Impact of Bacteria and Yeast with Probiotic Properties on Performance, Digestibility, Health Status and Gut Environment of Growing Pigs in Vietnam

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2010

Acta Universitatis agriculturae Sueciae
2010:78

ISSN 1652-6880
ISBN 978-91-576-7523-1

Print: SLU Service/Repro, Uppsala 2010

Impact of Bacteria and Yeast with Probiotic Properties and Microbial Enzymes on Performance, Digestibility, Health Status and Gut Environment of Growing Pigs in Vietnam

Abstract

This thesis aimed to evaluate the effects of six lactic acid bacteria (LAB) strains and *Bacillus subtilis* H4, *Saccharomyces boulardii* Sb, and a microbial enzyme mixture, supplemented to basal diets on the performance, diet digestibility, health status, and gut environment of growing pigs under Vietnamese conditions.

The results showed that three different 3-strain-LAB complexes, comprising combinations of *Enterococcus faecium* 6H2, *Lactobacillus acidophilus* C3, *Pediococcus pentosaceus* D7, *L. plantarum* 1K8 and *L. plantarum* 3K2 improved performance, digestibility, health status and gut environment of the LAB-supplemented piglets in the first two weeks post-weaning (Per I), but not in the following 3 weeks (Per II). Adding an LAB strain (*L. fermentum* NC1) alone or combined with the *Bacillus*, or combined with a *Bacillus* and yeast complex, to a 3-strain-LAB complex showed improvements in the performance, diet digestibility, health status and gut environment of the probiotics-supplemented piglets in both Per I and II. The inclusion of *Bacillus* resulted in higher nutrient digestibility, and the addition of *Saccharomyces* showed improvements in the scouring scores of the piglets. Supplementation with either a microbial enzyme mixture alone or a combination of a 3-strain-LAB complex and yeast in a weaner diet improved the performance and diet digestibility in piglets in Per I. In Per II, no changes in performance or digestibility were found in enzymes-fed piglets, while improved performance and diet digestibility were obtained in piglets fed the LAB-yeast diet. There was lack of response of piglets to this enzyme mixture when these enzymes were supplemented to the diet that contained the complex of LAB and yeast in both Per I and II. Dietary supplementation with the combination of *Bacillus, Saccharomyces* and 4-strain-LAB complex had positive effects on performance and digestibility in grower pigs, but not in finisher pigs, while supplementation with the *Bacillus* alone or combined with *Saccharomyces* did not affect the performance and digestibility in grower and finisher pigs. The results of these studies suggest that combinations of suitable strains of *Bacillus, Saccharomyces* and LAB can be used as an alternative to antibiotic feed additives in pig production under the conditions of Vietnam.

Keywords: Lactobacilli, Bacillus, Yeast, Enzymes, Probiotics, Pigs, Digestibility, Performance, Diarrhoea, Gut Bacteria, Gut Environment

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Dedication

To my parents
My husband *Le Huu Hoang*
My sons *Le Hoang Hung* and *Le Hoang An*

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Giang, H. H., Viet, T. Q., Ogle, B. and Lindberg, J. E. (2010). Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. *Livestock Science* 129 (1-3), 95-103.

II Giang, H. H., Viet, T. Q., Ogle, B. and Lindberg, J. E. Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with a potentially probiotic complex of lactic acid bacteria alone or in combination with *Bacillus subtilis* and *Saccharomyces boulardii* (manuscript).

III Giang, H. H., Viet, T. Q., Ogle, B. and Lindberg, J. E. (2010). Effects of microbial enzymes and a complex of lactic acid bacteria and *Saccharomyces boulardii* on growth performance and total tract digestibility in weaned pigs. *Livestock Research for Rural Development* 22 (10).

IV Giang, H. H., Viet, T. Q., Ogle, B. and Lindberg, J. E. (2010). Effects of supplementation of probiotics on the performance, nutrient digestibility and faecal microflora in growing-finishing pigs. *Asian-Australasian Journal of Animal Science* (in press).

Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
ATTD	Apparent total tract digestibility
CAD	Coefficient of apparent digestibility
CF	Crude fibre
CIAD	Coefficient of ileal apparent digestibility
CP	Crude protein
CTTAD	Coefficient of total tract apparent digestibility
Exp.	Experiment
FCR	Feed conversion ratio
GIT	Gastrointestinal tract
LAB	Lactic acid bacteria
OM	Organic matter
Per	Period
VFA	Volatile fatty acids

1 Introduction

Pig production is the most important sector of livestock production in Vietnam, and pork accounts for the highest proportion of total meat output, of about 76% in 2007 (MARD, 2008). The pig population has increased from 21.8 million in 2001 to 26.9 million in 2006, with an average annual growth rate of 4.3% (MARD, 2008). Recently, the Vietnamese Government issued Decision No 10/2008/QD-TTg dated 16/01/2008, approving a livestock development strategy to 2020, in which the number of pigs is predicted to increase to around 35 million by 2020 at an average rate of 2.0% per year, of which exotic pig herds reared in farms using industrial systems will increase from 14% in 2006 to 37% by 2020.

In order to improve pig performance, as well as to help the pigs to resist the common diseases occurring in the hot and humid climatic conditions, the feed regulations in Vietnam still allow the use of eight antibiotics as feed additives in pig diets (MARD, 2009). However, using antibiotics in animal feed is considered to promote bacterial resistance that may result in less efficient antibiotic treatments for human and animal diseases (Bates *et al.*, 1993; Levy, 1982). In addition, misusing antibiotics as feed additives for pig production can result in high antibiotic residues in pork, and the risk for development of the antibiotic-resistant bacteria is a controversial issue in Vietnam (Vo *et al.*, 2010; Vu *et al.*, 2007; Nhiem *et al.*, 2006). This situation has been pressing researchers and state officials in Vietnam to find alternative solutions, especially since the European Union decided to remove antibiotics from the list of feed additives which are allowed for food-producing animals.

Probiotics, that are defined as "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989), are suggested as one of the alternatives to the use of antibiotic feed additives. The commercial probiotics contain single strain or

multi-strains of lactic acid bacteria, *Bacillus* and yeasts (Hong *et al.*, 2005; Fox, 1988). Lactic acid bacteria (LAB) are considered as natural microflora of the gut, and probiotic LAB can adhere to and colonize the host's intestines (Mazza, 1994). *Bacillus* species are normally found in the soil (Mazza, 1994) and were not considered to be normal inhabitants of the intestine (Jonsson & Conway, 1992; Moore & Holdeman, 1974). However, *B. subtilis* was found to be able to survive during the passage through the mouse intestinal tract (Spinosa *et al.*, 2000) and had positive metabolic effects in the gastrointestinal tract of animals and man (Mazza, 1994), and also on the growth performance of pigs (Alexopoulos *et al.*, 2004). Yeasts, such as *Saccharomyces*, are normally found in plants, and in previous studies *in vitro* and *in vivo* have been found to act against various pathogens and to stimulate immune systems (Buts *et al.*, 2006; Czerucka *et al.*, 2000; Rodrigues *et al.*, 2000; Buts *et al.*, 1990). Probiotics containing different strains of micro-organisms have different efficacy (Weichselbaum, 2009). Multi-strain or multi-species probiotics have been found to have more effective and consistent functionality, as they are active against a wider range of the gut conditions than mono-strain or single-species probiotics (Timmerman *et al.*, 2004; Fuller, 1989).

In pig production, probiotics have been assessed as having some positive effects, such as improving growth rate, feed efficiency (Close, 2000; Abe *et al.*, 1995) and nutrient digestibility (Shen *et al.*, 2009; Chen *et al.*, 2006), by: increasing the gastrointestinal population of beneficial bacteria that may competitively exclude pathogenic bacteria belonging to the gram-negative flora such as *E. coli* and *Salmonealla* (Estienne *et al.*, 2005; Huang *et al.*, 2004); developing enzyme activities in the mucosal tissue of piglets (Collington *et al.*, 1990), and increasing the production of short chain fatty acids within the gut (Jadamus *et al.*, 2002). The effects of the probiotics appear to be more consistent and positive in piglets than in growing/finishing pigs (Vanbelle, 2001; Pollmann *et al.*, 1980), as the efficiency of probiotics is higher when the animals are confronted with challenge or stress (Estienne *et al.*, 2005; Jensen, 1998).

Recently, several commercial probiotic products of foreign companies have been imported into Vietnam, for example, from Alltech Co. Ltd. (USA), Bayer Co. Ltd. (Germany), Biomin Co. Ltd. (Austria), BGMP Co. Ltd. (Korea), and some Chinese companies. As far as we know, there are not as yet any commercial feed additive probiotics for pigs that originate in Vietnam. However, some *in vitro* studies have been carried out on the isolation, screening and evaluation of the probiotic properties of some beneficial bacteria and yeasts from different sources in Vietnam (Viet *et al.*,

2009; Viet *et al.*, 2006). Results of these studies have shown some strains of bacteria and yeasts to have probiotic properties. These include six strains of lactic acid bacteria (*Enterococcus faecium* 6H2, *Lactobacillus acidophilus* C3, *Pediococcus pentosaceus* D7, *L. plantarum* 1K8, *L. plantarum* 3K2 and *L. fermentum* NC1), one *Bacillus* strain (*Bacillus subtilis* H4) and one *Saccharomyces* strain (*Saccharomyces boulardii* Sb), that were selected for our present studies in pigs.

1.1 Objectives of the study

- To evaluate the effects of different lactic acid bacteria complexes on growth performance, nutrient digestibility, gut environment and health status in post-weaning piglets.
- To investigate the effects of dietary supplementation with a lactic acid bacteria complex alone or combined with *Bacillus*, or with a mixture of *Bacillus* and *Saccharomyces*, or with a complex of microbial enzymes on growth performance, nutrient digestibility, gut environment and health status in post-weaning piglets.
- To evaluate the effects of different combinations of an LAB complex, *Bacillus* and yeast, supplemented to a basal diet, on performance, digestibility and faecal bacteria counts in growing-finishing pigs.

