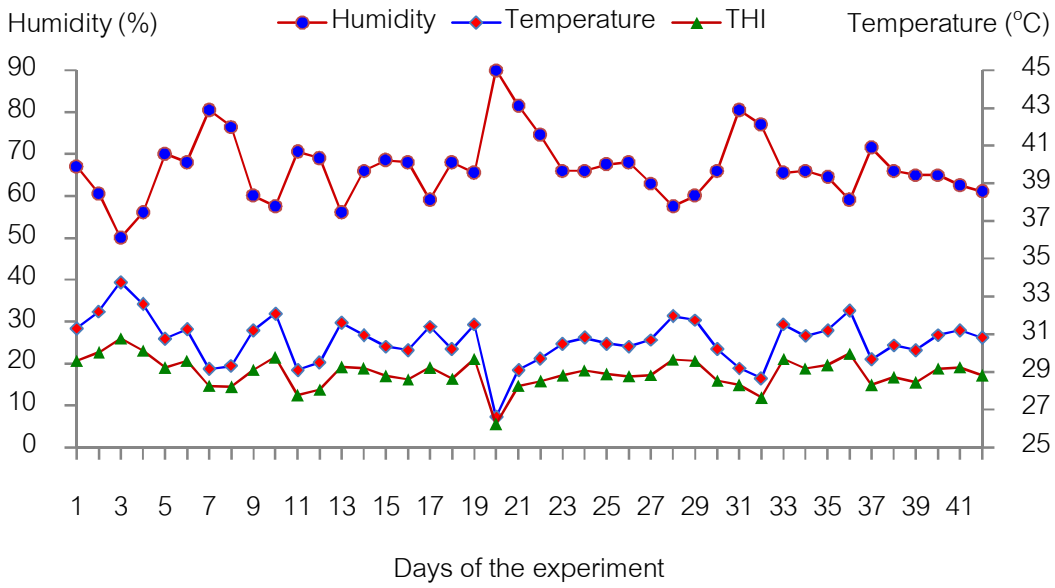

Figure 4.1 Average daily temperature, humidity, and temperature-humidity index (THI) of the experiment

4.2 Effects of probiotic supplement on feed efficiency and growth performance

Table 4.1 shows that the weaning rabbits supplemented with LA and BL had increased body weight at d42 and d70 as compared to the control animals ($P<0.05$). At d28-d42 period, the ADG was increased to 29.8 and 30.3 g/day in the LA and BL groups as compared to 25.0 g/day in the control group ($P<0.05$). The FCR was

reduced to 1.61 and 1.60 in the LA and BL groups when compared to 1.87 in the control group (P<0.05). However, there were no significant differences on the ADFI, ADG, and FCR at d42-d70 period. Overall, at d28-d70 period, the probiotic supplement had improved the ADG (P<0.05), the highest value in the LA group (28.1 g/day) versus the lowest value in the control group (24.0 g/day). The FCR was decreased from 2.85 in the control group to 2.53 in the LA group (P<0.05). Performance index of the animals was enhanced to 66.3 and 65.7 % in the LA and BL group as compared to 52.8 % in the control group (P<0.05).

Table 4.1 Effects of probiotic supplement on average daily feed intake (ADFI, g DM/day), average daily gain (ADG, g/day), feed conversion ratio (FCR, g DM/g gain), and performance index (PI, %) of weaning rabbits

