Dietary Modulation to Improve Pig Health and Performance

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Abstract

The aim of this thesis was to estimate the presence of *Salmonella* and enterotoxigenic *Escherichia coli* in piglets, the effect of fermented liquid feed and rice distiller's residue as a dietary modulation of the microbial population, the presence of microflora at different sites of the digestive tract, digestibility, and to evaluate the performance of pigs under small-scale farm conditions.

The first experiment showed that *Salmonella* and *E.coli* were found in faeces from piglets without and with diarrhoea. All *E. coli* antigens were isolated from piglets without and with diarrhoea. However, the frequency of antigen was much higher in piglets suffering from diarrhoea. Nutrient supply for sows and for piglets was low in comparison with feeding standards. In the second experiment, there were no differences in pH and butyric acid concentration of ileal digesta in pigs fed raw (R), cooked (C) or naturally fermented (F) diets. However, on diet F concentrations of acetic, lactic and propionic acid in ileal digesta were higher than on diets R and C. The ileal apparent digestibility of crude protein, crude fiber and NDF were higher in diet F than in diets R and C. The total tract apparent digestibility of crude protein was higher in diet F than in diets R and C. In conclusion, when compared with the diet in raw form, fermentation influenced the gut environment and improved the digestibility of some dietary components, while cooking prior to feeding had no measurable effects. In the third trial, a naturally fermental diet (FE) resulted in a lower pH than a diet basal on rice distiller's residue (RDR). Diet FE contained more organic acids, and had the lowest counts of *E. coli* and total coliforms, and the highest counts of lactic acid bacteria (LAB). Piglets fed diets FE and RDR had lower pH, and higher concentrations of organic acids in the stomach, ileum and mid-colon than piglets fed diet CO. Counts of LAB in stomach and ileum were higher in animals fed diets FE and RDR compared with diet CO, while the number of *E. coli* and total coliforms along the gastrointestinal tract was reduced. The ileal digestibility of crude protein and organic matter was improved in piglets fed diet RDR compared with piglets fed diet CO. Piglets fed diet RDR had a higher weight gain and a better feed utilization than piglets fed the other diets. In the final experiment DGGE analysis showed that animals fed diet FE had a much more uniform microbial flora in the stomach and the ileum compared to the other diets. Strains belonging to the genus *Pediococcus* were found in stomach and ileum of piglets fed diet FE and not on the other diets which probably arose from the feed.

Keywords: Diarrhoea, Feeding system, Management, Gut ecology, Organic acids, Piglets, Microflora, Feed fermentation; Rice distiller's residue, Vietnam.

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Dedication

To my parents
My husband Nguyen Van Hien
My daughter Nguyen Tran Thanh Phuong
My son Nguyen Dang Khanh

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 Tran Thi Thu Hong, Nguyen Quang Linh, Ogle, B. and Lindberg, J. E., 2006. Survey on the prevalence of diarrhoea in pre-weaning piglets, and on feeding systems as contributing risk factors in smallholdings in Central Vietnam. *Tropical Animal Health and Production* 38, 397-405.

II Tran Thi Thu Hong and Lindberg, J. E., 2007. Effect of cooking and fermentation of a pig diet on gut environment and digestibility in growing pigs. *Livestock Science* 109, 135-137.

III Tran Thi Thu Hong, Thai Thi Thuy, Passoth, V. and Lindberg, J. E., 2008. Gut ecology, feed digestion and performance of weaned piglets fed liquid diets. (Submitted).

IV Tran Thi Thu Hong, Passoth, V. and Lindberg, J. E., 2008. Effect of fermented liquid feed on the presence microflora at different sites of digestive tract in piglets. (In manuscript).

Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
CF	Crude fibre
CIAD	Coefficient of ileal apparent digestibility
CO	Control diet
CP	Crude protein
CTTAD	Coefficient of total tract apparent digestibility
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EE	Ether extract
EMB	Eosin methylene blue
ETEC	Enterotoxigenic *E. coli*
FCR	Feed conversion ratio
FE	Natural fermented diet
ME	Metabolisable energy
MRS	De Man Rogosa and Sharp
NDF	Neutral detergent fiber
OM	Organic matter
PCR	Polymerase chain reaction
RDR	Rice distiller's residue
T-RFLP	Terminal Restriction Fragment Length Polymorphism

1 Introduction

Animal production plays an important role for income of the farmers in Vietnam. The pig is the most important animal for livestock production (MARD, 2003). It has been estimated that 71% of the farmer households in Vietnam raise pigs with an average herd side of 5 pigs per household (MARD, 2003). Currently, about 80% of the meat production is pork, while the share of poultry meat is about 13% and other kinds of meat including beef, buffalo, and goat meat account for only about 7%. In North Central Vietnam about 97% of the farmers raise livestock in their households (MARD, 2003). Pig production in this area not only plays an important role to provide meat for human consumption, but also to provide pig manure for crop production and act as a form of savings and investment.

Pig production in Vietnam still has many problems; especially diarrhoea is common at both the pre-weaning and the post-weaning stages (Tuyen *et al.*, 2005). Diarrhoea caused by *Escherichia coli* (*E. coli*) was reported as a disease associated with industrialized pig production in Asia (Ranald *et al.*, 2000). This disease leads to considerable economic losses for producers. Piglets are often stunted, resulting in reduced performance and lowered income of the farmers. The main cause of diarrhoea pre-weaning and post-weaning is enterotoxigenic *E. coli* (ETEC) (Thuy *et al.*, 2006a). Thuy *et al.* (2006b) showed colonization of the F4-positive strains in duodenum, jejunum, and ileum in piglets with diarrhoea. The F4 (formerly K88) strain causes diarrhoea in neonatal, suckling and weaned pigs (Bertschinger, 1995). Early weaned piglets are more susceptible to enteric disease if the housing, environment, and nutrition are not optimal. In order to solve this problem, the pig producer can use antibiotics to treat piglets or use prophylactic vaccination of sows. In central Vietnam, smallholder farmers do not practice prophylactic vaccination of sows for the control of ETEC in piglets. The farmers often use antibiotics to treat piglets. This may have negative effects

on the environment and will contribute to the development of antimicrobial resistance (Levy, 1982; Bates *et al.*, 1993).

As mentioned above, both pre-weaning and post-weaning piglets can have problems with diarrhoea due to colonization of ETEC. They can proliferate and attach to the enterocytes which may lead to reduced absorption of nutrients and increase the amount of undigested feed in the small intestine. There are many practical measures that have been applied to prevent the occurrence of diarrhoea. These include nutrition management, addition of organic acids, addition of probiotic and prebiotic compounds, and using fermented liquid feed (Stein, 2002; Dirkzwager *et al.*, 2005; Hansen, *et al.*, 2007).

Fermented liquid feeds have been used in many studies to inhibit the growth of pathogenic bacteria such as *E. coli* in the feed and in the gastrointestinal tract of piglets. Fermented liquid feed is characterized by low pH, high concentration of lactic acid bacteria, and high concentration of organic acids. These characteristics of fermented liquid feed have been shown to inhibit the proliferation of pathogenic bacteria in the feed and in the intestinal tract of the pigs (Hansen *et al.*, 2000; van Winsen *et al.*, 2001a; van Winsen *et al.*, 2001b; Canibe and Jensen, 2003). Fermented liquid feed is of particular interest as a preventative tool in pig production as it can be easily applied under smallholder farm conditions. In addition, distiller's grain, a by-product from the ethanol industry, is also possible to use in pig production (Whitney and Shurson, 2004; Pedersen *et al.*, 2005). The chemical composition and nutritive value of distiller's grain are varying, depending on grain source, and processing and drying procedures (Cromwell *et al.*, 1993). It was shown that the use of distiller's grain in the diet for piglets reduced the frequency of diarrhoea (Pedersen *et al.*, 2005). In central Vietnam, alcohol production from rice is a source of income for the farmers. Therefore rice distiller's grain is available at level farm and is often used in the diet for post-weaning pigs.

In conclusion, there is a lot of scientific data showing that fermented liquid feed or distiller's grain fed to pigs during the post-weaning period has positive effects on pig performance and intestinal health, and may prevent the occurrence of diarrhoea. Therefore, dietary modulation based on local feed resources with emphasis on fermented liquid feed and rice distiller's grain for pre-weaning and post-weaning pigs at farm level can lead to greatly improved performance for pigs and improved income for the small scale pig producers in Central Vietnam.

Objectives:

The overall aim of this thesis was to estimate the prevalence of health problems in piglets in smallholdings in Central Vietnam and to study the impact of dietary modulation on the gut microflora, environment of digestive tract, digestibility, and performance of pigs under small-scale farm conditions.

The specific objectives were to:

- Monitor the prevalence of diarrhoea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings in Central Vietnam.
- Study the effect of cooking and fermentation of a pig diet on gut environment and digestibility in growing pigs
- Study the effect of fermented liquid feed and rice distiller's residue on gut environment, microflora, digestibility, and growth performance in weaned piglets.
- Study the impact of fermented liquid feed on the general gut microbial diversity and the presence of bacterial microflora at different sites of the digestive tract of piglets.