1.2 Hypotheses of the study

- Weaned piglets given diets supplemented with 3-strain-LAB complexes will have improved growth performance and reduced post-weaning diarrhoea compared with the control piglets.
- A complex of 4 LAB strains will be more effective with respect to the performance of weaned piglets than a 3-strain-LAB complex.
- Addition to the LAB complex with either *Bacillus* or yeast will improve the effectiveness of the microbial complexes with respect to performance and digestibility in weaned piglets.
- A mixture of an LAB complex, *Bacillus* and a *Saccharomyces* will be more effective than either the LAB complex or *Bacillus* alone, or either LAB-*Bacillus* complex or *Bacillus*-*Saccharomyces* complex with respect to the performance of weaner, grower and finisher pigs.
- The enzyme mixture will have positive effects on performance, and adding *Saccharomyces* alone or combined with the enzymes would have probiotic effects in weaned piglets during a longer period post-weaning than the 3-LAB complex only.

2 Background

2.1 Pig intestinal microbiota

At birth, the stomach and intestinal tract of pigs is sterile (Muralidhara *et al.*, 1977; Kenworthy & Crabb, 1963). Later in life pigs come in contact with their surroundings and colonization of the gastrointestinal tract by hundreds of different types of micro-organisms occurs (Vanbelle *et al.*, 1990). Within 2-3 hours after birth, at least four different groups of bacteria can be isolated from the stomach content of piglets; lactobacilli, streptococci, coliform bacteria and clostridia (Jensen, 1998). The major source of the intestinal flora in piglets is their dam, as it was found that the faecal flora of sows and their piglets were very similar three days post delivery (Katouli *et al.*, 1997). This similarity was lesser afterward and was lost in week 2, and piglets developed a new type of flora which differed from that of the sow (Katouli *et al.*, 1997). Recently, using 16S rRNA-based approaches, Konstantinov *et al.* (2006) found that ileal samples of 2-day-old piglets harboured a consortium of *E. coli*, *Shigella flexneri*, *Lactobacillus sobrius*, *L. reuteri*, and *L. acidophilus* related sequences. Differences in the composition of bacteria are found in different locations of the intestinal tract. The stomach is the first reservoir where the ingesta spend some time and that allows the microbiota to multiply. However, the proximal part of small intestine is not suited for bacteria proliferation in the healthy pig because the transit time is too rapid to allow time for microbial division (Jensen, 1998). The stomach and small intestine contain only a few species of bacteria because of the composition of the luminal medium (acid, bile, pancreatic secretion) and the phasic propulsive motor activity towards the ileal end, whereas the large intestine contains high densities of living bacteria (Guarner & Malagelada, 2003). During the suckling period, the *Lactobacilli* and *Streptococci* remain the dominant group of microbiota in the gastric and small intestine contents,

and the composition of the gut microbiota in piglets is fairly stable (Jensen, 1998; Fuller, 1989). Recently, a study found that a change in the diversity of the intestinal microbiota occurred three times (Inoue *et al.*, 2005), by using molecular microbial ecological techniques, temperature gradient gel electrophoresis, with sampling frequency of every 3 days of the rectal faeces during a 25-day-suckling period. However, when feeding piglets with creep feed, strict anaerobes such as *Bacteroides* become established in the large intestine, and this corresponds with a decline in the numbers of facultative anaerobe organisms (Konstantinov *et al.*, 2004). In general, microbiota in the gastrointestinal tract is unstable during the first week after weaning, and then it takes around a further two weeks before the microbiota has developed fermentative capacity in the hindgut (Jensen, 1998).

A diverse and stable gastrointestinal microbiota makes a vital contribution towards the health and productive performance of the host (Broom *et al.*, 2006; Fuller, 1989). Some beneficial effects of the gut microbiota can be obtained, such as production of metabolites, synthesis of vitamins B and K, stimulation of the intestinal immunoglobulin production, and increased animal resistance against enteropathogenic bacteria (Vanbelle *et al.*, 1990). The gut microflora are affected by a number of factors, such as age of pig, diet composition, growth promoting antibiotics, copper, use of probiotics, specific carbohydrates, organic acids and fermented feed (Jensen, 1998).

2.2 Digestive enzymes in pigs

2.2.1 Digestive enzymes in stomach

Hydrolysis of protein starts in the stomach under the active pepsins secreted in the gastric juice. The optimum pH of pepsins ranges from 2.0 to 3.9 (Corring, 1982). The acid secretion starts very early in the life of pigs, from birth in fact, and the capacity increases steadily with age during the first 4-5 weeks of life (Low, 1990). The gastric secretion capacity of pepsin is low in pigs up to about 3-4 weeks of age and then undergoes a very rapid increase (Cranwell, 1985). This author also reported that the pattern of development of pepsin secretory capacity was different from that for acid secretion. The maximal outputs of acid per unit stomach weight remained relatively constant, while the maximal output of pepsin per unit stomach weight and per unit body weight increased with age.

The stomach may be considered as the first place for hydrolysis of carbohydrates, because the feed ingested moves rapidly from the mouth to the gastric lumen, and thus the salivary amylase does not act (Corring, 1982). The salivary amylase can break down starch into maltose, maltotriose

and α-dextrins; however the pH for optimum activity of amylase is 6.9, while the pH value in the stomach is normally lower (Corring, 1982).

2.2.2 Digestive enzymes in small intestine

Brush-border enzymes in the small intestine for hydrolysis of carbohydrates are lactase, trehalase, sucrase, glycoamylase and isomaltase. Lactase activity is high at birth, remains high during the suckling period and decreases with age after weaning (Hampson & Kidder, 1986; Aumaitre & Corring, 1978), while the other enzymes generally increase with age (Kidder & Manners, 1980). The pattern of distribution of brush-border enzymes along the intestine changes with age in piglets, but approaches the adult pattern by 8 weeks of age and does not change in pigs over 10 weeks of age (Kidder & Manners, 1980).

Pancreatic enzymes present in the small intestine of pigs are the main source of protelytic enzymes (chymotrypsin, trypsin, elastase and carboxypeptidase, collagenase, nuclease), and also a source for starch (amylase) and lipid digestion (lipase and colipase) (McCracken & Kelly, 1993; Corring, 1982; Kidder & Manners, 1980). In the newborn piglet, the digestive enzymes are specialized for the milk diet, and contain a high lactase concentration in the small intestine, while the pancreatic enzymes are quite inactive (Hampson & Kidder, 1986). The development of enzymatic activities of the pancreas varies, and for example amylase activities increased after 21 days while lipase activities remained low until the 35th day after birth (Kitts *et al.*, 1956). Proteinases remained rather low (Lewis *et al.*, 1957) or increased in the first 8 weeks after birth (Hartma *et al.*, 1961). The total fresh weight of exocrine pancreas of piglets increased with age and the body weight; however, it appears that pancreatic function is stimulated from the 3rd to the 4th week of age (Corring *et al.*, 1978).

2.3 Challenges for weaned piglets

At weaning, the piglets have to cope with many changes, such as separation from their dams, the transition from milk to solid feed, changes in their physical environment and even mixing with piglets from other litters. The first two weeks post-weaning can be regarded as one of the most critical periods as a period of adaption and potential stress, regardless of whether the pigs are weaned at 2 or 6 weeks of age (McCracken & Kelly, 1993). These challenges include many changes in the gastrointestinal tract (GIT) physiology, microbiology and immunology.

2.3.1 Weaning and the GIT physiology and digestive enzymes

The stomach, which is an important component of both GIT motility and gut barrier function, may be affected by weaning, but little is known of this period (Lallès *et al*., 2007b). Lesniewska *et al*. (2000) found differences in myoelectric activities between suckling and weaned pigs, which resulted in increased gastric emptying rate and duodenal flow of digesta in weaned pigs. The piglets given access to creep feed in the pre-weaning period or after weaning had heavier stomachs and greater capacity of acid secretion than piglets only suckling (Cranwell, 1985).

In the small intestine, after weaning, villus atrophy and crypt hyperplasia and depressed activities of many brush-border digestive enzymes have been observed, which are more conspicuous when weaning earlier at 14 days rather than later at 28 days of age (Pluske *et al*., 1997). Hampson (1986a) found that within 24 hours after weaning (at day 21 of age), the villus height was reduced to around 75% of pre-weaning values; and then the reduction of the villus height continued until the fifth day after weaning at most sites along the gut, and was around 50% of the initial values found at weaning. In addition, the number of cells in the crypts increased steadily from day 3 to day 11 after weaning (Hampson, 1986a). Cera *et al*. (1988) reported that the villus surface area of the small intestine is dramatically changed, at least for the 7- to 14-day period post-weaning. The jejunum villus height was found to decline markedly at days 3 and 7 post-weaning, in both piglets weaned at 21 and 35 days of age; subsequently, the villus height increased by day 14 post-weaning.

The changes in the villus architecture are generally associated with reductions in the specific activity of the brush-border enzymes, and consequently the absorptive capacity of some nutrients of weaned piglets decreased (Pluske *et al*., 1997), and may influence the pathogenesis of diarrhoea after weaning (Nabuurs *et al*., 1993b). Hampson & Kidder (1986) found that lactase and sucrase activities decreased during the 11 days post weaning, with the minimum values recorded at days 4 and 5, coincident with a reduced ability to absorb xylose. Miller *et al*. (1986) reported that activities of sucrase, isomaltase and lactase fell by at least 50% five days after weaning in piglets weaned at 28 or 42 days of age.

Dietary manipulation can modify a number of the changes in GIT physiology that occur after weaning. For example regularly feeding a milk replacer reduced the increases in crypt depth in the distal small intestine, and a diet based on hydrolysed casein reduced the increases in crypt depth and the reductions in brush border enzyme activities in the mid- to distal small intestine (Hampson, 1986b). In addition, supplementation with live yeasts

for 3-4 weeks to weaned pigs improved villus height in the jejunum (Baum *et al*., 2002) and in the ileum (Bontempo *et al*., 2006). *Saccharomyces bourlardii* was able to alter the chemistry of jejunum enteric neurons in pigs (Kamm *et al*., 2004).

