Asian-Aust. J. Anim. Sci.
Vol. 24, No. 6 : 834 - 843
June 2011

www.ajas.info
doi: 10.5713/ajas.2011.10291

Bacterial Diversity at Different Sites of the Digestive Tract of Weaned Piglets Fed Liquid Diets

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ABSTRACT : Bacterial diversity was studied using PCR-DGGE, cloning and sequencing. DNA was isolated from digesta samples from stomach, ileum and colon of 28 weaned piglets (Large White×Mong Cai) fed dry control feed, naturally fermented liquid feed (FE) and a liquid diet with inclusion of rice distiller's residue feed. General bacterial diversity was described using DGGE analysis of the V3 region of 16S rDNA. The microbial populations in the stomach and the ileum were considerably influenced by the diet, while only marginal effects were observed in the colon. There was a large variation of the microbial flora in the stomach between individuals fed non-fermented diets. In contrast, animals fed diet FE had a more uniform microbial flora in the stomach and the ileum compared to the other diets. In total 47 bands from the DGGE profiles were cloned. In stomach, most frequently lactic acid bacteria were found. Feeding diet FE resulted in the occurrence of *Pediococcus* species in stomach and ileum. In pigs fed the other diets, *Lactobacillus gallinarum*, *Lactobacillus johnsonii* and *Lactobacillus fermentum* were found in stomach and ileum. Most of the sequences of bands isolated from colon samples and several from ileum matched to unknown bacteria, which often grouped within *Prevotellaceae*, *Enterobacteriaceae*, *Bacteroidaceae* and *Erysipelotrichaceae*. This study demonstrates that fermented liquid feed affects bacterial diversity and the specific microflora in stomach and ileum, which provides a potential to modulate the gut microflora with dietary means to increase the abundance of beneficial bacteria and improve piglets' health. (**Key Words :** Pig Intestine, Microbial Diversity, Fermented Liquid Feed, PCR-DGGE, Piglets)

INTRODUCTION

Diarrhoea caused by enterotoxigenic *Escherichia coli* (ETEC) was reported as a disease associated with industrialized pig production in Asia (Ranald et al., 2000; Thuy et al., 2006). This disease leads to considerable economic losses for producers. 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 producers 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 but frequently use antibiotics to treat piglets. This may have negative effects on the environment and will contribute to the development of bacterial strains that are resistance towards antibiotics (Levy, 1982; Bates et al., 1993).

Diet has an impact on the microbial flora in the pig intestine (Varel and Yen, 1997; Konstantinov et al., 2003; Konstantinov et al., 2004; Wang et al., 2007). Therefore, modulations of pig feed, such as using pre-biotic ingredients, supplementing with pro-biotics and organic acids, and fermenting liquid diets, have been applied in recent years to improve pig gut health status (Pluske et al., 2005). Fermented pig feed is generated by mixing solid carbohydrate-rich material with a liquid phase, water or a liquid co-product from food or ethanol production. During a period of incubation, microbial fermentation occurs resulting in a feed characterized by low pH, high concentration of lactic acid bacteria and organic acids. Fermented liquid feeds in particular have potential to improve pig health and performance by inhibiting the growth of pathogenic bacteria such as *E. coli* in the feed and in the gastrointestinal tract (Scholten et al., 1999; Hansen et al., 2000; van Winsen et al., 2001a; van Winsen et al.,

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Received August 12, 2010; Accepted December 28, 2010

2001b; Canibe and Jensen, 2003), and can easily be applied under smallholder farm conditions.

Hong et al. (2009) have recently shown that weaned piglets fed a naturally fermented liquid diet (FE) and a diet with inclusion of rice distiller's residue (RDR) feed had lower pH and higher concentrations of organic acids in the stomach, ileum and mid-colon than piglets fed a dry control diet. Moreover, in weaned piglets fed diets FE and RDR, counts of lactic acid bacteria in stomach and ileum were higher and counts of *E. coli* and total coliforms were lower than in those fed the dry control diet. Digesta samples collected from different sites of digestive tract of weaned piglets in the study by Hong et al. (2009) were used get a more detailed picture of the dietary impact on the bacterial diversity by using molecular methods previously applied in both humans and animals (Suau et al., 1999; Simpson et al., 2000; Donskey et al., 2003). The specific aim was to investigate the bacterial diversity at different sites of the digestive tract in weaned piglets by using the culture independent DGGE method combined with cloning and sequencing for microbial identification.