Hypothesis of this study:

- There is a relationship between the composition of the diet and the bacterial population of the gut, and growth performance when specific diets that contain fermented liquid feed are used.
- Fermented liquid feed can influence the general bacterial diversity and the presence of bacterial strains at different sites of digestive tract of piglets.
- The most promising diet formulations can be used to decrease Enterobacteria and to improve health in pigs.

2 Background

2.1 The role of pig production in Vietnam.

Livestock production plays an important role in providing meat, milk and eggs for human consumption. In addition, in developing countries livestock provide manure as source of fertilizer for crop production. According to information from the General Statistics Office of Vietnam (2006), livestock production is the second most important source of income after cultivation (Figure 1).

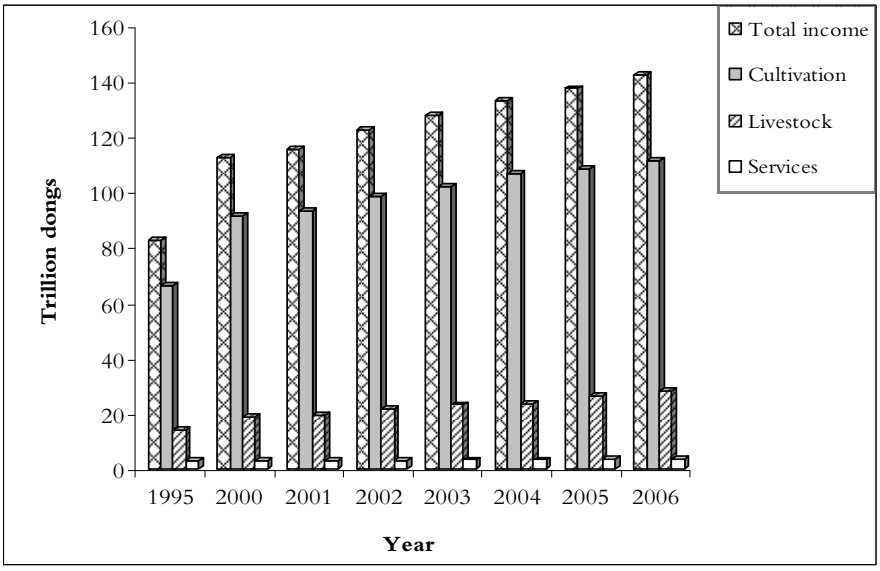

Figure 1: Output value of agriculture at constant 1994 prices
Source: General statistic office of Vietnam, 2006

In Vietnam, since 1990, the number of livestock has increased markedly. The only exception is poultry, because of the outbreak of avian influenza that occurred at the end 2003. Pig production has predominated in the livestock sector and pig numbers have increased rapidly in recent years; from 20.1 million head in 2000 to 27.4 million head in 2005 (Figure 2) (General Statistics Office of Vietnam, 2006).

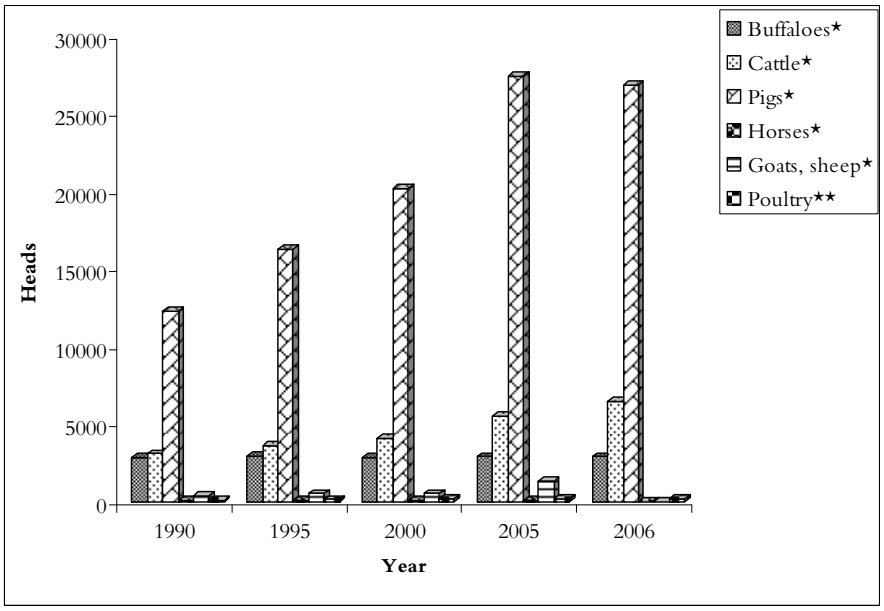

Figure 2: Livestock population
Source: General statistic office of Vietnam, 2006
* Thousand head
** Million head

Fresh pork meat is the most common animal protein source in the Vietnamese diet. The total pig population in Vietnam is predicted to reach 33 million head by 2010 (USDA, 2005). In Vietnam about 71% of pigs are kept under small-scale farm conditions, with an average number of 5 per household; the remainder is kept in large intensive farms (MARD, 2003). The quality of pork meat still is not sufficiently high for exportation. The lean meat ratio is only about 33.6 to 40.6 % in the North and 34.5 to 42.6% in the South of Vietnam, while international market requires a much leaner carcass (USDA, 2005).

In Vietnam, there are several good-quality imported pig breeds from the USA and from Australia, such as Landrace, Yorkshire and Duroc. These breeds will produce pork with lean meat ratios up to 56-60%. However, they are only suitable for large intensive farms because they need high investment for the breeding facilities, and a high quality feed. In contrast, the crosses between indigenous breeds and exotic breeds are suitable for small-scale farms with low input. The Mong Cai is one of the major local breeds in the northern part of Vietnam, particularly in the provinces of the North Mountains, the Red River Delta and the Northern part of Central Coastland. It has small to medium body size, a high prolificacy, and a good adaptation to poor quality feed and is resistant to several diseases. The Large White is a very common imported pig breed in central and northern Vietnam. The Large White has a large body and is a hardy breed that can withstand variations in climate and other environmental factors. Smallholder farmers often use Larger White boars crossed with Mong Cai sows to produce F1 crosses. The F1 crossbred (LW x MC) pig is adaptable to natural conditions and the management in central Vietnam. Under the same management conditions, the performance of crossbreeds is higher than that of native breeds.

2.2 Pre- and post-weaning diarrhoea syndrome

Diarrhoea in pigs is common and the occurrence is affected by many factors such as climate, age at weaning, nutrition, movement to a different environment and management (Mathew *et al.*, 1994; Martineau *et al.*, 1995). It causes considerable economic loss to the pig industry.

In Vietnam, pre-weaning diarrhoea is a major cause of economic loss in the pig industry. Results from a survey of pre-weaning diarrhea in five commercial intensive piggeries in North Vietnam showed that ETEC was responsible for 43% of the cases of diarrhoea in the first 4 days of life and 23.9% of remaining cases until weaning (Thuy *et al.*, 2006a). In South Vietnam diarrhoea in pre-weaning piglets was caused by ETEC (Khai *et al.*, 2002).

ETEC strains are important enteric pathogen bacteria in weaned pigs. These strains with fimbrial structures on the surface are the main cause of post-weaning diarrhoea. They are classified based on their antigenic properties and the most common antigens are denoted K88, K89, 987P and F107 (Imberechts *et al.*, 1992; Ramirez Santoyo *et al.*, 2001; La Ragione and Woodward, 2002). K88, K99 and 987P are antigens of E. coli strains isolated from intestinal contents of piglets affected by post-weaning

diarrhoea (Salajka *et al.*, 1992; Katsuda *et al.*, 2006). F4 (formerly K88) is commonly found in pigs with post-weaning diarrhoea (Frydendahl, 2002), while 987P is found in piglets from 3 weeks of age. Diarrhoea in piglets is affected by the prevalence of pathogens and the age of animal. ETEC were observed more frequently in weaned than in suckling piglets (Wieler *et al.*, 2001).

2.3 Enterotoxigenic *Escherichia coli* (ETEC)

The wall of small intestine has a mucosa with simple columnar epithelium, submucosa, smooth muscle with inner circular and outer longitudinal layers, and serosa. The absorptive surface area of the small intestine is increased by villi and microvilli. The digestive tract of all animals at birth is sterile, but contact between the young animal with microbes in the vagina, faeces, milk, and skin of the mother, as well as with microflora in the environment leads to establishment of a microflora (Conway, 1997; McDonald *et al.*, 2002). *E. coli* belong to the normal microflora in the digestive tract of animals but may also cause disease if unbalance occurs between pathogen and beneficial bacteria.