2.3.2 Weaning and the GIT microbiology and fermentation acids

Previous studies showed that the population of lactobacilli declined and concurrently coliform counts increased after weaning, indicating an inverse relationship between lactobacilli and coliform bacteria. For example, Mathew *et al*. (1996b) reported that regardless of whether piglets were weaned at 21 or 28 days of age, the total lactobacilli counts in the ileum were found to decrease at day 3 and recover at day 7 post weaning, while total coliforms in the ileum increased post weaning, then decreased by 41 days of age. This study also indicated greater fluctuations in the bacteria population in pigs weaned at 21 days of age compared with pigs weaned at 28 days of age. Similarly, Sutton & Patterson (1996) reported that in the intestines of piglets within 2 days of weaning, numbers of lactobacilli had decreased and *Escherichia coli (E. coli)* counts had increased. Compared with un-weaned piglets, piglets weaned at 21 days of age had lower lactobacilli in the ileum content at every sampling time, on day 23, 27 and 32 of age (Konstantinov *et al*., 2006).

The changes in GIT microbial population can be measured by investigation of the bacterial fermentation activities. The main products of fermentation include lactic acid and volatile fatty acids (VFA). Mathew *et al*. (1994) found that in the ileum of weaned piglets, the decreased LAB population and increased *E. coli* counts coincided with decreased VFA concentrations and increased lactate concentration. Other studies also found that VFA concentrations decreased in the GIT of young piglets subsequent to weaning, such as in the ileum (Mathew *et al*., 1996a), in all intestinal segments and in stomach, duodenum, caecum and colon (Mathew *et al*., 1993). Similarly, Konstantinov *et al*. (2006) reported decreased VFA concentration and LAB counts coincidently in the ileum content of weaned pigs during 12 days post weaning compared with un-weaned pigs.

2.3.3 Weaning and the GIT immune system and diarrhoea

The pig placenta does not transport maternal immunoglobin and thereby the newborn piglets get the maternal immunoglobulin from colostrum during the first 24 h to 48 h of life for immune protection, development and survival (Lallès *et al*., 2007a; Lallès *et al*., 2007b). Microbial colonization of the intestine drives the development of the immune system in several

ways; much of this is antigen-specific. The young piglet is capable of active immune responses to live virus and to dietary components at about 3 weeks of age (Bailey *et al*., 2004). The antigenic challenge at weaning, at around 3-5 weeks of age, occurs when the mucosal immune system has developed to the point of making active immune responses. In addition, the immunoregulatory and immunoprotective components of maternal milk are both removed (Lallès *et al*., 2007b).

As a consequence, post weaning diarrhoea commonly occurs, and while it is not associated with a particular age of pig, it is quite clearly linked to the event of weaning (Lallès *et al*., 2007b). The weaning diarrhoea in piglets happens commonly within day 3 to day 10 post weaning (Pluske *et al*., 1997) and is associated with enterotoxigenic *Escherichia coli* and rotavirus (Nabuurs *et al*., 1993a). In addition, other pathogens or other factors may contribute to the development of post-weaning diarrhoea (Hampson *et al*., 1985). For example, after weaning the net absorption of fluid and electrolytes in the small intestine of pigs is temporarily decreased, giving a condition that may initiate diarrhoea (Nabuurs *et al*., 1994); or the low absorptive capacity in the ileum could result in an increased risk of osmotic diarrhoea (Boudry *et al*., 2004). Furthermore, the reduction in small intestinal absorptive area and the appearance of a less mature enterocyte population resulted in diarrhoea in weaned pigs (Hampson, 1986a). Nabuurs *et al*. (1993b) observed that piglets that died of diarrhoea after weaning had shorter villi and deeper crypts than healthy piglets. However, there is a strong relation between the development of post-weaning diarrhoea and the presence of pathogenic strains of *E. coli* (Hinton *et al*., 1985).

2.4 Probiotics and their modes of action

2.4.1 Probiotics

Live bacteria supplements are widely used in human food and animal feed for promotion and improvement of the host health. Firstly, in 1907, Metchnikoff reported that ingested yoghurt altered the gut flora, and since then many studies have been carried to support this (Cole & Fuller, 1984). The word "probiotic" was used by Parker (1974) to describe animal feed supplements which had a beneficial effect on the host animal by affecting its gut flora, and also to indicate actions of microorganisms that were the opposite of antibiotics. Later, according to the definition by Fuller (1989), a probiotic was "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". According to Stanton *et al*. (2001) "probiotics can be described as single or mixed cultures

of live microorganisms that when applied to animals or humans beneficially affect the host by improving the properties of the indigenous microflora". The Food and Agriculture Organization (FAO/WHO 2002) suggested that "a probiotic is defined as a live microorganism which when administered in adequate amounts confers a health benefit on the host".

Lactobacilli and *bifidobacteria* have a long history of safe use in human food, and these strains are considered as inhabitants of the gastrointestinal microbiota (Servin, 2004; Salminen *et al.*, 1998; Saxelin *et al.*, 1996). These probiotics have been found to be beneficial to the host gut health, such as by their antimicrobial activities against gastrointestinal microbial pathogens *Salmonella* and *E. coli* (Servin, 2004), prevention and treatment of diarrhoea in infants and adults (Bezkorovainy, 2001; Heyman, 2000), and alteration of the composition and metabolism of the intestinal microbiota (Vandenbergh, 1993). In addition, probiotics can reduce the faecal activities of enzymes known to produce genotoxic compounds that act as tumour initiators in human beings (Guarner & Malagelada, 2003).

In animal nutrition, the types of microorganisms used as probiotics are lactobacilli, bifidobacteria, enterococci, *E. coli*, bacilli and yeasts (Ohashi & Ushida, 2009; Jensen, 1998; Vanbelle *et al.*, 1990; Fuller, 1989). The selection criteria in laboratories for screening microorganism strains to be used as probiotics are: (1) acid tolerance to ensure their survival during passage through the stomach and duodenum; (2) bile tolerance to ensure their survival during passage through the upper small intestine; (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 fimbriate to the brush-border cells; (7) immunological modulation; (8) heat tolerance in order to survive during pelleting of feeds; (9) tolerance of feed antimicrobials in order to combine with medicated feed (Nousiainen & Setälä, 1998; Vanbelle *et al.*, 1990).

The variation in efficacy of probiotics under different conditions may be attributable to the preparation of the probiotic, or may be caused by external conditions. There are a number of substances which may enhance the efficacy of probiotics, such as prebiotics, nutrients and growth factors, proteins, polyunsaturated fatty acids, organic acids and bacterial metabolites (Nemcová *et al.*, 2007).

2.4.2 Modes of action of probiotics

Alteration of microbiota composition: Probiotics can reinforce or re-establish the microbial balance, to correct the disturbance of the gut microbiota

(Vanbelle, 2001). In addition, the population of indigenous *Lactobacilli* in pigs was found to increase with oral administration of *Lactobacillus plantarum* strain Lq80 (Takahashi *et al*., 2007), or fermented milk prepared with *Lactobacillus casei* strain Shirota (Ohashi *et al*., 2001).

Competition for adhesion sites: Several previous studies *in vitro* in pigs found that *Lactobacilli* can compete with pathogens for adhesion sites in the gut. For example, *L. fermentum* 104R produced a proteinaceous component that could inhibit the adhesion of *E. coli* K88ab and K88ac fimbriae to ileal mucus by interacting with mucus components (Blomberg *et al*., 1993). Strains of *L. fermentum spp., L. acidophilus spp. and L. salivarius spp.* were found to affect the attachment of enterotoxigenic *E. coli* to porcine enterocytes by coaggregating with *E. coli* (Spencer & Chesson, 1994).

Production of antibacterial compounds: Lactic acid produced by both homofermentative and heterofermentative LAB reduce pH in the luminal contents, which can inhibit the proliferation of pathogenic bacteria (Nousiainen & Setälä, 1998; Vanbelle *et al*., 1990). In addition, acetic acid excreted by heterofermentative LAB and H_2O_2 may be toxic to some other bacteria (Nousiainen & Setälä, 1998). Furthermore, organic acid and H_2O_2 produced by LAB are inhibitory against coliforms, salmonella and clostridia *in vitro* (Nousiainen & Setälä, 1998). Several other antibacterials are also produced by LAB *in vitro*, such as acidophilin, acidolin or reuterin and nicin (Juven *et al*., 1991; Tagg *et al*., 1976).

Activity against rotavirus: In pathogen-free suckling rats infected with SA11 rotavirus, supplementation daily with milk fermented by *Lactobacillus casei* DN-114 001 increased brush-border enzyme activities in the small intestine (Thoreux *et al*., 1998). In the piglet developing naturally acquired diarrhoea, the administration of *Bifidobacterium lactis* HN019 decreased concentrations of faecal rotavirus (Shu *et al*., 2001).

Alteration of the digestive enzyme activity: Ingested LAB produce and release hydrolytic enzymes, which might aid digestion in farm animals, particularly during the early life of piglets and calves (Nousiainen & Setälä, 1998). For example, rats fed yogurt had increased β-galactosidase activity in their small intestine and the enzyme seemed to be of bacterial origin (Garvie *et al*., 1984). In addition, probiotics can increase some useful enzyme activities of the host. For example, Collington *et al*. (1990) fed piglets with diets supplemented with either antibiotic or LAB probiotic, and found increased lactase and sucrase activities in the small intestine mucosa with both treatments compared with the control.

Stimulation of immunity: Dietary supplementation with *Bacillus cereus var. toyoi* NCIMB 40112 increased intestinal IgA secretion in sows and piglets

(Scharek *et al*., 2007). Lessard *et al*. (2009) reported that the probiotics *Pediococcus acidilactici* and *Saccharomyces cerevisiae boulardii* supplemented to pigs might have the potential to modulate the establishment of lymphocyte populations and IgA secretion in the gut and reduce the bacterial translocation to mesenteric lymph nodes after *Escherichia coli* infection.