MATERIALS AND METHODS

Animals and experimental design

The experiment was performed at the experimental farm of the faculty of Animal Science, Hue Agriculture and Forestry University. Samples from a total of 28 pigs (Large White×Mong Cai) breed from four litters, with a body weight (BW) of 9.5 kg (SD 1.3 kg), were used in this study. At the start of the experiment (Day 0) four piglets, one from each litter, were exsanguinated and then eviscerated for collection of digesta samples. Digesta samples were collected to estimate the initial bacterial composition and general bacterial diversity. The remaining piglets from each litter were distributed randomly by sex into 3 dietary treatments, with four replicates of 2 pigs per replicate housed in the same pen, and were fed for a total of 42 days. Five hours after feeding, on the last day of the experiment, four piglets per treatment, one from each replicate, were exsanguinated by a blow to the head and then eviscerated immediately for collection of digesta samples to estimate the final bacterial composition and general bacterial diversity.

Feeds and feeding

Locally available feed resources were used to formulate three iso-nitrogenous experimental diets. Rice and maize were grinded through a 2 mm screen before mixing with the other feed ingredients. Rice distiller's residue was produced from the same batch of rice by a local rice wine producer and was delivered on a daily basis. The experimental diets were prepared in mash form and are shown in Table 1. The control diet (CO) was fed in raw form. The natural fermented diet (FE) had the same ingredient composition as diet CO and was prepared every day in a 20-liter tank by mixing the feed ingredients with warm boiled water (35-40°C) in a ratio of 1:1.5 to allow fermentation to take place. After 72 h of fermentation at room temperature (about 30°C) the fermented diet was fed to the piglets. In the diet RDR, cassava root meal, maize meal and soybean meal was replaced with rice distiller's residue. Chromium oxide was used as a digesta flow marker and was added at 5 g per kg dry matter of diet. Piglets were fed three times per day (06.00 h, 12.00 h and 18.00 h) and had free access to drinking water via automatic drinking nipples. The daily feed allowance was 4% of body weight.

Table 1. Dry matter (g kg^{-1}), ingredients (g kg^{-1} DM) and chemical composition (g kg^{-1} DM) of the experimental diets

Item	Diet		
	CO	FE	RDR
Dry matter	852	332	280
Ingredients			
Rice	100	100	100
Maize meal	295	295	255
Cassava root meal	100	100	0
Fishmeal	85	85	85
Soybean meal	200	200	120
Rice bran	210	210	230
Rice distillers residue[a]	0	0	200
Premix[b]	5	5	5
Cr_2O_3	5	5	5
Chemical composition			
Crude protein	201	201	203
Ether extract	81	81	79
Crude fiber	40	40	26
NDF	107	107	95
Ash	65	65	67

[a] DM 81 g kg^{-1}, CP 196 g kg^{-1} DM, acetic acid 740 mmol kg^{-1} DM and lactic acid 264 mmol kg^{-1} DM and pH 3.7

[b] Premix composition per kg (moisture content: 6%): 80 mg folic acid; 8 mg biotin; 18,000 mg Mn; 36,000 mg Fe; 36,000 mg Zn; 14,400 mg Cu; 324 mg I; 36 mg Co; 22 mg Se.

Digesta sampling

In order to estimate the general bacterial diversity at different sites of the digestive tract, digesta samples from the stomach, ileum and colon of each piglet were collected at day 0 and day 42. Immediately after collection, the samples were put on ice, froozen and kept at -20°C until further analysis.

Determination of general microbial diversity

DNA extraction: Digesta samples were taken from different parts (stomach, ileum, and colon) of the

gastrointestinal tract of piglets at day 0 and after 42 days feeding of the experimental diets collected into sterile tubes, put on ice and immediately transferred to the laboratory. All of samples were stored at -20°C until DNA isolation. The genomic DNA of the microflora was extracted using the QIAamp® DNA Stool Mini Kit. DNA concentrations were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, USA). Genomic DNA was stored at -20°C until analysis.

PCR amplification: To investigate the microbial diversity of weaning piglets, the V3 variable region of bacteria 16S rDNA was amplified by PCR using the forward primer 341F: 5'-CCTACGGGAGGCAGCAG-3' with GC clamp at the 5' end: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG and reverse primer 534R: 5'-ATTACCGCGGCTGCTGG-3' targeting the V3 region of the 16S rRNA genes (Muyzer et al., 1993; Simpson et al., 1999). Working solution of DNA templates were diluted to a final concentration of 30 ng μl^{-1}. PureTaq Ready-To-Go PCR Beads were used for PCR. The PCR mix contained 15 μM of each primer, 45 ng of DNA template in a final volume of 25 μl.