Items		Treatments				SEM	*P*-value
		Control	BS	LA	BL		
BW at d28, g		498	498	497	498	20.6	0.999
BW at d42, g		848[a]	887[ab]	914[b]	922[b]	28.1	0.007
BW at d70, g		1,506[a]	1,598[ab]	1,678[b]	1,669[b]	39.7	0.023
d28-d42	ADFI	46.8	48.4	48.1	48.5	1.48	0.657
	ADG	25.0[a]	27.8[ab]	29.8[b]	30.3[b]	0.80	0.013
	FCR	1.87[a]	1.74[ab]	1.61[b]	1.60[b]	0.05	0.042
d42-d70	ADFI	79.2	79.9	82.6	81.7	2.56	0.653
	ADG	23.5	25.4	27.3	26.6	0.60	0.102
	FCR	3.37	3.15	3.03	3.07	0.09	0.190
d28-d70	ADFI	68.4	69.4	71.1	70.6	2.17	0.615
	ADG	24.0[a]	26.2[ab]	28.1[b]	27.9[ab]	0.62	0.029
	FCR	2.85[a]	2.65[ab]	2.53[b]	2.54[b]	0.07	0.048
	PI, %	52.8[a]	60.3[ab]	66.3[b]	65.7[b]	1.85	0.020

BS: control diet + *B. subtilis*, LA: control diet + *L. acidophilus*, BL: control diet + *B. subtilis* + *L. acidophilus*.

BW: body weight.

[a, b] Means in a row with different superscripts are significantly different (P<0.05), n=8 per treatment.

4.3 Effects of probiotic supplement on fecal score and health risk index

At d28-d42 period, some rabbits had the wet soft feces (score 3). However, none of the animals in any group had diarrhea (score 4) (table 4.2). No animal in any group died during the study. Therefore, morbidity and health risk index were zero. At d28-d35 period, the probiotic supplement slightly affected the fecal score ($P<0.06$). At d35-d42 and overall period, the animals fed LA and BL diets had lower fecal score than the animals fed the control diet ($P<0.01$). However, no different effect was found on the fecal score in the animals fed BS diet as compared to the control group.

Table 4.2 Effects of probiotic supplement on fecal score of weaning rabbits

Items	Treatments				SEM	*P*-value
	Control	BS	LA	BL		
d28-d35	2.36^{a}	2.07^{ab}	1.91^{ab}	1.79^{b}	0.08	0.057*
d35-d42	2.02^{a}	1.66^{ab}	1.45^{b}	1.38^{b}	0.08	0.004
Overall, d28-d42	2.19^{a}	1.87^{ab}	1.68^{b}	1.58^{b}	0.07	0.006

BS: control diet + *B. subtilis*, LA: control diet + *L. acidophilus*, BL: control diet + *B. subtilis* + *L. acidophilus*.

$^{a, b}$ Means in a row with different superscripts are significantly different ($P<0.05$), *: $P<0.06$, n=8 per treatment.

4.4 Effects of probiotic supplement on coefficient of total tract apparent digestibility and nitrogen retention

Table 4.3 shows that probiotic supplement significantly affected the coefficient of total tract apparent digestibility of almost all nutrients and nitrogen retention. The coefficient of total tract apparent digestibility of DM, OM, CP, NDF, CF, and GE were greater in the weaning rabbits fed either LA or BL diets than the animals fed the control diet ($P<0.01$). Nitrogen retention was improved in the animals fed LA diet (1.45 g/day) as compared to the control animals (1.21 g/day) ($P<0.01$). However, no different effects were found in the animals fed BS diet as compared to the control animals.

Table 4.3 Effects of probiotic supplement on coefficient of total tract apparent digestibility and nitrogen retention of growing rabbits at 9 weeks of age

Items	Treatments				SEM	*P*-value
	Control	BS	LA	BL		
Coefficient of total tract apparent digestibility, %						
DM	65.4[a]	67.2[ab]	68.0[b]	67.8[b]	0.31	0.009
OM	66.4[a]	68.2[ab]	69.0[b]	68.7[b]	0.30	0.007
CP	67.8[a]	69.0[ab]	72.1[c]	71.3[bc]	0.51	0.004
NDF	40.1[a]	43.6[ab]	45.6[b]	44.9[b]	0.60	0.001
CF	27.6[a]	31.4[b]	33.0[b]	32.4[b]	0.63	<0.001
EE	78.0[a]	79.8[ab]	81.4[b]	81.2[b]	0.49	0.045[*]
NFE	76.0	77.4	77.3	77.2	0.35	0.501
TDN	51.6[a]	54.5[ab]	56.9[b]	56.2[ab]	0.99	0.043[*]
GE	62.2[a]	64.2[ab]	65.1[b]	64.7[b]	0.35	0.008
Nitrogen balance, g/day						
N-intake	2.31	2.36	2.44	2.40	0.05	0.561
Fecal-N	0.741	0.726	0.679	0.689	0.02	0.364
Urinary-N	0.355	0.338	0.306	0.331	0.02	0.764
N-retention	1.21[a]	1.30[ab]	1.45[b]	1.38[ab]	0.04	0.009