Improvement of intestinal barrier function: Administration of *Saccharomyces cerevisiae* spp. *boulardii*, or *Saccharomyces boulardii* and *Bacillus cereus* var. toyoi to weaned piglets for 3-4 weeks improved villus height epithelial cell proliferation and the numbers of macrophages at various sites of the small intestine (Bontempo *et al*., 2006; Baum *et al.,* 2002).

2.5 Influence of feeding exogenous enzymes in pigs

Johnson *et al*. (1993) indicated that successful use of enzymes in diets for monogastric animals can only be achieved when target applications in terms of animal physiology and feed chemistry are accurately and precisely identified. Feed enzymes have been targeted routinely at young pigs, which exhibit a relatively slow rate of development of the endogenous enzyme systems (Corring *et al*., 1978). However, inclusion of feed enzymes in conventional weaner pig feeds was, in many cases, not successful, as the diets contained highly digestible ingredients. Under these circumstances the addition of enzymes would not be expected to markedly improve nutrient availability, and responses would be difficult to detect in terms of growth performance (Johnson *et al*., 1993). In contrast, supplementation with enzymes has shown positive effects on growth performance in weaner pigs offered diets based on ingredients known to present problems in digestibility (Omogbenigun *et al*., 2004; Officer, 1995).

3 Materials and methods

3.1 Experimental sites

The experiments were carried out in a pig farm located in Tien Duong Commune, Dong Anh District, Hanoi City, Vietnam (Paper I, II & IV), in the Experimental Farm of the National Institute of Animal Sciences, Tu Liem District, Hanoi City, Vietnam (digestibility trial in Paper II) and in a pig farm in Phu Long Commune, Nho Quan District, Ninh Binh Province, Vietnam (Paper III).

3.2 Experimental animals and design

The experiments in Paper I, II and III were carried out with weaned piglets and the growth and digestibility trials in Paper IV were carried out with grower and finisher pigs. In Paper I, 96 weaned piglets from 12 litters, 48 females and 48 males, Landrace (L) × Yorkshire (Y), at 21-23 days of age with body weight (BW) of 6.6 ± 0.5 kg, were used. The piglets in Paper I were divided randomly into 4 groups balanced for sex, weight and litter origin, with 4 pens (replicates) per group, and 6 pigs per pen. In Paper II, the experiment was carried out with 128 weaned piglets from 16 L × Y litters at 26-28 days of age, 64 females and 64 males, whose BW was on average 7.7 ± 0.9 kg. The piglets in Paper II were also divided randomly into 4 groups, balanced for sex, weight and litter origin, with 4 pens per group, and 8 pigs per pen. In Paper III, a total of 96 weaned piglets, (L × Y) × (Duroc × Pietrain), 48 females and 48 males, from 12 litters, at 21-24 days of age and 7.4 ± 0.6 kg BW, was divided into 4 treatment groups, balanced in sex, weight and litter origin, with 3 pens per group and 8 pigs per pen. All the piglets in Paper I, II and III were introduced during suckling period to the creep feeds, which were without any probiotic or

antibiotic feed additives. These piglets were checked for health status during the pre-weaning period, and were healthy and had not been treated with antibiotics. The piglets were vaccinated against pasteurellosis, parathyphoid, asthma and hog cholera. Each of the experiments in Paper I, II and III lasted for 35 days with 2 periods recorded: the first 2-week period post-weaning and a following 3-week period. The ileal and total tract digestibility, organic acids and bacteria numbers in different gut segments were determined on the last day of each period (day 14 and 35) of the experiments in Paper I and II. Four piglets per treatment (one from each pen) were killed for sampling, resulting in a total of 16 piglets in each period. Thus, for the second period, there were 5 piglets (Paper I) or 7 piglets (Paper II) remaining in each pen.

In Paper IV, 80 pigs (L × Y, 32 females and 48 males), 28.7±0.9 kg BW, were selected for the performance experiment (Exp. 1), and another 16 male pigs (L × Y) with a BW of 29.2±0.8 kg were selected for the digestibility experiment (Exp. 2). In Exp. 1, the pigs were randomly assigned (based on sex and BW) to one of 4 treatment groups (5 pigs per pen, 4 pens per treatment). In Exp 2, the pigs were kept in individual pens and divided into 4 groups with 4 pigs as 4 replicates in each group.

3.3 Experimental diets

3.3.1 Bacteria, yeast and enzyme sources

Lactic acid bacteria strains: Five LAB strains that originated from the digesta of healthy fattening pigs were used in Paper I: three strains were isolated from the ileum content (*Enterococcus faecium* 6H2, *Lactobacillus acidophilus* C3, *Pediococcus pentosaceus* D7) and two strains were isolated from the colon content (*Lactobacillus plantarum* 1K8 and *Lactobacillus plantarum* K23). In Paper II, III and IV, three of the five LAB strains, *E. faecium* 6H2, *L. acidophilus* C3, and *Pediococcus pentosaceus* D7, were used. Another LAB strain, *Lactobacillus fermentum* NC1, which was isolated from a Vietnamese traditional fermented food (nem chua) was also used in Paper II and IV. The LAB strains were selected in previous tests, based on their resistance *in vitro* to heat, low pH, bile salts, and for antagonism with pathogenic bacteria such as *Salmonella* and *E. coli* (Viet *et al.*, 2006).

Bacillus strain: *Bacillus subtilis* H4 was isolated from the ileum digesta of a healthy fattening pig, and was tested *in vitro* for resistance to heat, low pH, bile salts, and enzyme activities (Viet *et al.*, 2009). This strain was used in Paper II and IV.

Yeast: *Saccharomyces boulardii* Sb was obtained from the Vietnam Type Culture Collection, Institute of Microbiology and Biotechnology, Vietnam

National University, Hanoi, Vietnam. This strain was tested for probiotic properties *in vitro* for resistance to heat, low pH, bile salts, and for antagonism with the pathogenic bacteria *Salmonella, Shigella* and *E. coli* (Viet *et al*., 2009). This strain was used in Paper II, III and IV.

Enzymes: protease was produced from a *Bacillus subtilis* UL-15 fermentation, and amylase from *Bacillus licheniformis* VTCC8B-768 fermentation. β-glucanase and xylanase were produced from *Rhodococcus fascian* A2026 fermentation, and cellulase was from *Aspergillus niger van tieghem var niger* VN06-F0329 fermentation. All the bacteria were obtained from the Vietnam Type Culture Collection, Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam. The enzymes selected were mixed together with rice bran as a carrier, and then dried at 45 ^{0}C until the dry matter reached 94%. These enzymes were used in Paper III.

3.3.2 Diets

In Paper I, three combinations of LAB strains were tested in weaned pigs: LAB complex 1 was comprised of *E. faecium* 6H2 (3×10^{8} CFU/g), *L. acidophilus* C3 (4×10^{6} CFU/g) and *Pediococcus pentosaceus* D7 (3×10^{6} CFU/g); LAB complex 2 was comprised of *E. faecium* 6H2 (3×10^{8} CFU/g), *L. acidophilus* C3 (4×10^{6} CFU/g) and *L. plantarum* 1K8 (2×10^{6} CFU/g); LAB complex 3 was comprised of *L. acidophilus* C3 (4×10^{6} CFU/g), *L. plantarum* 1K8 (2×10^{6} CFU/g) and *L. plantarum* 3K2 (7×10^{6} CFU/g). Each of the four groups of piglets was fed one of four diets: the basal diet **(Diet C)**, the basal diet supplemented with LAB complex 1 **(Diet C1)**, with complex 2 **(Diet C2)** and with complex 3 **(Diet C3)**. Each day, the LAB complexes were mixed into the basal diet at a level of 600 ppm (200 ppm per each strain). The LAB strains, prepared separately, were in meal form with whey powder as a carrier, and were stored in a refrigerator at 4 ^{0}C in separate bags until mixed with the basal diet.

Different combinations of LAB, *Bacillus* and yeast were tested in weaner, grower and finisher pigs (Paper II & IV). In Paper II, four diets were given to the four groups: the basal diet **(Diet C)**, the basal diet supplemented with a LAB complex **(Diet L)**, with a LAB-*Bacillus* complex **(Diet LB)**, and with a LAB-*Bacillus*-*Saccharomyces* complex **(Diet LBS)**. In Paper IV, a basal diet **(Diet C)**, or the basal diet supplemented with *Bacillus* alone **(diet B)**, or added to a mixture of *Bacillus* and *Saccharomyces* **(Diet BS)**, or added to a mixture of *Bacillus, Saccharomyces and LAB* **(Diet BSL)** was given to one of four groups in Exp. 1 & 2. The LAB complex in diet L, LB and LBS (Paper II) and diet BSL (Paper IV) was the same, and contained three strains of

complex C1 in Paper I and strain *L. fermentum* NC1. These bacteria and yeast strains were in culture form, prepared every 3 days with average density of colonies per ml culture of: *E. faecium* 6H2: 6×10^{9} CFU, *L. acidophilus* C3: 5×10^{9} CFU, *Pediococcus pentosaceus* D7: 4.9×10^{9} CFU, *L. fermentum* NC1: 6×10^{9} CFU, *B. subtilis* H4: 6×10^{11} CFU, *S. boulardii* Sb: 6×10^{10} CFU. These strains were stored in a refrigerator at 4 ^{0}C in separate bottles until added to the basal diets every day, at 2 ml (Paper II) or at 3 ml (Paper IV) of each bacteria strain, and of *Saccharomyces*, per kg feed.

In Paper III, each of the four piglet groups was fed one of four diets: Basal diet **(Diet C)**, the basal diet supplemented with 0.2% of enzyme complex **(Diet E)**, the basal diet supplemented with a 0.2% mixture of LAB and yeast **(Diet LY)**, and the basal diet supplemented with a complex of 0.2 % LAB-yeast and 0.2% enzymes **(Diet LYE)**. The LAB complex in Paper III contained 3 strains, the same as in LAB complex 1 in Paper I. The LAB strains and yeast were in meal form with dextrin and whey powder as carriers, and contained *E. faecium* 6H2 (4.8×10^{7} CFU/g), *L. acidophilus* C3 (5.2×10^{7} CFU/g), *P. pentosaceus* D7 (4.0×10^{7} CFU/g), and *S. boulardii* Sb (2.6×10^{8} CFU/g). The enzyme mixture was in meal form and contained amylase 2200 IU/g, protease 110 IU/g, cellulase 1100 IU/g, β-glucanase 200 IU/g, and xylanase 1000 IU/g. The supplements were stored in a refrigerator at 4 ^{0}C until mixed with the basal diet.