PCR was performed in MJ Mini™ Personal Thermal Cycler (BioRad) using the following program: 94°C for 5 min; 10 cycles of 94°C for 45 s; 60°C for 45 s; 72°C for 1 min (the annealing temperature was decreased of 0.5°C every cycle) and 16 cycles of 94°C for 45 s; 55°C, for 45 s; 72°C, for 1 min. PCR products were stored at 4°C before checking by electrophoresis (Sambrook and Russell, 2001). PCR products were stored at -20°C until DGGE analysis.

DGGE: Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-fragments with a GC-clamp was performed by using a DCode™ Universal Mutation Detection System (Bio-Rad), using a 7% polyacrylamid-gel in 1×TAE buffer and with 30-60% linear gradients of denaturant (Muyzer et al., 1993). Electrophoresis was performed at 60°C for 17 h at 130 V. After electrophoresis, DGGE gels were stained with SYBR® Gold dye for 30 min at room temperature, checked under UV light and photographed using the Gel DOC™ 2000 Gel Documentation System (Bio-Rad). Selected bands (see Results) were cut out from the gel using a sterilized scalpel and frozen until further analysis.

Isolation and amplification of DNA bands from the DGGE-gels

Fourty-seven DNA bands cut out from DGGE gel were cloned for sequencing. To elute the DNA from the gel, the frozen gel pieces (see above) were thawed at room temperature for 1 h, placed at -70°C for 1 h and finally thawed over night at 4°C. One μl of the eluate (about 10 ng of DNA) was used as a template in a PCR with the primers 341F and 534R (7.5 μM each) in a final volume of 12.5 μl.

PCR and detection of the PCR products was performed as described above. All PCR products were purified by using QIAquick Gel Extraction Kit of QIAGEN. PCR products were stored at -20°C until cloning.

Cloning of amplified DNA-molecules

The amplified molecules were cloned into the vector pCR® 4-TOPO® using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Stockholm, Sweden) and the resulting plasmids were transformed into chemically competent *E. coli* according to the supplier's manual. Blue-white selection was applied by adding 40 mg ml^{-1} X-gal to the LB medium.

Analyzing positive colonies

For each cloned band, five white colonies were randomly selected from the transformation plates. Cells from each selected colony were transferred to a PCR tube using a sterile toothpick. PCR was performed with PureTaq Ready-To-Go PCR Beads and the plasmid specific primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT-ATG AC-3'). For each tested colony also a frozen stock culture was prepared from an overnight culture in LBA medium (trypton 10 g L^{-1}, NaCl 5 g L^{-1}, yeast extract 5 g L^{-1}, ampicillin 100 mg L^{-1}) at 37°C, by mixing one culture volume with one volume of concentrated glycerol. Stock cultures were stored at -70°C.

PCR was performed in MJ Mini™ Personal Thermal Cycler (Bio-Rad) using the following program: 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. The program was finished by a final 7 minutes extension at 72°C. The final PCR products were checked in 2% agarose gels.

All PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany).

Sequencing

Sequencing was performed by MCLAB (Molecular cloning laboratories; South San Francisco, USA) using the purified PCR-product as template and the primer M13F or M13R.

Analyses of sequence data

All determined sequences were compared with known strains using NCBI-BLAST2 Nucleotide search (www.ebi.ac.uk/; http://blast.ncbi.nlm.nih.gov/). In cases where only matches to uncultured bacteria were obtained, the sequences were investigated in the database of the ribosomal database project (rdp, http://rdp.cme.msu.edu/). This at least allowed a determination to the family level, in some cases even to the genus or species level. If sequences