BS: control diet + *B. subtilis*, LA: control diet + *L. acidophilus*, BL: control diet + *B. subtilis* + *L. acidophilus*.

[a, b, c] Means in a row with different superscripts are significantly different (P<0.01), *: P<0.05, n=8 per treatment..

4.5 Effects of probiotic supplement on cecal pH and fermentative products

The table 4.4 shows that the animals fed LA diet had greater cecal acetic acid concentration (36.8 mmol/l) than the animals fed basal diet (32.9 mmol/l) (P<0.07). Whereas no different effects were found on cecal concentrations of propionic and butyric acids. Total cecal VFA concentration of the LA group (44.5 mmol/l) was greater than the control group (39.0 mmol/l) (P<0.06). There was no significant effect on the pH

of the cecal content in the current study. However, no different effect was found on the cecal VFA concentration in the rabbits fed BS diet as compared to the control animals.

Table 4.4 Effects of probiotic supplement on cecal pH and volatile fatty acids (VFA) concentration (mmol/l) of growing rabbits at 10 weeks of age

Items	Treatments				SEM	*P*-value
	Control	BS	LA	BL		
pH	6.51	6.52	6.48	6.50	0.02	0.804
Cecal VFA concentration, mmol/l						
Total	39.0^{a}	41.9^{ab}	44.5^{b}	42.3^{ab}	0.86	0.052*
Acetic acid	32.9^{a}	34.5^{ab}	36.8^{b}	34.6^{ab}	0.60	0.066**
Propionic acid	1.86	2.27	2.39	2.35	0.10	0.149
Butyric acid	4.26	5.16	5.31	5.41	0.27	0.194
Cecal VFA proportions, %						
Acetic acid	84.4	83.1	83.0	81.8	0.47	0.150
Propionic acid	4.75	5.16	5.30	5.54	0.17	0.485
Butyric acid	10.9	11.7	11.7	12.7	0.42	0.322

BS: control diet + *B. subtilis*, LA: control diet + *L. acidophilus*, BL: control diet + *B. subtilis* + *L. acidophilus*.

$^{a, b}$ Means in a row with different superscripts are significantly different (*: P<0.06, **: P<0.07), n=8 per treatment.

4.6 Effects of probiotic supplement on gut microbial populations

The table 4.5 shows that the rabbits fed BS diet had greater number of intestinal bacilli at 6.22 $\log_{10}$ cfu/g as compared to 5.56 $\log_{10}$ cfu/g in the control group (P<0.001). The number of intestinal lactobacilli were greater in the animals fed LA and BL diets (6.20 and 5.96 $\log_{10}$ cfu/g) as compared to the control animals (4.93 $\log_{10}$ cfu/g) (P<0.001). The average number of intestinal coliform population was reduced to 2.66 $\log_{10}$ MPN/g in the LA group as compared to 4.08 $\log_{10}$ MPN/g in the control group

(P<0.01). Ratio of intestinal Gram (+) to Gram (-) bacteria was higher in the rabbits fed LA diet (2.41) as compared to the control (1.39) and other probiotic groups (P<0.001).