The basal diets in Paper I, II and III were composed of extruded maize, extruded rice, soybean meal, extruded full fat soybean, soybean protein concentrate, milk replacer, whey powder, soybean oil, synthetic amino acids, and premix of vitamins and minerals, formulated to meet the nutrient requirements of exotic post-weaning piglets (TCN 651-2005) (in Paper I), or to meet NRC (1998) recommendations (Paper II and III). In paper IV, the basal diet consisted of maize meal, rice bran, cassava root meal, soybean meal, meat and bone meal, dicalcium phosphate, a vitamin and mineral premix, and synthetic amino acids, formulated following NRC (1998) recommended feeding standards for growing pigs (20-50 kg BW) and finishing pigs (> 50 kg BW). All the basal diets were in meal form and were without antibiotics or probiotics. Chromium oxide was used as marker added at 3 g/kg of the basal diet to determine digestibility of nutrients in Paper I, II and III.

3.4 Measurements

3.4.1 Performance

In Paper I, II, III, the piglets were weighed individually at the beginning of the experiment (at weaning), on day 14 and on day 35, and the feed offered and refused was weighed daily to calculate average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) in each period and overall. Similar measurements and calculations in Exp. 1 of Paper IV, ADFI, ADG and FCR were made in a 33-day growing period (28.8 - 54.2 kg BW) and a 42-day finishing period (54.2 - 89 kg BW). The pigs in the experiments had free access to the feed and water during experimental periods.

3.4.2 Health status

The health status of piglets in Paper I and II during the first 14 days after weaning was assessed by faecal consistency scoring using a four-grade system, where 0 corresponded to firm and dry; 1 to pasty; 2 to thick and fluid; and 3 to watery (Cupere *et al.*, 1992). The incidence of diarrhoea (%) was calculated as the sum of the total number of diarrhoeal piglets over the period divided by the number of piglet days in the period multiplied by 100. The faecal score was calculated as the sum of the diarrhoea scores over the period divided by the number of piglet days in the period.

3.4.3 Digestibility, organic acids and bacteria determinations

In Paper I and II, immediately after slaughter, the gastrointestinal tract was removed, and the digesta collected from stomach, ileum (1.2 m of intestine before the ileo-caecal ostium) and mid-colon (divided into 3 equal parts, with the digesta in the middle part being collected), and faeces was collected from the rectum. The samples were stored in separate sterile tubes on ice containers and transported to the laboratory shortly afterwards. The bacteria counts in the digesta samples were carried out the same day and other samples for determination of digestibility and organic acids were kept in a refrigerator at -20 ^{0}C until analysis. In Paper III, the apparent total tract digestibility was measured by using faeces samples collected daily during the sampling period from each pen. In Exp. 2 of Paper IV, the total tract digestibility and nitrogen retention were measured by using individual metabolism cages, with 5 days for adaptation and 5 days for collection. The pigs were fed *ad libitum* in the adaptation period to calculate mean feed intake, and then restricted to 85% of the mean feed intake in the collection

period. Total faeces and urine were collected twice per day at 08.00 h and 16.00 h.

The coefficients of apparent digestibility (CAD) of dietary components at the ileum (CIAD) (Paper I & II) and total tract (CTTAD) (Paper I, II & III) were determined using chromium oxide as indigestible marker and calculated according the equation (Sauer *et al.*, 2000):

$$CAD_D = 1 - (DC_F \times I_D) / (DC_D \times I_F)$$

where CAD_D is the coefficient of apparent digestibility of a dietary component, DC_F is the dietary component concentration in digesta or faeces, I_D is the indicator concentration in diet, DC_D is the dietary component concentration in diet, and I_F is the indicator concentration in digesta or faeces.

Bacterial counts: The digesta (Paper I & II) and faeces samples (Paper I, II & IV) were dissolved in sterile saline (0.9 %) in a 1:10 dilution. The stomach content samples were secondary diluted from 10^{-2} to 10^{-4} for *E. coli* counts and from 10^{-4} to 10^{-6} for total LAB counts. The further dilutions of the samples of ileal and colon digesta and of faeces taken from rectum were from 10^{-4} to 10^{-6} to estimate *E. coli* counts and were from 10^{-5} to 10^{-7} to estimate total LAB population. *E. coli* was cultured on MacConkey agar (MAC), under aerobic conditions for 24 h at 37 ^{0}C, and colonies with smooth, convex, circular shape, entire edges, and a pink colour were counted as *E. coli*. LAB was cultured on MRS agar (Mann, Rogosa and Sharpe) under anaerobic conditions for 48 h at 37 ^{0}C, and all colonies were counted as LAB.

3.5 Chemical analysis

Samples of feed, ileal digesta and faeces were analyzed for dry matter (DM), crude protein (CP), crude fibre (CF) and ash according to standard AOAC (1990) methods. Amino acid contents in the feed samples were determined using an ion exchange column (Amino Quant, 1990) (Amino Quant, 1990). The chromium content in feed, digesta and faeces was analyzed using an atomic absorption spectrophotometer (Spectr AA Perkin Elmer™, USA) (National Institute for Occupational Safety and Health, 1994). Acetic, propionic, butyric and lactic acid in samples from the stomach, ileum, colon and rectum were analyzed with high-performance liquid chromatography (HPLC Shimadzu, Japan) (Food Components Adhoc Committee, 2002).

3.6 Statistical analysis

The data of growth performance, digestibility, diarrhoea score and faecal bacteria were analysed statistically using the GLM of Minitab Software Version 14.1. Treatment means which showed significant differences at $P<0.05$ were compared using Tukey's pair-wise comparison procedure. In Paper I and II, the Chi-Square Test was used to compare the diarrhoea incidence between the treatments. The data of LAB and *E. coli* in Paper I, II and IV was transformed as $\log_{10}$ before statistical analysis. The data of LAB and *E. coli* counts ($\log_{10}$ CFU), and organic acid concentration, in Paper I and II, was analysed with a statistical model that included main effects (treatments and segments) and their interactions.

4 Summary of results

4.1 Growth performance (Paper I, II, III & IV)

In the first two weeks post-weaning, piglets fed diets C1, C2 and C3 (Paper I) and diets L, LB and LBS (Paper II) showed improved ADFI, ADG and FCR compared with the controls ($P<0.05$). There were no differences in ADFI, ADG and FCR among the different LAB complexes C1, C2 and C3 ($P>0.05$), and no differences in FCR among piglets fed diets L, LB and LBS were observed ($P>0.05$). Piglets fed diet LBS had higher ADFI than those fed diets L and LB ($P<0.05$), and had higher ADG than those fed diet L ($P<0.05$). In Paper III, the piglets fed diets E, LY and LYE had higher ADG ($P<0.01$) and lower FCR ($P<0.05$) compared to those fed the control diet, while the ADFI was not affected by treatment ($P>0.05$). There were no differences in ADG and FCR among piglets fed diets E, LY and LYE ($P>0.05$).

In the second period, from week 3 to week 5 post-weaning, the inclusion of LAB complexes in diets C1, C2 and C3 (Paper I) and inclusion of the enzyme mixture in diet E (Paper III) did not improve ADFI, ADG and FCR of the piglets compared with the controls ($P>0.05$). However, diets L, LB and LBS (Paper II) improved ADFI, ADG and FCR ($P<0.05$), and diets LY and LYE (Paper III) improved ADG and FCR ($P<0.05$) compared with the controls. There were no differences in the performance of piglets among diets L, LB and LBS or between diets LY and LYE.

In the growing period (Paper IV), pigs fed diet BSL had improved ADG and FCR ($P<0.05$) compared with those fed the control diet, while pigs fed diets B and BS did not show any improvement of performance compared with the control ($P>0.05$). In the finishing period (Paper IV), pigs fed diets B, BS and BSL did not show any improvement in performance compared with the control ($P>0.05$).

4.2 Nutrient digestibility (Paper I, II, III & IV)

In the first period, the coefficient of ileal apparent digestibility (CIAD) of organic matter (OM), CP and CF, and the coefficient of total tract apparent digestibility (CTTAD) of CP and CF were higher ($P<0.05$) in the piglets given LAB complexes compared with the control piglets (Paper I). However, there was no difference ($P>0.05$) in digestibility among the three LAB complexes (Paper I). The CIAD and CTTAD of OM and CP were higher ($P<0.01$) in piglets fed diets L, LB and LBS than in piglets fed the basal diet (Paper II). The CIAD of CP was higher ($P<0.05$) in piglets fed diet LBS than in those fed diet L, and the CTTAD of CP and OM was higher ($P<0.05$) in piglets fed diets LB and LBS than in piglets fed diet L (Paper II). The apparent total tract digestibility (ATTD) of CP, CF and OM was higher ($P<0.001$) in piglets fed diets E, LY and LYE compared with the piglets fed the control diet, and there was no difference ($P>0.05$) in ATTD of CP, CF and OM among diets E, LY and LYE (Paper III).

In the second period, there was no effect ($P>0.05$) of LAB complex supplementation on the CIAD or CTTAD of OM, CP and CF (Paper I). Diets L, LB and LBS (Paper II) had higher CIAD and CTTAD of OM and CP than the control diet ($P<0.01$), and diets LB and LBS had higher CIAD and CTTAD of OM and CP than diet L ($P<0.05$), while there were no differences in digestibility between diets LB and LBS ($P>0.05$). In Paper III, diets LY and LYE had higher ATTD of CP and OM than diet E and the control ($P<0.05$), while diet E improved only ATTD of CP compared with the control ($P<0.05$).