Escherichia coli with the presence of the filamentous antigen called fimbriae or pili allow adhesion to the enterocyte brush borders of pig small intestine (Candy *et al.*, 1980). Fimbriae or pili play an important role in the pathogenesis of ETEC and most ETEC produce adhesion antigen to adhere to villi and are associated with diarrhoea in pigs (Moon *et al.*, 1980).

The pili are made up by specific proteins, called lectins. There are many specific oligosaccharide receptor sites on the membrane cells of the gut wall. Fimbriae of harmful bacteria, such as ETEC, will attach to specific oligosaccharide receptor sites and lead to reduced nutrient absorption ability in the animal. Especially, young animals are sensitive and are suffering from digestive disturbances more often than older animals. It has been suggested to use beneficial lactic acid bacteria to compete with adhesion sites for *E. coli* on the gut wall (McDonald *et al.*, 2002). In addition, providing fermented liquid feed containing lactic acid bacteria strains as a starter is also an interesting strategy to improve pig health. It has been demonstrated that fermented feed improves performance and gut health of pigs (Geary *et al.*, 1996; Scholten *et al.*, 1999). It has been shown that fermented liquid feed that contained high concentrations of lactic acid, a high number of lactic acid bacteria and low pH (Canibe and Jensen, 2003; Canibe *et al.*, 2007b; Canibe *et al.*, 2008; Lyberg *et al.*, 2008) prevented the growth of harmful

bacteria, which may lead to reduced incidence of diarrhoea (McDonald *et al.*, 2002).

2.4 Growth inhibition of coliform bacteria

Fermented liquid feed has been used as method to reduce harmful bacteria in pigs (van Winsen *et al.*, 2001a; Canibe and Jensen, 2003). Fermentation of pig feed can start by inoculation of lactic acid bacteria, such as *Lactobacillus plantarum*, *Lactococcus lactics* subsp. *cremoris* 303, *Pediococcus acidilactici* (Geary *et al.*, 1999; van Winsen *et al.*, 2001 a&b; Lawlor *et al.*, 2002), or by natural fermentation without starter culture (Canibe and Jensen, 2003; Højberg *et al.*, 2003). Normally the fermented feed is prepared by mixing meal and water in a 1:2 or 1:2.5 ratio in a closed tank for 3-4 days before it is offered to the pigs. During fermentation pH decreases to around 4 and lactic acid and other organic acids are produced in high concentration. This can inhibit the growth of *Enterobacteriaece* in the feed. The mode of action for reducing the presence of harmful bacteria in an environment with low pH is related to an uncoupling mechanism (Russell and Diez-Gonzalez, 1998; van Winsen *et al.*, 2001a). Lactic acid and acetic acid are weak acids, and can not dissociate in an environment with low pH. Therefore undissociated fermentation acids can pass across the membrane of the cell. They will dissociate in the more alkaline interior of the cell and this will lead to an accumulation of the anionic species, and this accumulation is dependent on the pH gradient across the membrane. Gram-positive bacteria such as lactic acid bacteria can survive in environments with low pH, while Gram-negative bacteria, such as *E. coli* or *Salmonella*, are very sensitive to an acidic environment and can not tolerate a low pH (Russell and Diez-Gonzalez, 1998).

2.5 Digestive processes

Food sources for animals include three major nutrient components: carbohydrates, proteins and lipids. These nutrient components are macromolecules that must be broken down to simple compounds such as glucose monomers, amino acids and fatty acids before passing through the mucous membrane of the digestive tract. The digestive process to break these fractions is based on mechanical and chemical mechanisms. Mechanical mechanisms include chewing and grinding of food into smaller particles in the mouth, and on contractions of smooth muscle of the stomach wall. The

main chemical actions are based on secretion of enzyme in saliva for digestion of starch, enzymes in the wall of stomach or in the wall of small intestine, or enzymes from pancreas for digestion of carbohydrates, proteins and fats, and bile from liver for digestion of fats. After breakdown nutrients will pass along digestive tract and will be absorbed into the blood and lymph system. The digestive process of the main components can be summarized as below.

2.5.1 Digestion of protein

The proteins in the food are digested first in the stomach by pepsin. From birth until about an age of five weeks the digestibility of protein in the small piglet is different to the adult animal. The stomach of piglets lack HCl and pepsinogen, and only chymosin can break the protein of milk into simple peptides. As the piglet develops, pepsinogen and HCl increase in the stomach (McDonald *et al.*, 2002). Therefore, pig pepsinogens are hydrolysed to pepsin in weak acid conditions of pH 2 to 3.5. The products of protein digestion in the stomach are mainly polypeptides and a few amino acids. The chains of polypeptides pass down small intestine and continue the digestive processes by enzymes of pancreas such as trypsin and chymotripsin. These enzymes are activated under pH conditions of 7-9.

2.5.2 Digestion of carbohydrates

Carbohydrates are divided into two major groups, sugars and the non-sugars. The sugar fraction consists of mono- and oligosaccharides, while non-sugars include polysaccharides and complex carbohydrates (McDonald *et al.*, 2002). Starch is a polysaccharide and is present in most plant materials, particularly cereals. For example, sucrose and glucose are absorbed in small intestine while oligosaccharides such as fructo-oligosacchrides and transgalacto-oligosaccharides have a high pre-caecal digestibility. These oligosacchrides are considered to be prebiotics to stimulate the growth or activity of one or a limited number of bacteria in the colon, and improve host health (Bach Knudsen and Jørgensen, 2001). Digestibility of starch depends on type and structure of starch and most of the starch has been absorbed at the small intestine (Bach Knudsen and Jørgensen, 2001).

The first digestive process of starch is by enzyme amylase in the mouth. This enzyme hydrolyses the α-(1-4)-glucan links in polysaccharides containing three or more α-(1-4)-linked D-glucose units. In the small intestine, α-amylase of pancreas is similar in function to the saliva amylase and attacks α-(1-4)-glucan links in starch and glycogen.

In addition, the digestibility of non starch polysaccharides in the pig is very low because there are no endogenous enzymes secreted into the stomach and small intestine in pig. Therefore, the main site for non starch polysaccharides degradation is by microflora in the large intestine to create short chain fatty acids, H_2, CO_2 and CH_4.

2.5.3 Digestion of lipid

Lipid is the main form of energy storage in animals. The energy from the complete oxidation of fat is higher than from oxidation of glycogen. Lipids will be split into fatty acids and glycerol by the enzyme lipase of pancreas. Bile salt is secreted by the liver and passes to the duodenum through the bile duct and it plays an important part in digestion of lipids by activating pancreatic lipase.

2.6 Determination of digestibility

Digestibility of a feed can be determined with direct or indirect methods. The most reliable method used to determine digestibility is the direct method where the feed consumed and all faeces excreted are quantitatively collected during a certain number of days (Guevara *et al.*, 2008). Most commonly, male pigs are used because it is easier to collect faeces and urine separately than in female pigs. During time of total collection animals are confined in metabolism cages, to facilitate the collection of faeces and urine (McDonald *et al.*, 2002).

If direct methods can't be applied, an indirect method has to be used to determine digestibility. These methods are based on spot sampling of faeces (or digesta) and the use of an indigestible marker to allow calculations of digestibility. The marker can be added to the feed (external) or could be a natural component of the feed (internal). The marker should be evenly distributed in the feed and faeces (and/or digesta), it should not be absorbed or be toxic to the animal and it should be easy to analyse. Using an internal marker for estimating apparent digestibility coefficients has a number of advantages in comparison to the total collection method (Sales and Janssens, 2003). There are many different markers that have been used for digestibility measurements. The most commonly used internal marker is acid-insoluble ash (van Keulen and Young, 1977) and the most common external markers are titanium dioxide (TiO_2) and chromic oxide (Cr_2O_3) (Weatherup and McCracken, 1998; McDonald *et al.*, 2002; Lyberg *et al.*, 2006).

Total tract digestibility

Digestibility of the any feedstuff is calculated from the food given to the animal and the output of faeces. All the feed offered, feed refused and the outputs in faeces are measured. The formula for calculation of digestibility coefficients is shown below:

$$\frac{\text{Nutrient consumed} - \text{Nutrient in faeces}}{\text{Nutrient consumed}}$$

Ileal digestibility

The value of ileal digestibility of feedstuffs provides a more accurate estimate of the animals' utilization of the feed than total digestibility. The pig is monogastric, and therefore small intestine is the main site for absorption of nutritients. The loss of nutrients between feed offered and feed refused is the portion digested and absorbed. The remainder of undigested food is excreted as faeces. However, microorganisms in the caecum and colon of digestive tract of pigs and cell walls of large intestine also are excreted into the faeces. Therefore the concept of ileal digestibility of feedstuffs is a better measure than digestibility in the whole tract of pig. In order to collect ileal digesta at the end of ileum, the cannula technique has been used. The equation for calculation of digestibility is described below:

CADD = 1- (DCF x ID)/(DCD x IF)

where

CADD = coefficient of apparent digestibility of dietary component in the assay diet

DCF = dietary component concentration in ileal digesta or faeces (g kg-1)

ID = indicator concentration in the assay diet (g kg-1)

DCD = dietary component concentration in the assay diet (g kg-1)

IF = indicator concentration in ileal digesta or faeces (g kg-1)

2.7 Determination of bacterial diversity

Until recently, the analysis of the microbial community was based on the use of traditional culturing methods on suitable media. Nowadays, the development of molecular techniques has made it possible to understand in more detail the relationship between the microflora of the intestinal tract and the host (Tannock, 1999).