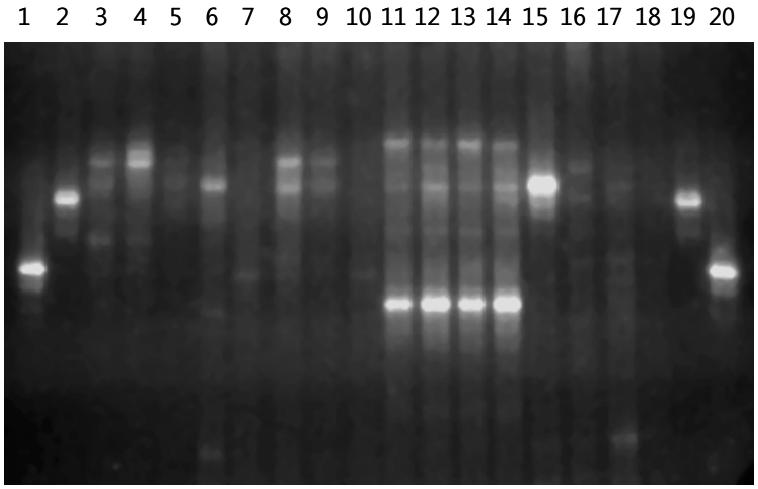

Figure 1. DGGE profiled of V3-16S rDNA fragments, amplified from bacterial genomic DNA extracted from stomach samples of piglets before starting the diets (day 0) and after 42 days feeding with the different diets. Lanes 3-6, day 0; lanes 7-10 control diet (CO); lanes 11-14 natural fermented diet (FE); lanes 15-18 rice distiller's residue diet (RDR). Lanes 1, 2, 19, 20: Calibration bands generated from genomic DNA of pure bacterial cultures (non-identified isolates) AB 1 (lanes 1, 20) and AB 6 (lanes 2, 19).

of known species were found in the rdp-search, they were aligned to the identified sequences to determine the degree of identity. Multiple alignments were performed using the ClustalW-programme (www.ebi.ac.uk/).

RESULTS

General microbial diversity in different parts of the digestive tract

Amplification of the V3 region yielded clear products without by-products, for all samples with a size of approximately 200 bp as determined by electrophoresis in 2% agarose gels.

DGGE-profiles of the bacterial populations were successfully generated and in Figure 1 the profile obtained from the stomach samples is shown. As can be seen, some bands were dominant in the profile, while others were faint and thus difficult to see. We checked the gels at different exposure times to visualise also weaker bands. The results of all samples from stomach, ileum and colon are schematically summarised in Figure 2.

In the stomach (st) samples, a clear impact of the diet on the microbial population was seen (Figure 1 and Figure 2A). One striking feature of the general microbial flora in animals fed diets with non-fermented material (samples prior the experiment started (day 0), CO and RDR) was the strong variation between the different individuals. In contrast, the four individuals fed FE showed a uniform banding pattern. The figure also shows that the different diets influenced the general microbial diversity in the stomach. At least the upper and the lower band of the four clearly visible bands in the DGGE-profile of the animals fed FE-diet seem to represent species that were not or only to a low amount present in individuals fed the other diets. This impression was confirmed by sequencing the according bands (see below).

In ileum (il) samples also an influence of the diets was found. Although there were more individual differences than in the colon, animals fed the FE diet had similar banding patterns, three patterns were almost identical by strong DNA bands on DGGE (e.g. il11, il12, il13) (Figure 2B). Cloning and sequencing showed that in some cases bands with similar migration pattern represented the same species, but there were a number of exceptions (see below).

The colon (co) 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 to the animals before starting the diets (day 0). These individuals also showed a substantial difference to each other (Figure 2C).

Identification of microorganisms represented by bands in the DGGE-gels

To obtain more detailed information about the microflora in the intestine, bands were excised from the gels, cloned and sequenced. In total 47 dominant DNA bands were investigated. For each band, five independent clones were sequenced. In ten of fifteen cases of DNA bands of stomach samples (e.g. st1, st2, st5, st7, st8, st10, st11, st12, st13, st14, Figure 2, Table 2) only one sequence per band was found. However, a substantial number of bands contained more than one sequence (Tables 2, 3 and 4).

In the stomach, most of the bands were formed from lactic acid bacteria. Several bands at similar positions in the gel but obtained from different animals had identical sequences like st1, st3, st5 and st7 in stomach, which all