Table 4.5 Effects of probiotic supplement on microbial populations in different intestinal segments of growing rabbits at 10 weeks of age

Items		Treatments				SEM	*P*-value
		Control	BS	LA	BL		
Bacilli	Ileum	5.50	6.07	5.54	5.75	0.09	0.042*
	Cecum	5.64[a]	6.34[b]	6.01[ab]	6.21[b]	0.07	<0.001
	Colon	5.55[a]	6.25[b]	5.78[ab]	6.01[ab]	0.08	0.003
	Average	5.56[a]	6.22[b]	5.77[ab]	5.99[ab]	0.06	<0.001
	SEM	0.08	0.05	0.09	0.09	-	-
	P-value	0.812	0.103	0.057	0.099	-	-
Lactobacilli	Ileum	4.69[a]	4.76[a]	6.13[b]	5.85[b]	0.16	<0.001
	Cecum	5.09[a]	5.37[a]	6.36[b]	6.03[b]	0.11	<0.001
	Colon	5.01[a]	5.33[a]	6.12[b]	6.00[b]	0.12	<0.001
	Average	4.93[a]	5.16[a]	6.20[b]	5.96[b]	0.12	<0.001
	SEM	0.12	0.13	0.10	0.09	-	-
	P-value	0.218	0.097	0.586	0.699	-	-
Coliform	Ileum	3.68	3.24	2.59	3.13	0.18	0.137
	Cecum	4.30[a]	3.83[a]	2.70[b]	3.43[ab]	0.18	0.005
	Colon	4.26[a]	4.09[a]	2.70[b]	3.75[ab]	0.19	0.005
	Average	4.08[a]	3.72[a]	2.66[b]	3.43[ab]	0.16	0.004
	SEM	0.22	0.22	0.07	0.19	-	-
	P-value	0.299	0.083	0.773	0.450	-	-
Total Gram (+)[1]		5.69[a]	6.29[b]	6.41[b]	6.36[b]	0.06	<0.001
Gram (+) / Gram (-)[2]		1.39[a]	1.69[a]	2.41[b]	1.85[a]	0.09	<0.001

BS: control diet + *B. subtilis*, LA: control diet + *L. acidophilus*, BL: control diet + *B. subtilis* + *L. acidophilus*.

[1]: total gut bacilli and lactobacilli populations. [2]: coliform population.

[a, b, c] Means in a row with different superscripts are significantly different (P<0.01), *: P<0.05, n=8 per treatment.

CHAPTER V
DISCUSSION

The temperature-humidity index (THI) at 29.0 as found in this study was the starting point of heat stress for the growing rabbits (Marai et al., 2001). There was a strong negative correlation between rabbit feed efficiency, body weight gain and thermal comfort level of the habitat, but there was a positive correlation between THI and rabbit respiration rate (Marai et al., 2001). An increase environmental temperature resulted in low body weight gain, feed efficiency, and high respiratory rate in the animals. The adverse effect of high ambient temperature on rabbit performance might relate to a decrease in feed consumption, animal dehydration, and tissue catabolism (Abo-Elezz et al., 1984). In addition, more energy could be consumed by the increase respiratory frequency. Therefore, low metabolizable energy left for growth (Habeeb et al., 1993).

The greater ADG and lower FCR in the rabbits fed diets supplemented with *L. acidophilus* alone or the complex of *B. subtilis* and *L. acidophilus* compared to the control group demonstrated the remarkably positive effects of *L. acidophilus* on the growth performance of weaning animals. These could be due to the greater nutrient digestibility and nitrogen retention in the LA- and BL-supplemented animals. The positive effects of probiotic supplement on growth performance and feed efficiency of weaning rabbits clearly showed in the first two weeks feeding, while no significant difference was observed in the last four weeks. Jensen (1998) found that the intestinal microflora become stable, and normal gut functions has been re-established (Pluske, 2001) during two to three weeks of post-weaning period. Therefore, the effect of probiotic supplements on animal growth performance could be expected to be less importance after weaning for 3 weeks (Huang et al., 2004). These findings were supported by earlier studies, which showed positive effects on the ADG and FCR in weaning rabbits supplemented with microbial complexes during a 14-day perod after