Identification of the microflora is based on sequence data of the 16S ribosomal RNA gene (16S rDNA). This gene is ubiquitous and conserved in all bacteria and is considered one of the best genes for identification of new species (Tannock, 1999). In order to apply molecular techniques for

studying bacterial diversity, the polymerase chain reaction (PCR) has been used as a first step for further study. PCR is a laboratory technique used to detect nucleic acids in minute amounts by amplifying a single molecule to provide numerous copies of specific DNA segments in the cells quickly and accurately. PCR is a simple method and is widely used to determine 16S rRNA gene sequences. Three major steps are involved in a PCR. These are denaturation, annealing, and extension. During PCR, at high temperature the double strand DNA is separated into single strand DNA. After that, two oligonucleotide primers join to regions of denatured DNA and a heat-stable DNA polymerase with nucleotides creates a new strand of DNA by extending the primer, using the complementary strand as a template. A new cycle begins as this new double-stranded molecule is denatured again (Spiegelman *et al.*, 2005).

There are several molecular methods available that can be used to study bacterial diversity. However, each method has different advantages and disadvantages (Kirk *et al.*, 2004). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two similar methods for studying microbial diversity. Both methods separate DNA molecules according to their resistance against denaturation, which is mainly determined by the GC-content of the sequence. Faeces samples, digesta samples, or food samples can be used to extract genomic DNA from bacterial strains. This DNA mixture was used as a template in PCR amplifications of particular variable DNA regions. All of these DNA have the same size but different sequences and DNA fragments will be separated on a DGGE gel. From the separated DNA fragments information about the species can be obtained; the DNA bands can be isolated from the gel and re-amplified for sequencing. The sequence of the isolated fragments can be compared with according databases and by doing this, the species reflected by the band can often be identified (Tannock, 1999; Ercolini, 2004). In addition, terminal restriction fragment length polymorphism (T-RFLP) has been used for studying bacterial diversity (Leser *et al.*, 2000; Canibe *et al.*, 2007a).

Recently, molecular biotechnology methods such as DGGE or T-RFLP have been used to study bacterial diversity in the digestive tract of humans and animals (Simpson *et al.*, 1999; Donskey *et al.*, 2003; Konstantinov *et al.*, 2003; Canibe *et al.*, 2007a; Janczyk *et al.*, 2007). Simpson *et al.* (1999) concluded that DGGE can be applied effectively to the pig gastrointestinal tract to monitor changes in bacterial populations through the gut.

3 Material and methods

3.1 Location of the study area

This research included two parts. The first part (Paper I) was performed as a survey to evaluate the prevalence of diarrhea in piglets and feeding systems for pigs under small-farmer conditions. The second part included experiments designed to study the effect of fermented feed on gut environment and microflora, and digestibility and performance in weaned pigs.

The survey (Paper I) was performed in three different areas of Thua Thien Hue Province; Huong Van village and Huong Toan village in Huong Tra District, and Thuy Duong village in Huong Thuy District.

- Huong Van village is in an upland area of Huong Tra District, Thua Thien Hue Province. Huong Van is 20 km North of Hue City. Livestock and crop production are the main livelihood activities of the local people. At present, the farmers in this area use local feed resources from agricultural products and by-products for feeding livestock, such as ensiled cassava leaves, ensiled cassava root meal and groundnut leaves.
- Huong Toan village is in a lowland area of Huong Tra District, Thua Thien Hue Province. It is located about 20 km from Hue city. Pig production is also a main income source of the farmers. In addition, the farmers in this area have off-farm activities, such as producing alcohol from rice, and rice distiller's grain is used as feed for pigs.
- Thuy Duong village is 12 km to the South of the City. Thuy Duong is an intensive rice growing area with high yields. The farming system is based on rice-pig production. Pigs are the main domestic animal in this area, and pig manure is used as a fertilizer.

The experiments in Paper II, Paper III and Paper IV were performed at the experimental farm of Hue University. The samples of Paper IV were collected and analyzed at the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

3.2 Experimental design (Paper I, II, III and IV)

In Paper I a cross-sectional survey on the prevalence of diarrhea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings was carried out in Thua Thien Hue province. Huong Van village and Huong Toan village, in Huong Tra district and Thuy Duong village in Huong Thuy district were selected as study sites. In total 100 smallholder pig farms that keep sows were randomly chosen for this survey, in which the number of smallholders involved in Huong Van, Huong Toan and Thuy Duong were 36, 30 and 34, respectively. The surveys were carried out in July (dry season) and in December (rainy season), 2004. The questionnaires were designed to collect information on the prevalence of diarrhea, feeding system and animal management from each farm.

In Paper II, the experiment was designed as a double 3 x 3 Latin square. The experiment lasted 60 days and included three periods. Each of the three experimental periods was 20 days, of which 12 days was for adaptation and 5 days for collection of faeces to determine total tract digestibility, and the remaining 3 days for collection of ileal digesta to determine ileal digestibility.

In Paper III and Paper IV, the experiment was arranged as a completely randomized design. Of a total of 32 crossbred piglets (Large White x Mong Cai) were used, 8 piglets were slaughtered at the beginning of the experiment for collection of digesta samples for counting microflora, analysis of bacterial diversity, and the presence of bacterial strains in different sites of gastrointestinal tract of piglets. The remaining piglets were allocated randomly by sex into three treatments with four replications of two piglets each. The experiment lasted 42 days.

3.3 Animals and management (Paper II, III and IV)

In the present study (Paper II, Paper III, and Paper IV) F1 crossbred pigs between Mong Cai sows and Large White boars were used. Animals in Paper II were castrated male pigs from the same litter with an initial body weight of 29.8 (±2.3) kg. All pigs in Paper II were selected when they were around 45 days of age with an initial body weight around 10 kg and were kept until 29.8 kg. At this body weight, six pigs were fitted with post-valve

T-ceacum cannulas and kept for 2 weeks to recover from surgery before experimental diets were introduced. These pigs were kept individually in pens (1.6 m x 0.8 m) with floors made from concrete.

In Paper III and IV, a total of 32 F1 crossbreds between Large White and Mong Cai pigs were selected from four litters and balanced for sex. The piglets used were 42 days of age and weighed on average 9.5 kg.

Before beginning the experiments, the piglets were vaccinated against pasteurellosis, parathyphoid, asthma and hog cholera. The animals in Paper II were adapted to the experimental diets for at least 12 days. In Paper III and IV, the animals were adapted to new feeds for at least 7 days before recording feed intake.

The piglets in Paper III were weighed every two weeks and feed refusals were recorded daily and were used for correction of the feed intake data. All animals in these experiments had free access to water via automatic drinking nipples.

3.4 Diets and feeding (Paper II, III and IV)

The diets used in these experiments were uncooked (raw form; Paper II, III, and IV), cooked (Paper II) or fermented feed (Paper III, and IV), and rice distiller's residue at a level of 20% of the basal diet (Paper III, IV). Rice distiller's residue was collected daily from an alcohol producing local farmer.

In Paper II, III, and IV raw feeds were prepared based on local feed resources such as cassava root meal, rice bran, maize meal, fishmeal, and soybean meal (Paper III and IV). Cooked feed (Paper II) had the same chemical composition as the raw feed (Paper II) but was prepared by mixing feed with boiled water at a 1:2 ratio and was cooked for 10 minutes. The cooked diet was prepared every day.

In Paper III and IV rice distiller's residue was used to replace 20% of the dry matter of basal diet.

In Paper II, III and IV, fermented feed was prepared every day in a 15-20 liter tank by mixing the feed ingredients with warm water (35-40 $^{\circ}$C) in a ratio of 1:1 (Paper II) and 1:1.5 (Paper III and IV).

In the experiments in Paper II feed was offered two times per day in the morning at 07.00 h and in the evening at 18.00 h. In the experiments in Paper III and IV feed was offered three times per day, in the morning at 06.00 h, in the afternoon at 12.00 and in the evening at 18.00 h.

The daily feed allowance was 4% of body weight (Paper II, III and IV).
In the present study (Paper II, Paper III). 5 g of chromic oxide per kg of feed was used.

3.5 Sample collection (Paper I, II, III and IV)

In the survey (Paper I), faeces samples were collected directly from rectum of pre-weaned piglets with and without diarrhoea to study the prevalence of diarrhoea in piglets and occurrence of *E. coli* antigens K88, K89 and 987P and the presence of *Salmonella* sp.. The samples were put in sterile tubes, placed in a cool box during transportation to the laboratory.