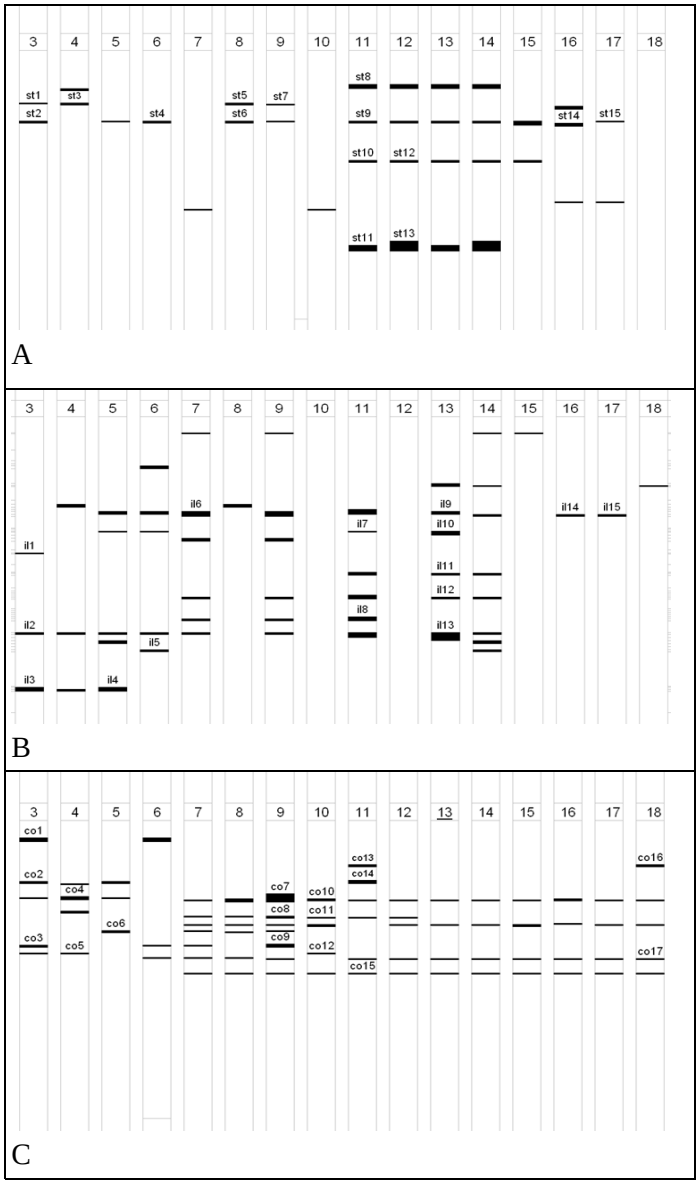

Figure 2. Schematic picture of DGGE of amplified V3 regions of the bacterial 16S rDNA. Bands were amplified using bacterial genomic DNA isolated from different sites of the intestine (A: Stomach; B: Ileum; C: Colon) as template. Labelled bands were excised for cloning. Lanes 3-6, day 0; lanes 7-10 control diet (CO); lanes 11-14 natural fermented diet (FE); lanes 15-18 rice distiller's residue diet (RDR).

matched to *Lactobacillus acidophilus*/*L. gasseri*. These closely related species cannot be distinguished according to their V3-sequence (Berger et al., 2007). However, band st9 that was found in an individual fed FE matched to *Pediococcus pentosaceus*. Band st4 from an animal at t_0 was formed by two organisms, *Staphylococcus gallinarum* and *L. jensenii*. It has earlier been shown that sequences of different strains can show a similar running pattern in DGGE (e.g. Ercolini et al., 2001). We also found that bands that were isolated from different positions in the gel contained the same sequence. This includes the bands st8, st9 and st10, st11, st12 and st13. Bands st8 and st9 both included molecules that matched most closely to *P. pentosaceus*, although band st9 also included *L. gallinarum* and band st8 matched only to 96% to *P. pentosaceus*. Band st10, st11, st12 and st13 matched to *P. acidilactici* in the Blast searches. The results from the Blast searches with the sequences of the isolated bands in stomach are summarised in Table 2.

Among the bands from the ileum some extreme cases of different migration patterns of identical sequences were observed (Table 3 and Figure 2B). Bands il1, il2 and il3 were all formed from identical *Enterobacteriaceae* and *Prevotellaceae* sequences, although situated at three different positions in the gel. Similarly, bands il7, il8 and il9 showed different migration distances although formed by

Table 2. 16S rRNA gene sequences of the strong DNA bands from stomach detected by DGGE and cloning technique