In grower pigs (Paper IV), diets B and BS did not affect the total tract digestibility of CP and OM compared with the control ($P>0.05$). However, pigs fed diet BS had higher digestibility of CF ($P<0.05$) and pigs fed diet BSL had higher digestibility of CP, CF and OM ($P<0.05$) compared with pigs fed the basal diet. Nitrogen retention was not affected by treatment ($P>0.05$). However, there was a tendency to higher nitrogen retention on diet BSL ($P=0.058$) compared with the control.

4.3 Diarrhoea (Paper I & II)

During the first week after weaning, the diets that contained LAB complexes, C1, C2 and C3 (Paper I), did not result in any improvements in diarrhoea incidence and faecal score compared with the control ($P>0.05$). In

Paper II, diets L, LB and LBS did not improve the incidence of diarrhoea compared with the control ($P>0.05$); however, piglets fed diet LBS had lower faecal scores than piglets fed the control diet ($P<0.05$).

In the second week and overall, piglets fed diets C1, C2 and C3 (Paper I) and diets L, LB and LBS (Paper II) had significantly lower diarrhoea incidence and faecal score than the control groups ($P<0.05$). No difference among the LAB treatments (Paper I) was observed ($P>0.05$), while diet LBS was more effective ($P<0.05$) than diets L and LB (Paper II).

4.4 Bacteria counts (Paper I, II & IV)

On day 14 after weaning, the LAB population in the stomach and ileum was increased ($P<0.05$) by all three LAB complexes supplemented in diet C1, C2 and C3, while there was no difference in *E. coli* counts among treatments at all sites of the digestive tract ($P>0.05$), although there was a tendency towards lower *E. coli* counts in piglets fed LAB complexes (Paper I). Higher LAB counts ($P<0.05$) were found in all four segments (stomach, ileum, colon and rectum) and lower *E. coli* counts were found in the stomach and ileum ($P<0.05$) in piglets fed diets L, LB and LBS than in piglets fed the basal diet (Paper II).

On day 35 post-weaning, LAB counts were increased ($P<0.05$) at all sites of the digestive tract by the inclusion of the three LAB complexes in diet C1, C2 and C3, and *E. coli* counts were lower ($P<0.05$) in the ileum of piglets given the LAB complex supplemented in diet C1 (Paper I). Piglets fed diets L, LB and LBS (Paper II) had higher ($P<0.05$) LAB counts in the stomach, ileum and colon and had lower ($P<0.05$) *E. coli* counts in the ileum than those fed the control diet.

In grower pigs, diet BSL increased the faecal LAB count compared with diets C, B and BS ($P<0.01$), and diets BS and BSL decreased ($P<0.05$) faecal *E. coli* counts compared with diets C and B (Paper IV). There were no effects ($P>0.05$) of diet on faecal LAB and *E. coli* counts in the finisher pigs (Paper IV).

4.5 Gut environment (Paper I & II)

Overall, across all treatments (Paper I & II), lactic acid concentration was the highest ($P<0.05$) in the ileum, and the concentration of total volatile fatty acids (VFA) was the highest ($P<0.05$) in the colon on both days 14 and 35. In Paper I, overall average concentrations of lactic and acetic acid were higher ($P<0.05$) in piglets fed diets C1, C2 and C3 than in control pigs. The

inclusion of LAB complexes (Paper I) increased LAB counts in the ileum and colon ($P<0.05$), while no effects of treatment on propionic and butyric acid concentrations at all sites of the gut were found on both day 14 and 35 ($P>0.05$). There was no difference ($P>0.05$) in organic acid concentrations in the pig gut among diets C1, C2 and C3 (Paper I). In Paper II, piglets fed diets L, LB and LBS had higher contents of total VFA and organic acids (VFA plus lactic acid) in the ileum and colon ($P<0.01$) than piglets fed the basal diet, while no treatment effects on the contents of organic acids and total VFA were found in the stomach and rectum ($P>0.05$) on both day 14 and 35. On day 14, there were no differences ($P>0.05$) among piglets fed diets L, LB and LBS in the content of individual VFA and total organic acids (Paper II). However, on day 35 (Paper II), pigs fed diet LBS had the highest ($P<0.05$) total VFA concentration in the ileum compared with the other treatment groups.

5 General discussion

5.1 Effects of probiotics and enzymes on performance and digestibility

5.1.1 Effects of supplementation with LAB complexes (Paper I, II & IV)

Effects of dietary supplementation with the 3-strain LAB complexes (diets C1, C2 and C3 in Paper I) improved the performance and nutrient digestibility during the first two weeks after weaning (Per I) but did not affect them in the following 3-week period (Per II), while the 4-strain LAB complex alone (diet L in Paper II) improved the performance and digestibility over a longer period (both Per I and II). Supplementation of LAB to weaned pig diets has been found to give positive effects on performance in previous studies (Mallo *et al.*, 2010; Lessard *et al.*, 2009; Guerra *et al.*, 2007; Huang *et al.*, 2004; Pollmann *et al.*, 1980). The improvement of growth performance and feed efficiency in piglets fed diets supplemented with LAB complexes in the current study could have resulted from the higher nutrient digestibility in these diets than in the un-supplemented basal diets. The higher organic acid concentration in the ileum and colon of LAB-fed piglets in the current study should be expected to lower pH (Högberg & Lindberg, 2006), and a low gut pH has been shown to have a beneficial effect on nutrient digestibility (Lyberg *et al.*, 2006; Canibe & Jensen, 2003). In addition, the higher gut LAB count in LAB-fed piglets could increase the activity of various hydrolytic enzymes, resulting in better nutrient utilization (Nousiainen & Setälä, 1998; Fuller, 1989). Collington *et al.* (1990) found increased activity of brush-border enzymes (lactase and sucrase) in weaned piglets fed diets with a LAB probiotic added. Furthermore, the lower scouring frequency in piglets fed diets supplemented with the LAB complexes in the current study could also have contributed to the higher digestibility. Higher digestibility in piglets

fed diets supplemented with a LAB complex *(L. gasseri, L. reuteri, L. acidophilus* and *L. fermentum)* after weaning was also reported by Huang *et al.* (2004).

The two-week period after weaning represents a period of adaptation and potential stress, regardless of whether the pigs are weaned at 2 or 6 weeks of age (McCracken & Kelly, 1993). As the efficacy of probiotics is higher when the animals are confronted with challenge or stress (Estienne *et al.*, 2005; Jensen, 1998) this may explain the clear positive effects of the LAB complexes during the first two weeks post-weaning in Paper I of the present study. Freter (1992) presented strong evidence that indicated that probiotics containing one or a few bacterial strains will have limitation in their broad action and their effectiveness in the gut. Thus, diet L (Paper II) contained four LAB strains and could be expected to act in a wider range of the gut conditions and be more effective than the three 3-strain LAB complexes in Paper I. In addition, with dietary supplementation with 0.1% or 0.2% of a probiotic, the higher level was found to improve efficacy in growing pigs while the lower level did not show any effect (Chen *et al.*, 2005). Thus, diet L (in Paper II) was more effective for a longer period (5 weeks post-weaning) than the diets C1, C2 and C3 (in Paper I) and this might also have resulted from the higher LAB density in the diet (4.4×10^7 CFU compared with around 4.1×10^4 CFU per kg).

In grower and finisher pigs (Paper IV), while dietary supplementation with *Bacillus* alone (diet B) or combined with *Saccharomyces* (diet BS) did not affect the performance and nutrient digestibility, the addition of the 4-strain LAB complex to the mixture of *Bacillus* and *Saccharomyces* (diet BSL) improved the performance and digestibility in grower pigs, but did not affect these parameters in finisher pigs, compared with the un-supplemented diets (diets B, BS and control). These results suggest that the LAB complex had some effects, dependently or independently of the *Bacillus* and yeast, that improved digestibility, and consequently improved the performance of the grower pigs. Previous studies on grower and finisher pigs reported a lack of positive effects of single-strain LAB and of multi-strain LAB probiotics supplementation (Chen *et al.*, 2006; Apgar *et al.*, 1993; Harper *et al.*, 1983; Pollmann *et al.*, 1980). However, in a study on dietary supplementation with mixtures of *B. subtilis, S. cervisae and L. acidophilus*, Chen *et al.* (2005) found improved ADG in grower pigs, which supports the results in the current study.

5.1.2 Effects of supplementation with Bacillus (Paper II & IV)

Addition *B. subtilis* H4 to the LAB complex (diet LB in Paper II) increased the diet digestibility and had a tendency to improve ADG in Per I (week 1-2 post weaning). It also increased both nutrient digestibility and performance in Per II (week 3-5) compared with the LAB complex alone (diet L in Paper II), indicating an additional effectiveness of *Bacillus*. *B. subtilis* can germinate in the upper part of the intestine, especially when the gastrointestinal flora is disturbed during gastrointestinal disorders (Mazza, 1994), and *B. cerus var. toyoi* can germinate in both the upper and lower part of the intestine of pigs (Jadamus *et al.*, 2001). It has been found that *B. subtilis* is able to produce useful enzymes, such as α-amylase, arabinase, cellulase, dextranase, levansucrase, maltase, alkaline protease, neutral protease and β-glucanase (Priest, 1977). In addition, *B. cereus* is able to stimulate the rate of glucose transport throughout brush border vesicles from porcine jejunum *in vitro* (Breves *et al.*, 2000). These actions of *Bacillus* could have resulted in the improved nutrient digestion and utilization of feed, contributing to the improved performance. In agreement with the current study, *Bacillus* probiotics have been found to improve feed intake, weight gain and feed efficiency in weaned piglets in previous studies (Taras *et al.*, 2005; Kyriakis *et al.*, 1999; Zani *et al.*, 1998; Kirchgessner *et al.*, 1993).