In order to determine the effect of cooking and fermentation on ileal digestibility in growing pigs (Paper II) digesta samples from each pig were taken during two days of collection in each experimental period. On each day of collection, digesta samples were taken every 2 h during the 12 h period between the morning and afternoon feedings. The digesta were frequently removed from the tube and the container and were kept on ice during sampling. The samples were kept frozen at -20 °C. At the end of each experimental period ileal digesta were thawed and mixed within pigs and periods.

In Paper II, the post-valve T-caecum (PVTC) cannulation technique was used to collect digesta at the end of the ileum for calculation of ileal digestibility. In Paper III, ileal digesta at ileum were collected by slaughtering the piglets at the end of experiment. For the determination of total tract digestibility (Paper II and III) faeces were collected two times per day for 5 days and were kept frozen at –20 °C. At the end of each experimental period (Paper II) and at the end of experiment (Paper III) faeces samples were thawed and mixed within pigs and periods.

In Paper III, the digesta samples from different sites of intestinal tract of piglets were collected into sterile tubes and were immediately prepared for culturing of *E. coli*, coliforms and lactic acid bacteria, and for analyzing the content of organic acids. In addition, feed samples were collected for measurement of organic acids and pH (Paper II and III).

The bacterial diversity and the presence of bacterial strains in piglets fed fermented feed and rice distiller's residue were analysed along the gastrointestinal tract at different sites using samples collected in stomach, ileum and colon. The samples were stored frozen at – 20 °C until analysis (Paper IV).

3.6 Measurements and chemical analysis (Paper I, II, III and IV)

To estimate the presence of *E. coli* and *Salmonella* spp. in faeces of piglets with and without diarrhea, EMB agar, selenite broth, MacConkey agar (colourless) and brilliant green agar (red) were used (Paper I). After isolation and biochemical testing, all *E. coli* colonies were used to determine the

occurrence of antigens for fimbriae K88, K99, and 987P using a serological method (Paper I).

In the enumerative experiment of harmful bacteria (*E. coli*, and total coli forms) in feed and digesta (Paper III), EMB agar and MacConkey agar were used. The samples of diets and different sites of digestive tract were diluted with sterile 0.9 % NaCl solution and incubated under aerobic conditions for 24 h at 37 °C. For total number of lactic acid bacteria, the suspensions were spread on MRS agar, and incubated for 48 h at 37 °C in a 2.5 L anaerobic jar.

The bacterial profile at different sites of the digestive tract was analysed using the DGGE method (Paper IV). The presence of microflora at different sites of the digestive tract of piglets fed fermented liquid feed was estimated by cloning technique. All DNA bands from the DGGE analysis were cut out and reamplified using V3 primer without GC-clamp. Then the bands were cloned into a plasmid using the TOPO TA Cloning® Kit for sequencing (Invitrogen). From the resulting recombinant plasmids the inserts were sequenced and bacteria present at different sites of digestive tract of piglets were identified (Paper IV).

In the total tract digestibility studies in Paper II and III, the feed offered and refused was recorded and faeces samples were taken two times per day for 5 days and then pooled for the whole collection period. For the ileal digestibility in Paper II and III, ileal digesta samples were collected at ileum using the PVTC cannulation technique (Paper II) and at ileum on day 42 from 1 m of the small intestine proximal to the ileo-caecal valve (Paper III). All samples were immediately frozen at –20 °C pending analysis. Chromium oxide was used as a digesta flow marker and was added at 5 g/kg DM of diet (Paper II and III).

For gut environmental measurements, digesta samples at different sites were collected. These samples were used for analysis of pH and organic acids, such as lactic acid, acetic acid, butyric acid and propionic acid at ileum (Paper II), and only lactic acid and acetic acid at stomach, ileum and colon in Paper III.

In order to calculate performance of piglets, they were weighed every two weeks. Feed refusals were recorded daily and were used for correction of the feed intake data (Paper III). The feeds in Paper II and III were collected for analysis of pH and organic acids.

The samples in experiments in Paper II, III and IV were dried at 40 °C for 24 hours and were milled through a 1 mm screen before analysis. Dry matter (DM), ash, crude protein (CP), ether extracts (EE) and crude fibre (CF) were analyzed in dry samples according to standard AOAC methods

(AOAC, 1990). Neutral detergent fiber (NDF) was analyzed according to Robertson and Van Soest (1977). Digesta samples from stomach, ileum and colon in Paper III were analysed for acetic acid (Cat. No.: 10 148 261 035) and lactic acid (Cat. No.: 10 139 084 035) using commercial test kits (R-Biopharm GmbH, Darmstadt, Germany). All measurements were done following the manufacturers instructions using a UV/VIS Spectrometer (Model: Lambda 25; Perkin Elmer, USA).

3.7 Statistical analyses (Paper I, II, III and IV)

Fisher's exact test of SPSS 11.5 was used to analyse the data collected for frequency of bacteria in faecal samples. The chemical composition and nutritive value of the feed was analysed by the display descriptive statistics procedure of the MINITAB Reference Manual Release 14 (Paper I).

The General Linear Models (GLM) Procedure of the MINITAB Reference Manual Release 14 was used. Least-Squares Means were compared statistically using Tukey's pairwise comparison procedures Test ($p<0.05$) (Paper II and III).

The software from website: http://www.ebi.ac.uk/; http://rdp.cme.msu.edu/; http://blast.ncbi.nlm.nih.gov/ was used to analyse sequencing data of DNA to recognize bacterial strains at stomach, ileum and colon in piglets fed fermented liquid feed (Paper IV).

4 Summary of results

4.1 Prevalence of diarrhoea and feeding management for pigs (Paper I)

The prevalence of diarrhoea was highest for piglets at the age of 3-5 weeks and lowest for piglets at the age of 1-2 weeks. In general, the prevalence of diarrhoea in the different study sites was higher in the rainy season (37.7%, 65.1% and 32.0%, respectively) than in the dry season (29.7%, 20.6% and 24.3%, respectively).

The occurrence of the *E. coli* antigens K88, K99 and 987P in piglets with diarrhoea was higher than in piglets without diarrhoea in the dry season (p = 0.046, p = 0.004 and p = 0.035, respectively) and for antigens K99 and 987P in the rainy season (p = 0.05, p = 0.028, respectively).

Local feeds such as rice, rice bran, maize meal, cassava root meal, and vegetables were mainly used for sows. All farmer households used rice in sow diets, while the proportion of farmer households that used rice bran was from 87 to 94%. The proportion of farmer households that used concentrate and fish meal in sow diets ranged from 47-87%, 37-62%, respectively, at the three study sites. Most farmers reared pre-weaning piglets with high proportion of rice in the diets (from 34-42%). In contrast, there was a low proportion of rice in the diets of post-weaning piglets (10-13%). The proportion of rice bran in the diets post-weaning pigs was 49-68% and for pre-weaning pigs 4-29%.

4.2 Characteristics of fermented feeds (Paper II and III)

The pH of fermented feed was from 4.1 (Paper II) to 4.2 (Paper III). Acetic acid, lactic acid and butyrate acid concentrations were highest (1.25, 0.90 and 0.20 mol/kg DM, respectively) for the fermented diet in comparison with the cooked and uncooked diet (Paper II). As with fermented feed in Paper II, fermented feed in Paper III had highest content of organic acids (429.7 mmol/kg DM), which was almost twice as high (213.2 mmol/kg DM) as in the rice distiller's residue (diet RDR).

The microflora in the diets was affected by fermentation. The fermented diet (diet FE) had the lowest counts of *E. coli* and total coliforms ($P<0.05$), and the highest counts of lactic acid bacteria ($P<0.05$) (Paper III).

4.3 Environment and microbiology in the digestive tract in piglets fed liquid feed (Paper II and III)

There were no differences in pH in ileum of growing pigs fed fermented feed compared with pigs fed raw and cooked diets ($P >0.05$) (Paper II). In contrast, fermented liquid feed affected pH in stomach, ileum and rectum of piglets. For example, pH at those segments was lower than in piglets fed a control diet ($P<0.05$), while there were no differences in pH in caecum and mid-colon between piglets at day 0 and day 42 (Paper III).

Growing pigs and piglets fed fermented liquid feed had higher concentrations of acetic acid and lactic acid in stomach, ileum and colon (Paper II, III) ($P<0.05$). There were significant differences in concentration of both acetic acid ($P<0.001$) and lactic acid ($P<0.05$) between segments (Paper III).

Fermented liquid feed affected the number of *E. coli*, total coliforms and lactic acid bacteria in the digestive tract of piglets. Piglets fed fermented feed (FE) and rice distiller's residue (RDR) had lower ($P<0.05$) counts of *E. coli* and total coliforms in the stomach, ileum, caecum and rectum than piglets fed the control diet (CO). There were differences in the number of lactic acid bacteria in stomach and ileum of piglets fed fermented feed and rice distiller's residue compared with piglets fed the control diet. The number of lactic acid bacteria in caecum and mid-colon was higher in piglets fed CO than in those fed diets FE and RDR ($P<0.001$) (Paper III).