DNA band	Diet	Closest sequence	Accession no.	Identity (%)	Length of DNA
st1	Day 0	*Lactobacillus acidophilus/L. gasseri* [a]	M99704	100	160
st2	Day 0	*Lactobacillus acidophilus/L. gasseri* [a]	M99704	100	160
st3	Day 0	*Lactobacillus acidophilus/L. gasseri* [a]	M99704	100	160
		Flavobacteriaceae [b]	AJ518814	96	160
st4	Day 0	*Lactobacillus jensenii*	AB289172	100	160
		Staphylococcus gallinarum	AY211158	100	160
st5	CO	*Lactobacillus acidophilus/L. gasseri* [a]	M99704	98	159
st6	CO	*Lactobacillus crispatus*	AJ421224	100	160
		Flavobacteriaceae [b]	AJ518814	99	160
		Erysipelotrichaceae [b]	AY237191	98	160
st7	CO	*Lactobacillus acidophilus/L. gasseri* [a]	M99704	97	160
st8	FE	*Pediococcus pentosaceus*	ATCC33316	96	160
st9	FE	*Pediococcus pentosaceus*	EU082196	99	160
		Lactobacillus gallinarum	ATCC33199	99	160
st10	FE	*Pediococcus acidilactici*	YDW17	98	160
st11	FE	*Pediococcus acidilactici*	YDW17	98	160
st12	FE	*Pediococcus acidilactici*	YDW17	98	160
st13	FE	*Pediococcus acidilactici*	YDW17	98	160
st14	RDR	*Lactobacillus gallinarum*	ATCC33199	100	160
st15	RDR	*Lactobacillus gallinarum*	ATCC33199	100	160
		Lactobacillus jensenii	AY262342	100	160

[a] *Lactobacillus acidophilus* and *L. gasseri* could not be distinguished (Berger et al., 2007).
[b] Determination according to the rdp search.

identical sequences, similar to the sequence of uncultured *Turicibacter*, family *Erysipelotrichaceae* as well as il11 and il13 that represented *P. acidilactici* sequences. Many of these bands were obviously formed by more than one sequence, including bands in ileum and colon. Sequences matching to *E. coli* as those of bands il1, il2 and il3 cannot be distinguished from related enterobacteria, including *Shigella flexneri* or even a group of plant pathogens because sequences of the amplified V3-region of these organisms are identical (Naum et al., 2008).

In the colon samples, bands co4, co7, and co10 running to similar positions in the gel had identical sequences matching to *Prevotellaceae* (Figure 2C and Table 4). On the other hand, bands co11 and co12 contained identical sequences, matching to *L. johnsonii*. However, both co11 and co12 bands also contained sequences belonging to *Prevotellaceae* (Table 4).

The number of samples that matched to sequences of un-cultured and not further classified bacteria increased from stomach to ileum to colon. Band il8 in ileum (91% identity), and bands co3 and co6 in colon (85 and 94% identity, respectively) contained sequences that had sequence identities to the closest Blast hits of clearly less than 98%, i.e. where the sequences were isolated from strains that do not belong to any known bacterial species (Leser et al., 2002).

DISCUSSION

This study aimed to investigate the impact of fermented feeds on the gastrointestinal flora in piglets. We analysed the bacterial flora in groups of animals fed different diets by culture independent DGGE-analysis. There is yet little known about the presence of bacterial strains in different parts of the digestive tract of pigs fed different diets (Leser et al., 2002; Konstantinov et al., 2003; Pedersen et al., 2005; Wang et al., 2007). Most studies have focussed on enumeration of harmful and beneficial bacteria in the digestive tract of piglets but not on their identification (van Winsen et al., 2001b; Scholten et al., 2002; Canibe and Jensen, 2003; Højberg et al., 2003). However, Leser et al. (2002) found that *Lactobacillus amylovorus*, *L. johnsonii*, and *L. reuteri* were the most common in pigs while *L. sharpeae* were common in the ileum and cecum of pigs fed fermented feed. Pedersen et al. (2005) showed that feeding wet wheat distiller's grain to piglets affected the composition of the lactic acid bacteria flora in their intestine. Canibe and Jensen (2003) reported that feeding fermented liquid cereal grain feed changed the bacterial population of the stomach.