However, in grower and finisher pigs, addition of *Bacillus* alone to the diet (diet B) did not affect digestibility and performance compared with the un-supplemented control (Paper IV). In accordance with the current results, (Kornegay & Risley, 1996) reported that supplementation of a mixture of *Bacillus subtilis* and *B. licheniformis,* or a mixture of *B. subtilis*, *B. licheniformis* and *B. pumilus* in a finisher pig diet, did not result in any improvement in ADFI, ADG and feed efficiency. Moreover, another study by Wang *et al.* (2009) did not find any improvement in the performance of grower pigs fed diets supplemented with 0.05% or 0.1% of a commercial *Bacillus* probiotic, but ADFI increased significantly and ADG tended to increase when the level of probiotic was 0.2%.

5.1.3 Effects of supplementation with yeast (Paper II, III & IV)

The inclusion of *S. boulardii* Sb in the 3-strain-LAB complex (diet LY in Paper III) had probiotic effects on the performance and digestibility in piglets during the 5-week period after weaning, while inclusion of the 3-strain-LAB complex alone (diet C1 in Paper I) had a probiotic effect only in the 2-week period after weaning. The 3 LAB strains were the same in these two diets, and the density of LAB in diet C1 was 6.1×10^4 CFU and in diet LY it was 2.8×10^5 CFU per kg. The higher LAB density might have partly

contributed to the effectiveness of diet LY, as higher levels of probiotics supplemented to diets have been found to improve their efficacy (Wang *et al.*, 2009; Chen *et al.*, 2005). However, *Saccharomyces* are rich sources of enzymes, nutrients and growth factors (Kornegay *et al.*, 1995) and supplementation with the yeast in weaner diets has been reported to improve the performance of weaned piglets in previous studies (Bontempo *et al.*, 2006; Li *et al.*, 2006; Mathew *et al.*, 1998; Jurgens *et al.*, 1997). Furthermore, probiotics containing multi strains of more than one species are more efficient than those of multi strains of one species, as the mixed bacteria/strains can complement each other to act in the host's gut environmental conditions (Timmerman *et al.*, 2004). Thus, diet LY (Paper III), that contained a mixture of a yeast and a 3-strain-LAB complex, could be expected to be more effective with respect to performance and digestibility than diet C1 (Paper I), which only contained the 3-strain-LAB complex. These positive effects of the yeast in weaned piglets were confirmed in Paper II, as the results showed that adding the yeast to the diet which contained a mixture of *Bacillus* and LAB (diet LBS) increased feed intake compared with the diet that contained the mixture of *Bacillus* and LAB (diet LB) and increased the digestibility compared with the diet that contained the LAB complex only (diet L) during the first two weeks after weaning. The improvement in the performance of LBS-fed piglets could result from the lower diarrhoea score and incidence in piglets fed that diet. A higher effectiveness with respect to ADG and ADFI of weaned piglets of supplementing *Bacillus* and an active yeast complex was found by Min *et al.* (2004) compared with either *Bacillus* or the active yeast alone. In grower and finisher pigs (Paper IV), the addition of the yeast to the diet (diet BS) did not affect performance and digestibility compared with the un-supplemented diets (diets C and B). These results were similar to those found by Veum & Bowman (1973) when supplementing a *Saccharomyces cervisiae* culture to a grower pig diet.

5.1.4 Effects of supplementation with enzymes (Paper III)

In the current study (Paper III), addition of microbial enzymes (amylase, proteases, cellulase, β-glucanase and xylanase) (diet E) improved the ADG and FCR and digestibility of CP, crude fibre and OM in the first two weeks post-weaning compared with the control. The proteolytic and amylolytic digestive system of pigs is not well developed until 4 or 6 weeks of age (Kidder & Manners, 1978). In addition, weaning is associated with reductions in the activities of brush-border enzymes (Pluske *et al.*, 1997). Lactase and sucrase activities decreased during the 11 days post weaning

(Hampson & Kidder, 1986), and pancreatic enzyme (trypsin, chymotrypsin, amylase and lipase) activities decreased during the first week post-weaning (Lindemann *et al.*, 1986). Moreover, the microbiota in the gastrointestinal tract is not stable during the two or three weeks after weaning (Jensen, 1998), meaning a lack of microbial capacity to degrade the non-starch polysaccharides (NPS) found in the current dietary ingredients (maize, rice and soybean meal). Furthermore, exogenous enzymes can be utilized successfully by young, stressed or ill animals if they have similar properties to those in the animal's endogenous secretions and microbial enzymes (Wenk, 2003). Thus, the inclusion of the microbial enzyme mixture in the current study could have helped the piglets to compensate for their poor digestive capability in the two-week period after weaning, and improved nutrient digestibility, resulting in better growth performance. These results are generally in line with previous studies (Dierick, 1989; Collier & Hardy, 1986).

In the following period, the pigs' digestive capacity is more developed, with respect for example to fermentative capacity of microbiota (Jensen, 1998), activities of maltase and sucrase (Aumaitre & Corring, 1978), and levels in pancreatic tissue of the enzymes lipase, α-amylase, chymotrypsin and trypsin (Manners, 1976). In addition, Wenk (2003) reported that in adult animals, the successful utilization of exogenous enzymes depends mainly on the feed composition, while the basal diet and enzyme-supplemented diets in the current study had the same chemical composition, contained highly digestible ingredients and were formulated meet the nutrient recommendations of NRC (1998). Furthermore, Chesson (1993) reported that supplementation with enzymes to a cereal-based weaner diet improved the digestion of starch and protein immediately after weaning, and that improvement decreased as the endogenous enzyme production recovered. This could explain the lack of response to our enzyme mixture in diet E during weeks 3-5 post-weaning. No additional effect of the enzymes supplemented to the diet that contained the LAB-yeast complex (diet LYE) (Paper III) was found, which suggested that the poor digestive capacity of weaned piglets had been compensated for by the complex of LAB and yeast.

5.2 Effects of probiotics on diarrhoea in weaned piglets (Paper I & II)

The inclusion of LAB complexes in diets C1, C2 and C3 (Paper I) and diets L, LB and LBS (Paper II) resulted in lower diarrhoea incidence and faecal

score compared with the controls in the two weeks post weaning. These results can be explained by the higher LAB population and organic acid concentrations, and the lower *E. coli* counts in the intestinal segments of the piglets fed the supplemented diets. LAB in the gut can reduce the harmful effects of pathogens by production of organic acids and hydrogen peroxide, which may be toxic and inhibitory against *Coliforms*, *Salmonella* and *Clostridia* (Nousiainen & Setälä, 1998; Lidbeck & Nord, 1993). The higher organic acids in the gut could be expected to reduce pH, and the lower pH can inhibit the proliferation of pathogenic bacteria (Nousiainen & Setälä, 1998; Mathew *et al.*, 1993). In addition, the higher VFA concentration in the colon of the LAB-fed piglets in the current study could also have contributed to the lower diarrhoea incidence, as VFA provide a powerful driving force for the movement of water and sodium out of the colonic lumen (Cummings & Macfarlane, 1991; Cummings, 1983; Argenzio & Whipp, 1979). Furthermore, post-weaning diarrhoea associated with *Escherichia coli* is a major problem in pig production during the first 2 weeks post-weaning (Katouli *et al.*, 1999). Thus, the lower total *E. coli* counts in the LAB-fed piglets in the present study might be associated with the reduced post-weaning diarrhoea. Previous studies also found decreased diarrhoea levels in weaned piglets fed fermented feeds that contained LAB (Pedersen *et al.*, 2005; Mikkelsen & Jensen, 1997) or weaner diets supplemented with probiotic LAB (Taras *et al.*, 2006; Huang *et al.*, 2004).

Both LAB (*Enterococcus faecium* NCIMB 10415) and the *Bacillus* (*B. cereus* var. toyoi NCIMB 40112) were found to have positive effects on the health status of piglets, but by different mechanisms; the LAB changed the intestinal colonization while the *Bacillus* increased mucosal IgA levels (Scharek *et al.*, 2007). Kyriakis *et al.* (1999) and Taras *et al.* (2005) also found that *Bacillus* supplements reduced the incidence and severity of diarrhoea in post-weaned piglets, while Kirchgessner *et al.* (1993) did not find any effect of *Bacillus cereus* on the diarrhoea frequency in post-weaned piglets. The inclusion *Bacillus* in the diet that contained the LAB complex in the current study (diet LB in Paper II) did not result in any additional improvements with respect to piglet diarrhoea.

Saccharomyces have been found to be effective in controlling diarrhoea due to their antagonistic action to *E. coli* (Buts *et al.*, 2006; Czerucka *et al.*, 2000), or stimulation of immune systems (Rodrigues *et al.*, 2000; Pothoulakis *et al.*, 1993; Buts *et al.*, 1990), or inhibition of bacterial toxins (Castagliuolo *et al.*, 1999). In addition, *Saccharomyces* decreased levels of potential pathogens in the ileum of weaned piglets (Bontempo *et al.*, 2006). In the present study, addition of the yeast to the combination of *Bacillus* and

LAB complex (diet LBS in Paper II) resulted in an additional effect, with reduced diarrhoea incidence and faecal score compared with the diets that contained LAB alone (diet L) or the LAB and *Bacillus* complex (diet LB) overall two weeks after weaning.

5.3 Effects of probiotics on gut bacteria and environment (Paper I & II)

In Paper I and II of the current study, supplementation of the basal diet with different LAB complexes alone (diets C1, C2, C3 and L) or combined with *Bacillus* (diet LB) or with the *Bacillus* and *Saccharomyces* complex (diet LBS) increased the overall LAB population, coincidently with higher overall lactic acid and total VFA concentrations across the intestinal segments (stomach, ileum, colon and rectum) on both day 14 and 35 after weaning. There are consistent results showing that supplementation with probiotic LAB increased the intestinal LAB population in piglets (Takahashi *et al.*, 2007; Huang *et al.*, 2004; Tortuero *et al.*, 1995). Probiotic LAB and indigenous LAB were found to have positive effects which could also enhance the indigenous lactobacilli population in pigs (De Angelis *et al.*, 2007; Ohashi *et al.*, 2007; Ohashi *et al.*, 2001). The higher number of intestinal LAB in the current study could have resulted in higher lactic acid and VFA concentrations in the intestine of these pigs, as LAB may either produce lactic acid alone or both lactic and acetic acid (Cummings & Macfarlane, 1991). In an *in vitro* study, Sakata *et al.* (1999) also found that addition of probiotic preparations (containing *Bifidobacterium*, *Enterococcus* or *Lactobacillus*) increased production of lactic acid and short chain fatty acids in the caecum of pigs. In addition, production of lactic and acetic acids by fermentation of lactobacilli inhibited the growth of *E. coli in vitro* (Jin *et al.*, 2000). Hong *et al.* (2009) found that higher levels of organic acids were associated with lowered total *E. coli* counts along the intestinal tract (stomach, ileum, caecum, colon and rectum) of pigs on day 42 post-weaning, while in the current study higher organic acid levels associated with lower *E. coli* were found in the stomach and ileum, but not in the colon and rectum of weaned piglets.