4.4 Digestibility and performance (Paper II and III)

The fermented liquid feed improved ileal and total tract digestibility in growing pigs (Paper II). The ileal digestibility of CP, crude fibre, and NDF

was higher ($P<0.05$) on the fermented diet than on the raw and control diets. The total tract digestibility of CP was higher ($P<0.05$) on the fermented diet than on the other diets (Paper II). However, the fermentation of diets did not improve the digestibility in piglets (Paper III).

Piglets offered diet RDR had a lower average daily feed intake (ADFI) during the period from week 1-2 ($P<0.05$) than piglets offered other diets. There were no differences between diets week 3-4, while higher ADFI ($P<0.05$) was recorded for diets FE and RDR week 5-6. However, there were no differences ($P>0.05$) in ADFI for the whole experimental period. Piglets offered diet RDR had higher average daily gain (ADG) ($P<0.05$) at week 5-6 and for the whole period than piglets offered diets FE and CO. There were no differences in ADG between experimental diets ($P>0.05$) week 1-2 and week 3-4. As a consequence, the feed conversion ratio (FCR) was highest ($P<0.05$) for diets CO, FE and RDR in descending order, and in the whole period FCR was lowest for diet RDR (Paper III).

4.5 Bacterial diversity at different sites of gastrointestinal tract (Paper IV)

In Paper IV, PCR products obtained from genomic bacterial DNA isolated from stomach, ileum and colon as template were clear and without by-product. The final PCR products length was approximately 200 bp, as determined in 2% agarose gels.

DGGE profiles were produced from samples collected in the stomach, ileum and colon of piglets at day 0 (n=4) and day 42 (n=4 for each treatment) of the different feeding experiments. One striking feature of the general microbial flora in animals fed diets with non-fermented material (samples prior the experiment started-day 0, and samples from diets CO and RDR) was the very high difference between individuals. In contrast, the four individuals fed FE showed a uniform banding pattern. The different diets influenced the general microbial diversity in the stomach. At least the upper and the lower band of the five clearly visible bands in the DGGE-profile seem to represent species that were not present in individuals fed the other diets.

Ileum samples were more diverse in all diet groups. Two DGGE-patterns in individuals fed the FE-diet were apparently identical, while all other samples showed an individual banding pattern, demonstrating that different feeds had a pronounced impact on the microbial flora in the ileum.

The colon samples looked less diverse than those from the other intestinal regions. Although there were some differences between the individuals,

most of the bands that were obtained in one treatment were also found in individuals fed other diets. Considerable differences were found compared before starting to feed the diets (day 0). These individuals also showed a substantial difference from each other.

4.6 The presence of bacterial species at different sites of digestive tract of piglets (Paper IV)

Three experimental diets were used in this study to identify microbial communities at different sites of digestive tract of piglets fed fermented liquid feed. Lactic acid bacteria were found to dominate in the stomach of piglets fed different diets. For example, *Lactobacillus acidophilus johnsonii, Staphylococcus gallinarum, Pediococcus pentosaceus,* and *Pediococcus acidilactici* were identified. Specifically, *Pediococcus pentosaceus* and *Pediococcus acidilactici* were only found in piglets fed the FE diet.

In the ileum of piglets day 0, before starting the experiment, *Escherichia sp.* and *Treponema sp.* were found, while *Escherichia sp.* appeared in ileum of piglets fed CO diet after 42 days of experiment. *Lactobacillus murinus*, *Lactobacillus jensenii, Lactococcus lactics, Weissella cibaria* and *Pediococcus acidilactici* were found in ileum of piglets fed FE. *Lactobacillus fermentum and Lactobacillus johnsonii* were identified in ileum of piglets fed RDR diet day 42.

Many band sequences found in ileum and colon matched to those of uncultured bacteria that were closely related with bacteria of the families *Prevotellaceae, Enterobacteriaceae, Bacteroidaceae, Lachnospiraceae, Spirochataceae, Helicobacteraceae, Pasteurellaceae* or *Erysipelotrichaceae*.

5 General discussion

5.1 Pre-weaning and post-weaning diarrhoea in piglets, and impact of management and feeding

Pre-weaning and post-weaning diarrhoea are the most important diseases in piglets worldwide (Nagy *et al.*, 1990; Wieler *et al.*, 2001). They result in large economic losses to the pig industry due to poor performance and increased veterinary costs. In severe cases, an outbreak of diarrhoea may cause death in piglets. The main cause of this disease is infection by ETEC strains (Fairbrother *et al.*, 2005; Thuy *et al.*, 2006a). The aetiology of the disease is often complex and includes interactions between sow (litter size, parity, postpartum dysgalactia syndrome), piglet (birth weight, birth order, genotype), environment (temperature, crate/pen, season) and management (stockmanship, attendance at farrowing, vaccination) (Martineau *et al.*, 1995).

In the current study (Paper I), diarrhoea was prevalent at all ages in piglets, although the incidence was highest at 3–5 weeks of age and lowest at 1–2 weeks of age in both seasons. The high frequency of diarrhoea at 3–5 weeks of age can be related to the introduction of piglet feed, which occurred at 28 days after birth on all farms studied. This could be due to a lack in the capacity to produce sufficient amounts of gastric acid and digestive enzymes (Cranwell, 1995), which makes them more susceptible to dietary changes. Hampson (1986) and Pluske and colleagues (1997) showed that the change of nutrition at weaning may lead to changes in the histology and biochemistry of the small intestine, which cause reduced digestive and absorptive capacity. Indigestible food in the lower gut allows pathogenic strains to multiply, which may lead to diarrhoea (Hampson *et al.*, 2001).

In general, ETEC strains such as K88 (F4), K99 (F5) and 987P (F6), with fimbrial structure attach to villus epithelium of the small intestine, which is an important factor in the pathogenesis of diarrhoea (Supar *et al.*, 1991; Salajka *et al.*, 1992; Khai *et al.*, 2002; Kwon *et al.*, 2002; Katsuda *et al.*, 2006; Thuy *et al.*, 2006a). In Paper I, the results showed that antigen K99 (F5) was found more often than antigen K88 (F4) and 987P (F6) in piglets with diarrhoea in the dry season, while antigen 987P (F6) was found more often than antigen K88 (F4) and 987P (F6) in piglets with diarrhoea in the rainy season. Evans and colleagues (1986) reported a higher prevalence of K99 (F5) than of K88 (F4) and 987P (F6) in piglets with enteric colibacillosis. Supar *et al.* (1991) reported that most cases of diarrheoa associated with *E. coli* 987P (F6) occurred within the first 3 weeks of life in piglets. In contrast, Wilson and Francis (1986) found that the prevalence of K88 (F4) was higher than that of K99 (F5) and 987P (F6) in pigs with colibacillosis. Runnels and colleagues (1980) found that the prevalence of K99 (F5) was lower in piglets at 6 weeks of age than in piglets at 3 weeks of age. Thus, it appears likely that piglets with diarrhoea in the present survey (Paper I) suffered from enteric colibacillosis and that the *E. coli* strains involved differed between seasons. According to Katsuda *et al.* (2006), the frequency of enteric pathogens that cause diarrhoea disease by *E. coli* at different of ages in pigs were varying and *E. coli* was found more often in weaned piglets with diarrhoea than in suckling piglets, and the fimbrial gene F4 (K88) was dominant.

The feeding system for pig production under small scale conditions was found to be based on local feed resources such as rice, maize meal, cassava root meal or by-products from agricultural production (Paper I). The proportion of these feedstuffs used in the pig feed was different among the different categories of pigs. Rice, maize meal, rice bran and concentrate were the most commonly used feedstuffs in sow diets. The contents of crude protein, lysine, and calcium for pigs at different periods were low in comparison with recommended levels (NRC, 1998). The lactation diets were low in protein and deficient in lysine and calcium compared to recommended levels for lactating sows (NRC, 1998). Tokach *et al.* (1992) showed that a low lysine level in the diet affects milk production and milk composition. During pregnancy, the poor nutrient supply to sows may have a negative influence on piglet immunity (Chandra, 2002), as well as on colostrum quantity (Tokach *et al.*, 1992) and quality (Darragh and Moughan, 1998).

The balance of nutrients in the diet for piglets after weaning plays an important role in reducing the occurrence of diarrhoea. Diets with low

protein level may lead to reduced villus growth, which may affect the absorption of nutrients, and lead to diarrhoea. On the other hand, if the dietary crude protein level is too high, this also may affect the absorptive capacity of the piglet, which can result in nutritional diarrhoea (Gu and Li, 2004). The diets for piglets were too low in protein and were deficient in lysine and calcium (Paper I) compared to recommended levels (NRC, 1998). The dietary crude protein (CP) values for pre-weaning and post-weaning piglets under smallholder farm conditions ranged from 12 to 15%, and 13 to 15%, respectively (Paper I). The low lysine supply will be a major limiting factor for piglet growth and may also negatively influence the piglets' immune status. In humans, protein–energy malnutrition will lead to a reduction in the number of antibody–producing cells and in amounts of immunoglobulin secreted (Chandra, 2002).