The strongest impact of the diet in the current study was seen in the stomach, where all investigated groups showed different banding patterns. Sequencing also confirmed that

Table 3. 16S rRNA gene sequences of the strong DNA bands from ileum detected by DGGE and clonning technique

DNA band	Diet	Closest sequence	Accession no.	Identity (%)	Length of DNA
il1	Day 0	*Escherichia sp.*	DQ856894	100	160
		Streptococcus sp.	AF313406	99	160
		Prevotellaceae [a]	AY806910	96	154
il2	Day 0	*E. coli/Shigella* [b]	V00350	100	160
		Prevotellaceae [a]	AY919880	100	154
il3	Day 0	*Shigella sp.*	FJ193356	100	160
		Prevotellaceae [a]	AB237859	96	155
il4	Day 0	*Enterobacteriaceae* [a]	DQ856894	100	160
		Prevotellaceae [a]	DQ795950	100	155
il5	Day 0	*Treponema sp.*	EU794268	99	160
		Prevotellaceae [a]	AY695689	100	155
		Erysipelotrichaceae [a]	AY237191	99	160
		Helicobacteraceae [a]	AF334681	100	135
il6	CO	*Escherichia sp.*	BR04AA06	100	160
il7	FE	*Erysipelotrichaceae* [a]	EF365175	98	160
il8	FE	*Erysipelotrichaceae* [a]	AY242739	91	160
		L. acidophilus/L. gasseri [c]	M99704	99	160
il9	FE	*Erysipelotrichaceae* [a]	DQ374468	100	160
		Lachnospiraceae [a]	AY343186	99	135
		Pasteurellaceae [a]	AY938978	98	160
il10	FE	*Prevotellaceae* [a]	AB237894	99	155
		Spirochaetaceae [a]	AB433133	100	160
il11	FE	*Lactobacillus murinus*	AY186045	99	160
		Pediococcus acidilactici	YDW17	99	160
		Bacteroidaceae [a]	EU573805	85	155
il12	FE	*Lactobacillus jensenii*	AY339169	99	159
		Weissella cibaria	AY244624	98	160
il13	FE	*Pediococcus acidilactici*	YDW17	99	161
		Lactococcus lactics	AF323179	99	161
		Peptostreptococcaceae [a]	DQ178969	99	135
il14	RDR	*Lactobacillus johnsonii*	ATCC33200	100	160
		Lachnospiraceae [a]	AF153855	97	135
		Erysipelotrichaceae [a]	DQ374468	100	160
il15	RDR	*Lactobacillus fermentum*	AY244629	99	160
		Erysipelotrichaceae [a]	DQ374468	100	160

[a] Determination according to the rdp search.
[b] *E. coli* and *Shigella* could not be distinguished on the basic of the V3 sequence (Naum et al., 2008).
[c] *Lactobacillus acidophilus* and *L. gasseri* could not be distinguished (Berger et al., 2007).

there were different microorganisms in the intestine of animals fed different diets. Interestingly, the diet consisting of fermented feed had the highest impact on the microbial flora in the animals tested in our study. In the stomach of piglets fed fermented feed the microbial flora was more uniform than in animals fed the other diets. It has earlier been shown that the supply of nutrients in the feed, especially the content of fermentable carbohydrates and of fibre, can influence the bacterial flora in the intestine (Durmic et al., 1998; Konstantinov et al., 2003). However, diet CO and FE in the current study had the same ingredient and nutrient composition. Thus, the intestinal flora was obviously mainly influenced by the microbial flora that developed on the feed. Probably, it was only in the FE diet that a number of microorganisms was reached that was high enough to substantially influence the microbial flora in the intestine. Organisms belonging to the genus *Pediococcus* were only found in animals fed the diet with fermented feed (FE). We have earlier shown that bacteria belonging to this genus can dominate feed fermentations (Olstorpe et al., 2008). Thus, it is likely that these organisms arose from the naturally fermented feed. In the ileum, some influence of

Table 4. 16S rRNA gene sequences of the strong DNA bands from colon detected by DGGE and clonning technique

DNA band	Diet	Closest sequence	Accession no.	Identity (%)	Length of DNA
co1	Day 0	*Prevotellaceae* [a]	AF018512	97	155
		Lachnospiraceae [a]	EU473780	97	136
co2	Day 0	*Veillonellaceae* [a]	AY919972	96	155
		Prevotellaceae [a]	AB237881	98	155
co3	Day 0	*Prevotellaceae* [a]	EU573805	85	155
co4	Day 0	*Lactobacillus johnsonii*	RP3AY186044	100	155
		Prevotellaceae [a]	AB237881	100	155
co5	Day 0	*Prevotellaceae* [a]	DQ795745	99	155
co6	Day 0	*Prevotellaceae* [a]	AB237849	100	155
		Bacteroidaceae [a]	EU462150	94	155
co7	CO	*Prevotellaceae* [a]	AY695689	100	155
		Bacteroidaceae [a]	AY695659	96	155
co8	CO	*Prevotellaceae* [a]	AY806606	96	155
co9	CO	*Prevotellaceae* [a]	AB099767	99	155
co10	CO	*Prevotellaceae* [a]	AY696689	100	155
		Lachnospiraceae [a]	AB064744	100	135
co11	CO	*Lactobacillus johnsonii*	RP3AY186044	99	155
		Prevotellaceae [a]	AB237881	99	155
co12	CO	*Lactobacillus johnsonii*	RP3AY186044	100	155
		Prevotellaceae [a]	DQ795745	98	155
co13	FE	*Prevotellaceae* [a]	AY850419	98	155
co14	FE	*Prevotellaceae* [a]	AB237881	97	155
co15	FE	*Prevotellaceae* [a]	DQ795963	99	155
		Lachnospiraceae [a]	AF153857	100	135
co16	RDR	*Prevotellaceae* [a]	AY850419	99	155
co17	RDR	*Prevotellaceae* [a]	AF018512	97	155