Effects of dietary supplementation with *Bacillus* or *Saccharomyces* on gut environments are inconsistent in previous studies. For example, Jadamus *et al.* (2002) found that supplementation of the diet with *B. cereus var. toyoi* increased lactic acid concentrations in the middle and last parts of jejunum and tended to increase SCFA concentrations at the end of jejunum, and in ileum, caecum and colon of piglets at day 4 post weaning. However,

Kirchgessner *et al*. (1993) reported that *B. cereus* decreased lactate and VFA concentrations in the small intestine, and acetic and propionic acid in the caecum of post-weaned piglets. Marinho *et al*. (2007) found that inclusion of *S. cerevisiae* in the diet of weaned piglets decreased SCFA concentration in the colon, while live yeast additions did not influence *E. coli* and LAB counts and total VFA concentration along the intestinal tract (stomach, duodenum, ileum, caecum and colon) of piglets on day 14 post-weaning (Li *et al*., 2006) or on day 41 post-weaning (Mathew *et al*., 1998). In the current study, addition to the LAB complex of *B. subtilis* H4 only (diet LB) or *B. subtilis* H4 and *S. boulardii* Sb (diet LBS) did not result in any clear additional effects on the intestinal LAB population, or on lactic acid and total VFA concentrations.

6 General conclusions and implications

6.1 Conclusions

- Three 3-strain LAB complexes which were tested in Paper I were clearly shown to have probiotic properties in weaned piglets. Dietary supplementation with the LAB complexes improved the performance, digestibility, health status and gut environment in the first two weeks post-weaning. In the following three weeks, although the LAB complexes had effects on the gut bacteria and environment, they did not result in any improvement in the performance and diet digestibility of piglets.
- Adding a LAB strain (diet L in Paper II) and *Saccharomyces boulardii Sb* (diet LY in Paper III) to the 3-strain LAB complex enhanced the probiotic effectiveness of the microbial complexes in weaned piglets, which resulted in improvements in the performance and diet digestibility over a longer period (5 weeks post-weaning).
- The inclusion of *Bacillus subtilis* H4 in the 4-strain LAB complex (diet LB in Paper II) resulted in improved diet digestibility in weaned piglets compared with the 4-strain LAB complex alone (diet L in Paper II).
- The addition of *Saccharomyces boulardii* Sb in the complex of 4-strain LAB and *B. subtilis* H4 (diet LBS in Paper II) resulted in a lowered incidence of diarrhoea in weaned piglets compared with the 4-strain LAB alone or combined with the *Bacillus* (diets L and LB in Paper II).
- Supplementation of the weaner pig diet (diet E in Paper III) with microbial enzymes improved the performance and diet digestibility only in the first two weeks post-weaning. There was lack of response of the piglets to this enzyme mixture when they were supplemented to the diet

that already contained the complex of LAB and yeast (diet LYE in Paper III) during 5 weeks after weaning.

- Dietary supplementation with a combination of *B. subtilis H4, S. boulardii* Sb and the 4-strain LAB (diet BSL in Paper IV) had positive effects on growth, feed conversion and nutrient digestibility in grower pigs, but not in finisher pigs, while supplementation with *B. subtilis* H4 alone or combined with *S. boulardii* Sb did not have any effects on the performance and diet digestibility in grower and finisher pigs.

6.2 Implications and further research

By combining suitable probiotic strains of *Bacillus, Saccharomyces* and LAB, positive effects on growth, feed conversion and nutrient digestibility can be obtained in weaner and grower pigs. The current results suggest that these microbial complexes can be used as an alternative to antibiotic feed additives in pig production under the conditions of Vietnam.

However, storage of the microbes, yeast and enzymes, as well the techniques for mixing the additives to the basal diet used in the current study would not as yet be practical. Thus, further studies on different methods of preservation of microbes, yeast and enzymes need to be conducted in order to find the optimum techniques for producing the commercial products. In addition, studies on the effects of different densities of each microbial strain and yeast in the supplements on pig performance should be carried out. Regarding microbial enzymes, we expect, in studies planned for the future, that the complex of enzymes and LAB-yeast will clearly show positive effects when added to diets containing poorly digestible ingredients.

7 References

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Acknowledgements

This study was funded by Sida-SAREC Mekong Basin Animal Research Network (MEKARN). I am very grateful for their financial support.

Many people have been involved for different periods of time in helping me to complete this thesis. To all of them I owe a great debt of gratitude. However, I would like to specially mention and thank the following persons:

Professor Brian Ogle, my main supervisor, as he was one who gave me the opportunity to take part in my PhD studies. He was also my main supervisor during my Master studies. During these years, I received a lot of valuable advice, encouragement and support, as well as corrections to my papers and thesis. Under his supervision, all of my difficulties become easier as he always gave reasonable solutions that made me feel confident and believe that a bright future is coming. His kindness also made me comfortable when I was in Sweden. I am deeply indebted to him.

Professor Jan Erik Lindberg, my co-supervisor. He is the one who suggested to me to study probiotics, about which I understood very little at that time. With his valuable professional guidance, strong encouragement and support during my studies, my papers and thesis have been completed. A four-year-study period is a short period in a life, but his supervision during this time made me change a lot, in my way of thinking, the methods of study, the way to explain the findings, and especially never to feel disappointed when facing difficulties. I am particularly thankful to him.

Professor Inger Ledin, our group (MEKARN students) called her "mother". She is the one who interviewed me and then I was selected as a MEKARN Master student. During these years, I received a lot of encouragement, support and sympathy from her.

Dr. Tran Quoc Viet, my local supervisor, who always cooperated with me on my studies. Without his help, my experiments could never have been carried out successfully.

My former boss, Dr. Le Hung Quoc, former Director of Department of Agriculture, who was the one who signed the contract that allowed me to attend the 4-year PhD education, and since then my studies were given priority by the Department. I am grateful to him.

My other former boss, Dr. Nguyen Dang Vang, former Director of Department of Livestock Production, who allowed me to go abroad for studying during the time he was the Director.

My present boss, Dr. Hoang Kim Giao, Director of Department of Livestock Production, who allowed me to go abroad for studying. I received a lot of encouragement and support from him.

My special advisor, Dr. Bui Thi Oanh, former Head of Feed Division, who always understood me, and supported and encouraged me during my studies. I would like to express my deep and sincere gratitude to her.

I also would like to thank Dr. Ngo Van Man and Mr. Nguyen Van Cong and the members of SAREC/MEKARN committee for their support.

I am indeed grateful to the Directorial Board of the National Institute of Animal Sciences, Hanoi, Vietnam for considering me as one of their staff, so that I could carry out experiments as well as analyse the samples in the lab there.

I also would like to thank the many people who have helped me to complete my experiments: researchers at the Department of Animal Nutrition and the staff of the laboratory of the National Institute of Animal Sciences, Hanoi, Vietnam for assistance in carrying out the studies and chemical analyses; Dr. Cu Huu Phu, Dr. Do Ngoc Thuy and their staff in Department of Microbiology, National Institute of Veterinary Research, Hanoi, Vietnam, for assistance in slaughtering piglets, digesta sampling and bacteria quantification; Dr. Duong Van Hop, the Director, and his staff at the Institute of Microbiology and Biotechnology, Vietnam National University Hanoi, Vietnam, for assistance in preparing the microbial culture; Mrs. Huong and Mr. Trong in the Institute of Food Industry, Hanoi, Vietnam for their discussions with me in analytical methods of the organic acids.

I wish to thank Mr. Tuan who introduced me with the owner of the pig farm, Mr. Doan, since then I could carry out my experiments in his farm. I also would like to take this opportunity to thank Mr. Doan and his family for cooperation and kindness during my experiments carried out there.

I also would like to thank my colleagues at my home office: Khu, Len and Hoan, they have taken over my work duties in the Department during my studies. Furthermore, I would like to give my special thanks to Len who always considered me as her younger sister; she gave me a lot of special care, encouragement and supports in my studies.

My friends at SLU from Vietnam: Hong, Thuy, Tram, Van, Thang, Thieu, Lam, Quang, Teddy, Khai, Xuan, Trien, Tham, Thi Da; other foreign friends: Malin, Alice, Kerya, Milogo, and all other friends who are/were PhD students on the MEKARN program, who came to Sweden in the same time as me. Thanks for your friendship and help during my stays in Sweden.

I would like to express my sincere gratitude to the lecturers, researchers, staff and PhD students at our Department in Ultuna for friendship and kindness during my times in Sweden.

My sincere thanks go to my parents in law, sisters and brothers in law, for their support and encouragement during my studies.

My beautiful younger sister Hoang Thi Thanh Thuy, and your family, you bring second home to my sons, especially when I was away for studies. Thank and love all of you.

I owe my loving thanks to my husband, Le Huu Hoang. Without your agreements, I could never have dared to leave our sons at home to study overseas. During my long period of studies, you never gave a "sigh"; you always encouraged and supported me. My thanks cannot be enough for your contribution. Thanks to my sons, Le Hoang Hung and Le Hoang An, you are so lovely - you are my life!

Last but not least, I would like to give my deepest thanks to my mum Hoang Thi Ngoi and my dad Hoang Liem. You have taken care of me since I was born, and then you also have taken care of my sons since they were born, which let me be free to study. You are always available, giving me strong encouragement and support. You are in me for my whole life.