weaning (Ayed and Saïd, 2008; Giang et al., 2010). However, the weaning animals fed diet supplemented with *B. subtilis* alone at $1x10^6$ cfu/g feed did not significantly affect the ADG and FCR as compared to the animals fed basal diet for the current study. These finding results were in agreement with the study of Trocino et al. (2005) that weaning rabbit diet supplemented with *B. cereus* var. *toyoi* at $1x10^6$ cfu/g feed had no significant effect on growth performance and feed efficiency as compared to the control group. There might be the synergistic effects between *B. subtilis* and *L. acidophilus* since supplement mixing *B. subtilis* and *L. acidophilus* at half dose of each ($0.5x10^6$ and $0.5x10^7$ cfu/g feed) showed similar results in almost all parameters as *L. acidophilus* supplement alone at a full dose. Lee and Salminen (2009) found that *L. acidophilus* survival was able to release peptides, which could help the growth of typically weakly proteolytic probiotics as *Bacillus* spp. Moerover, bacterial activities of *Lactobacillus* strains were increased after co-culture these bacteria with *Bacillus* spp., which indicated that *Bacillus* spp. could stimulate biosynthetic capacities of *Lactobacillus* strains (Røssland et al. 2004). Futher investigation should be performed in order to confirm their activities.

The fecal score in current study was used as one parameter to determine fecal status of the weaning rabbitls. The lower fecal score in the rabbits supplemented with *L. acidophilus* might relate to the greater total VFA concentration in the intestine of these animals, as VFA provide a powerful driving force for the movement of water and sodium out of the colonic lumen (Cummings and Macfarlane, 1991). The result in current study was in agreement with the previous studies (Giang et al., 2010; Giang et al., 2012). However, the supplementation of *B. subtilis* alone did not result in any improvement in the fecal score of weaning rabbits in this study. The similar results was also found in the study of Giang et al. (2012).

In the current study, weaning rabbits fed diets supplemented with *L. acidophilus* showed better nutrient digestibility and nitrogen retention than animals fed the un-

supplemented basal diet after 5 weeks feeding. The rabbits fed diets containing *L. acidophilus* had greater number of intestinal lactobacilli population, which could enhance intestinal hydrolytic enzyme activity in these animals resulting in increase nutrient digestibility and feed efficiency utilization (Fuller, 1989). Moreover, gut function might have been improved by feeding diet supplemented with *L. acidophilus* due to the increase of lactase and sucrase activities in the small intestinal mucosa (Collington et al., 1990). In addition, the lower fecal score and greater intestinal VFA concentration in the rabbits fed diets containing *L. acidophilus* could contribute to improve nutrient digestibility (Giang et al., 2012). However, the weaning animals fed diet supplemented with *B. subtilis* alone did not improved nutrient digestibility and nitrogen retention. The similar results were also found in the study by Kornegay and Risley (1996).

The greater total cecal VFA concentration of the LA-fed weaning rabbits could be due to the greater lactobacilli activity in the cecum (Cummings and Macfarlane, 1991). An increase of the total cecal VFA concentration in the rabbits supplemented with *L. acidophilus* was expected to reduce cecal pH, which may exert adverse effect to the intestinal pathogens (Högberg and Lindberg, 2006), and a low gut pH has been shown to have a beneficial effect on nutrient digestibility (Lyberg et al., 2006). The rabbits fed LA diet had greater acetic acid concentration in the cecal content than the animals fed basal diet, while no significant differences were found on cecal concentrations of propionic and butyric acids. This might be due to heterofermentative lactobacilli strains under strictly anaerobic condition of rabbit cecum leading to produce mainly acetic acid (Lee and Salminen, 2009).