5.2 Characteristics of fermented feed

Fermented liquid feed can be produced by natural fermentation by mixing fresh feed and water or by addition of specific starter cultures to give better control over the fermentation process (Geary *et al.*, 1999; van Winsen *et al.*, 2001 a&b; Lawlor *et al.*, 2002; Canibe and Jensen, 2003; Højberg *et al.*, 2003). In Paper II and III, fermented feed for pigs was prepared by mixing fresh feed with warm water (35-40 °C) followed by fermentation for 72 h. Fermented feeds had a low pH (Paper II and III), high level of organic acids (Paper II), high levels of acetic and lactic acids (Paper III), high numbers of lactic acid bacteria (Paper III), and low numbers of *E. coli* and total coliforms (Paper III). The general characteristics of the fermented diet (FE) used in the present study (Paper II and III) were in agreement with other published data (Scholten *et al.*, 2002; Brooks *et al.*, 2003; Canibe and Jensen, 2003; Canibe *et al.*, 2007a&b; Canibe *et al.*, 2008; Lyberg *et al.*, 2008). In a previous study, Russell *et al.* (1996) reported that fermented feed with low pH will have reduced numbers of coliform bacteria compared with non-fermented feed. During fermentation of liquid feed, numbers of lactic acid bacteria increased and also production of lactic acid, which led to a reduction of pH in the feed (van Winsen *et al.*, 2001a). The fermentation time and the difference in temperature affected the pH value in the fermented feed. The high concentration of lactic acid and low pH reduced the number of *Salmonella* in the feed after fermentation (Beal *et al.*, 2002). The lower numbers of *E. coli* and total coliforms in diet FE (Paper III) could be explained by high content of fermentation acids and a low pH (Russell and Diez-Gonzalez, 1998; van Winsen *et al.*, 2001a). Due to the potential to reduce the occurrence of

harmful bacteria in the gut of pigs and the ban on the use antibiotic growth promoters, there has been an increasing interest in using fermented liquid feed in the European pig industry (Scholten *et al.*, 1999).

5.3 Effect of fermented liquid feed on the environment and microbiology of digestive tract in piglets

The microflora has an important role in the digestive tract of the animal. The diversity of the bacterial population within a particular ecosystem is directly related to the number of limiting nutrients, as a limiting nutrient will support the one bacterial species or strain that is most efficient in utilizing it (Gaskins, 2001). Fermented liquid feed is one kind of feed that has been shown to alter the population of the microbiota in the intestine and influence volatile fatty acid levels (Pluske *et al.*, 2002). In the current study (Paper II) the results showed that there was no effect of fermented liquid feed on pH at ileum but there were effects on organic acids at ileum (Paper II). Fermented liquid feed affected pH in stomach, ileum and colon in piglets (Paper III), and increased the concentration of acetic and lactic acid in stomach, ileum and mid-colon (Paper III). The result in the Paper II was in agreement with previous findings by Moran *et al.* (2001) and Højberg *et al.* (2003). They reported no difference in pH along the gastrointestinal tract of pigs fed fermented feed. In contrast, our study (Paper III) showed lower pH in the stomach, ileum and colon of piglets fed fermented liquid feed compared with piglets fed dry feed. A lowered stomach pH, as a result of feeding fermented diets, has also been reported by Mikkelsen and Jensen (1998), van Winsen *et al.* (2001b), Canibe and Jensen (2003) and Hansen *et al.* (2000).

Feeding fermented liquid feed to piglets has been shown to reduce the number of *E. coli* and coliforms and increase the count of lactic acid bacteria along the gastrointestinal tract (Paper III). In piglets fed the diets FE and RDR (Paper III) the numbers of *E. coli* and total coliforms were markedly reduced in the stomach and ileum compared with piglets fed the control diet. In accordance, there were also lower numbers of *E. coli* in the caecum and rectum of piglets fed diets FE and RDR, and in the mid-colon of piglets fed diet FE. Piglets fed diet FE had lowest number of total coliforms in the caecum, mid-colon and rectum. These effects on the gut microflora may be explained by the lower pH and higher concentration of lactic acid and acetic acid in diets FE and RDR. The low pH, caused by the production of lactic acid and short-chain fatty acids, plays a very important role in the elimination of many pathogens, which are not able to tolerate

low pH conditions (Jin *et al.*, 2000; Gopal *et al.*, 2001). The results in Paper III are in agreement with other studies (Hansen *et al.*, 2000; van Winsen *et al.*, 2001b; Demeckova *et al.*, 2002; van Winsen *et al.*, 2002; Canibe and Jensen, 2003). According to Mikkelsen and Jensen (1998), feeding a fermented liquid diet to weaned piglets will reduce both pH and the number of coliforms in the stomach compared with feeding a non-fermented diet.

Demeckova *et al.* (2002) found that the concentration of short chain fatty acids (SCFA) increased in the faeces of sows fed fermented liquid feed. This could be due to the ingested SCFA or to more extensive hindgut fermentation on the fermented liquid diet. As mentioned above, fermented liquid feed with low pH stimulated growth of lactic acid bacteria in the stomach and ileum of piglets (Paper III). The highest content of lactic acid was found in the proximal part of the digestive tract, while the highest content of acetic acid was found in the distal parts (Hansen *et al.*, 2000; van Winsen *et al.*, 2001b; Scholten *et al.*, 2002; Canibe and Jensen 2003, Højberg *et al.*, 2003). Piglets fed diets FE and RDR had higher contents of acetic acid in the stomach, ileum and mid-colon than piglets fed the control diet. Similarly, the content of lactic acid in stomach and mid-colon was higher in piglets fed diets FE and RDR than in piglets fed the control diet. Both acetic acid and lactic acid content was high in the diets FE and RDR (Paper III). This may in part be due to the ingested lactic acid and acetic acid with the fermented liquid feed (Scholten *et al.*, 1999). In agreement with earlier reports (Højberg *et al.*, 2003; Canibe and Jensen, 2003), the current studies support the contention that high concentrations of lactic acid and acetic acid in fermented liquid feed reduce the number of *E. coli* and coliforms along digestive tract of piglets.

5.4 Digestibility and performance

Most studies that reported on the use of fermented liquid feed to pigs have focused on the effect on bacterial numbers in the digestive tract and growth performance. So far, there are very few experiments published on the effect of fermented liquid feed on digestibility of protein and other nutrients (Lyberg *et al.*, 2006).

The results presented in Paper II and III showed that fermentation of a diet improved the coefficient of ileal apparent digestibility (CIAD) of CP, crude fibre and NDF, and the coefficient of total tract apparent digestibility (CTTAD) of CP in growing pigs (Paper II). In general, the results obtained are in good agreement with previous studies that reported that fermentation

of pig diets improved CTTAD of CP and OM (Dung *et al.*, 2005), as well as CIAD of OM, minerals and amino acids (Lyberg *et al.*, 2006). This result was in agreement with earlier studies and may be due to the impact of microbial activities during fermentation on the diet prior to feeding, in particular affecting the dietary fiber fraction (Pedersen and Lindberg, 2003). According to Kemme *et al.* (1999), Lawlor *et al.* (2002) and Lyberg *et al.* (2006), diet acidification or fermented liquid feed may indirectly stimulate protein digestion by reducing gastric pH, leading to an improved nutrient utilization. In contrast, in Paper III, fermentation did not improve the digestibility of the diet.

Despite the beneficial characteristics of fermented liquid feed on gut environment and gut microflora compared with dry or non fermented feed, the effects of fermented liquid feed on feed intake and growth performance are variable (Russel *et al.*, 1996; Lawlor *et al.*, 2002; Canibe and Jensen 2007; Canibe *et al.*, 2008). In Paper III, the piglets in all treatments had similar dry matter intake (DMI) from week 1 to 4. In the first weeks of the experiment, the pigs in our study had a low DMI. Similar finding was reported by Lawlor *et al.* (2002). According to Brooks *et al.* (2001) low pH combined with high concentration of lactic acid or acetic acid in fermented liquid feed has been hypothesized to impair palatability of fermented liquid feed. However, DMI in our study increased with both FE and RDR diets from week 5 to 6. In the present work (Paper III), DMI was not affected by fermented liquid feeding. A similar result was reported by Lawlor *et al.* (2002). However, according to Scholten *et al.* (2002) piglets fed a fermented wheat diet had improved ADG compared with liquid feed, and the fermented feed had a positive influence on gut architecture. Canibe and Jensen (2007) showed that fermented feed reduced piglet feed intake compared to dry feed, but without any effects on FCR between treatments, while fermented liquid cereal grain feed improved daily feed intake and daily body weight gain compared to the fermented liquid feed. Canibe and Jensen (2003) found no effect on growth performance of feeding a fermented feed compared to a dry feed. Plumed-Ferrer *et al.* (2005) found no weight differences between pigs fed with fermented feed or non-fermented feed. Recently, Canibe *et al.* (2008) showed that fermented liquid feed with addition of *L. plantarum* improved growth performance without affecting the gastrointestinal ecology of piglets.