The animals fed diets containing *L. acidophilus* had increased number of intestinal lactobacilli at 10 weeks of age. Lactobacilli are generally absent in GIT of normal adult rabbits (Penney et al., 1986) due to highly acidic environment in the stomach. An increase of the cecal lactobacilli population in the animals supplemented with *L. acidophilus* leaded to increase the cecal acetic acid and total VFA concentration

and reduce intestinal coliform population (Jin et al., 2000). Acetic acid has been shown to penetrate into the bacterial cytoplasm resulting in reduce internal bacterial pH and collapse the electrochemical proton gradient, leading a bacteriostasis and death of susceptible bacteria as cecal coliform (Eklund, 1989). The decrease of the intestinal coliform population could contribute to reduce the gastrointestinal problems in the weaning animals (Oglesbee and Jenkins, 2012). An increase of Gram positive and Gram negative bacteria ratio in the intestine of LA-fed animals suggested that the intestine was predominantly colonized by non pathogenic bacteria, and toxic substances of intestinal pathogens were inhibited by gut beneficial bacteria. These results were supported by the previous studies (Bónai et al., 2008; Giang et al., 2010, Giang et al., 2012) which demonstrated an increase of intestinal beneficial bacteria population with a decrease of intestinal pathogens when weaning animal diets were supplemented with probiotics.

In conclusion, the results from this study indicated that supplementation of *L. acidophilus* alone at $1x10^7$ cfu/g feed could enhance number of gut beneficial bacteria populations, nutrient digestibility, cecal fermentation, feed efficiency, and growth performance. Moreover, gut coliform population and fecal score were reduced in the animals supplemented with only *L. acidophilus*. However, There were no significant effects on growth performance and feed efficiency of weaning rabbits when the animals were supplemented with *B subtilis* alone at $1x10^6$ cfu/g feed. The combination of *B. subtilis* and *L. acidophilus* at half dose of each showed similar results as the supplementation of *L. acidophilus* alone at the full dose. These present results suggest that the *L. acidophilus* has the potential benefits in terms of probiotic effects in the weaning rabbits.

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APPENDIX

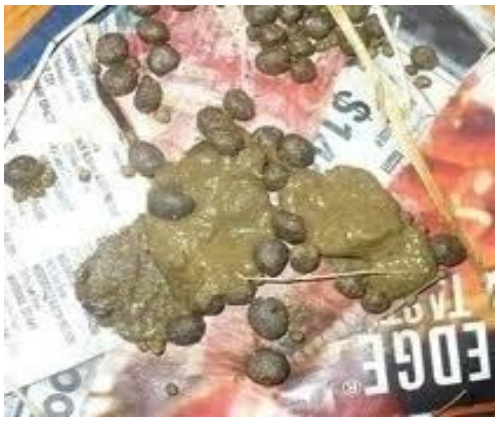
APPENDIX

Fecal scores of growth rabbits during 28-42 days of age

score 1: normal, hard pellets

score 2: soft formed pellets

score 3: mixed soft pellets and moisture



score 4: shapeless feces, completely liquid

BIOGRAPHY

Mr Lam Phuoc Thanh was born on 28 February 1984 in Tra Cu district, Tra Vinh province, Viet Nam. He attended to University in 2002 and got his Bachelor degree in Animal Husbandry that was awarded on 10 May 2007 by the Rector of Can Tho University, Can Tho city, Viet Nam. After graduation, he was employed by the Department of Animal Husbandry, College of Agriculture and Applied Biology, Can Tho University to work as a researcher for rabbit and ruminant production in the MEKARN project funded by Swedish Government on "Sustainable livestock Systems in the Tropics". In 2010, he applied and was awarded a master scholarship from Chulalongkorn University, Thailand in the program of "Scholarship Programs for Neighboring Countries". He studied in the field of Animal Nutrition at Department of Animal Husbandry, Faculty of Veterinary Science, Chulalongkorn University from June 2010 to May 2012. In order to fulfill requirements for his master degree, he carried out the thesis entitled "The effects of probiotic supplement (*Bacillus subtilis* and *Lactobacillus acidophilus*) on feed efficiency, growth performance and microbial population in distal gastrointestinal tract of weaning rabbits" as a partial need.