5.5 Effect of fermented liquid diets on general diversity of bacteria at different sites of gastrointestinal tract

DGGE was applied to estimate bacterial diversity in stomach, ileum and colon of piglets fed fermented liquid feed (Paper IV). The results showed differences in the DNA profile of the gastrointestinal microflora of piglets fed fermented liquid feed compared to piglets fed the other diets. In general, the DNA profile of piglets fed fermented feed was more stable than those from piglets fed the other diets. Obviously the microflora that was formed in the feed during fermentation influenced the flora of the intestine. The bacterial communities in the colon of piglets fed fermented feed and rice distiller's residues were more stable compared with piglets at day 0 and piglets fed control diets at day 42.

DGGE and T-RFLP analysis has been used to estimate the effect of different diets on microflora communities in the digestive tract of pigs. In these studies, feed sources such as non-digestible carbohydrate (Leser *et al.*, 2000; Konstantinov *et al.*, 2003) and fermented liquid feed (Canibe *et al.*, 2007a) have been used, as well as diets with addition of lactic acid bacteria strains (Davis *et al.*, 2007). The addition of non-digestible, fermentable carbohydrates (sugar beet pulp and fructooligosaccharides) has resulted in a higher bacterial diversity in faecal samples and a more rapid stabilization of the bacterial communities compared with piglets fed diets without fermentable carbohydrates (Konstantinov *et al.*, 2003). According to Simpson *et al.* (1999), there were differences in banding patterns in the gastrointestinal tract of piglets of different ages and fed different diets. In addition, based on traditional culturing methods (Durmic *et al.*, 1998), and using terminal restriction fragment length polymorphism (T-RFLP) (Leser *et al.*, 2000), it has been shown that the type of diet can influence the bacterial community in the colon of pigs. Canibe *et al.* (2007a) reported that terminal restriction fragments of stomach samples were different among piglets fed dry feed, fermented liquid cereal grains and fermented liquid feed. Furthermore, the T-RFLP profiles of digesta samples from mid colon showed a difference between the dry and fermented liquid cereal grains groups, whereas the fermented liquid cereal grains and fermented liquid feed groups showed similar profiles. Davis *et al.* (2007) showed that supplementation of *Lactobacillus brevis* to piglet diets resulted in a new unique band within the jejunum compared with other diets. From this study (Paper IV), it appears reasonable to assume that the use of fermented liquid feed in a pig diet can improve the stability of the bacterial communities in stomach and ileum.

5.6 Effect of fermented liquid diets on the presence of microflora at different sites of gastrointestinal tract

PCR-DGGE was used to describe the general bacterial diversity in piglets fed fermented liquid feed (Paper IV). DNA bands were excised from DGGE gels for identification of microbial communities at different sites of gastrointestinal tract of piglets (Paper IV). Strong visual DNA bands that appeared on the DGGE gels were excised, re-amplified, cloned and sequenced in order to identify bacterial communities along the gastrointestinal tract (Paper IV).

The result of the cloning showed that lactic acid bacteria are very frequent in the stomach of piglets fed different diet. This result agrees with Barrow *et al.* (1977), who isolated microbes in the stomach of piglets by traditional culturing methods. They found that *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Streptococcus salivarius* were the most common lactic acid bacteria in the stomach of piglets. In contrast, Yin *et al.* (2005) reported that *Lactobacillus ruminis* was dominant in the stomach of pigs fed a corn-soybean diet based on sequencing of 16S rDNA. Normally, the number of bacteria in stomach is low because of low pH and rapid digesta flow, and in piglets the dominant bacteria in stomach tend to belong to the genera *Lactobacillus* and *Streptococcus* (Pluske *et al.*, 2002). In the present study (Paper IV), *Pediococcus pentosaceus* and *Pediococcus acidilactici* were identified in the stomach of piglets fed diet FE. In contrast, no *Pediococcus* were found in the stomach of piglets fed the other diets. Other studies have reported that *Pediococcus* has been found in forage crops, traditional fermented food, and by-products of wet wheat distiller's grains (Cai *et al.*, 1999; Ercolini *et al.*, 2001; Jamuna *et al.*, 2004; Olstorpe, *et al.*, 2008). It appears likely that *Pediococcus* strains proliferated in the naturally fermented feed and were transferred to stomach via the diet consumed by the piglets.

According to Mäyrä-Mäkinen *et al.* (1983), *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* are the most frequently found lactobacilli in the faeces, small intestine and colon contents of pigs. These lactobacilli can adhere to columnar epithelial cells and can tolerate low pH and bile acids, which is important for survival under conditions in the stomach and intestine. In our study (Paper IV), *Lactobacillus murinus, Lactobacillus jensenii, Lactococcus lactics, Weissella cibaria* and *Pediococcus acidilactici* were found in ileum of piglets fed diet FE. *Lactobacillus fermentum* and *Lactobacillus johnsonii* were identified in ileum on day 42 in piglets fed diet RDR.

In the colon, most of the DNA bands of piglets fed different diets that were isolated and sequenced matched to sequences of uncultured bacteria.

This indicates that most of these sequences may have arosen from unknown species that have not been identified yet by traditional methods. Konstantinov *et al*. (2003) and Wang *et al*. (2007) found uncultured bacteria in faeces samples of pigs. Most uncultured bacteria from this experiment were closely related to *Prevotellaceae, Enterobacteriaceae, Bacteroidaceae, Lachnospiraceae, Spirochataceae, Helicobacteraceae* and *Erysipelotrichaceae*. The bands sequenced in the ileum and colon in this study were most closely to bacteria previously identified in the gut of pigs, including species within *Lactobacillus, Bacteroides, Prevotella*, and *Enterobacteriaceae* (Vaahtovuo *et al*. 2007; Gong *et al*. 2008).

6 General conclusions and implications

6.1 Conclusions

- Diarrhoea disease often occurs in piglets on small-holder farms in Central Vietnam, especially in suckling and weaned piglets and the main cause is infection by enterotoxigenic *E. coli* (ETEC) such as K88, K99 and 987P. The prevalence of antigen K99 and 987P was more frequent in piglets in the dry season and rainy season (37 and 32%, respectively).
- Feeding systems for pig production were mainly based on local feed resources such as rice, maize meal, cassava root meal or by-products from agricultural production. The proportions of these feedstuffs were different among type of pigs.
- The characteristics of fermented liquid feed were low pH, low counts of harmful bacteria, high counts of beneficial bacteria and high content of organic acids, and therefore it can be used to improve pig health.
- Fermented liquid feed affected pH, and increased concentration of acetic and lactic acid in stomach, ileum and mid-colon. Moreover, it reduced the number of *E. coli* and coliforms, and increased the counts of lactic acid bacteria along the gastrointestinal tract of piglets.
- The digestibility of nutrients was improved in growing pigs fed fermented feed. Fermented liquid feed had no effect on dry matter intake, but improved the growth performance of weaned piglets.
- Using natural fermented feed in piglet diets improved the stability of bacterial communities in stomach and ileum. Identification of microflora in stomach and ileum of piglets fed fermented feed leads to the conclusion that feeding natural fermented feed may increase theoccurence of *Lactobacillus* and *Pediococcus* in stomach and ileum.

6.2 Implications

Diarrhoea in piglets is a common disease under small-scale farm conditions in Vietnam, mainly due to infectious enterotoxigenic *E. coli*. Using fermented liquid feed can improve pig health and performance. From the current study, it is suggested that piglets given natural fermented feed do not change performance in comparison with other diets. In practical pig production under small-scale conditions, natural fermented feed can be prepared every day by mixing the feed ingredients with warm water (35-40 ^{o}C) in a ratio 1:1 or 1:1.5 and allowing 72 h for fermentation.

6.3 Further study

- The characteristics of fermented liquid feed, especially the diversity of lactic acid bacteria in natural fermented feed should be further studied to identify the dominant beneficial lactic acid bacteria strains in the natural fermented feed, with characteristics of resistance to low pH and tolerance of bile salts.
- In order to understand the role of lactic acid bacteria in the digestive tract of piglets in terms of controlling diarrhoea disease, lactic acid bacteria strains in natural fermented liquid feed should be identified and chosen for studies on their ability to inhibit growth of enterotoxigenic *E. coli* in the digestive tract of piglets.
- Feeding fermented feed should be carried out on a larger scale to evaluate the prevalence of diarrhoea disease and performance of piglets under small-scale farm conditions.

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