[a] Determination according to the rdp search.

the feed was observed. Again, diet FE seemed to have the highest impact on the bacterial flora. Although there were greater individual differences and there were several bands in the DGGE pattern in individuals fed the FE-diet that were also observed in individuals fed other diets, some bands were unique for the FE-diet, and some identified organisms were only found in the FE-group. This again included bacteria belonging to the genus *Pediococcus*. The colon samples showed the smallest diet dependent differences. Differences seemed rather to be due to the individual than to diet. This is in accordance with earlier studies of the faecal flora of pigs, where it has been shown that individual differences in the microflora are often bigger than those due to the nutrition (Simpson et al., 2000).

Lactic acid bacteria have frequently been found in the stomach of pigs (Fuller et al., 1978; Henriksson et al., 1995; Leser et al., 2002), which is confirmed by our results. The occurrence of *Pediococcus* species in the pig intestine has rarely been reported and no sequences arising from this genus have been found in a clone library made of 16S rDNA sequences isolated and amplified from pig intestine (Leser et al., 2002). This again indicates that these organisms were introduced by the diets into the intestine of the pigs. Some clones matched to species that have been earlier described to be abundant in the pig intestine, including *L. acidophilus*, *E. coli*/*Shigella* and bacteria belonging to the *Prevotella* group (Leser et al., 2000; Leser et al., 2002). We also found a considerable number of organisms that did not match to any known bacterial species. This has been described in other studies using molecular detection methods and supports the contention that great parts of the microbial flora in the pig intestine are still unknown (Leser et al., 2002).

DGGE is a method that has frequently been applied to analyze the gastrointestinal microflora in pigs (Simpson et al., 1999; Simpson et al., 2000; Collier et al., 2003; Konstantinov et al., 2003). The method is relatively easy to perform and provides a good general survey about the microbial flora in the intestine. However, there are also limitations. Due to the short length of the amplified sequences several organisms cannot clearly be identified. This became obvious in the case of bands that matched to the *E. coli*/*Shigella* group. In this case it is impossible to distinguish between *E. coli*, which is a common bacterium

in the intestine of mammals, and *Shigella flexneri*, which is supposed to be pathogenic (Naum et al., 2008). The occurrence of chimeric bands, due to recombination processes has also been described (von Wintzingerode et al., 1997; Leser et al., 2002). However, we did not find such bands, which might also be due to the low molecular weight of the amplified molecules. Co-migration of bands with different sequences was found for 28 of the 47 cloned bands in the current study, a phenomenon that has been observed earlier (Ercolini et al., 2001; Gafan et al., 2005). Multiple bands have been observed when molecules with attached GC-clamps were amplified (Nübel et al., 1996), which might also account for multiple bands of the same sequence in our data (Figure 2). Anyway, our results show that the method was appropriate to detect population changes as a result of feeding fermented diets. Especially in the stomach, a great impact of feeding fermented diet on the banding pattern was observed. Sequencing revealed the presence of strains belonging to *P. acidilactici* and *P. pentosaceus*, which were not found in piglets fed the other diets.

P. acidilactici has been used as a probiotic in weaning piglets resulting in improved weight gain and increased resistance against infections (Giancamillo et al., 2008). *Pediococcus* strains have been shown to produce bacteriocines, which may have a potential against pathogenic bacteria. Thus, our study strongly suggests that the use of a fermented diet with a microbial flora dominated by *Pediococcus* strains may be preferable to traditional diets.

ACKNOWLEDGMENT

We wish to thank the MEKARN project and Ministry and Science and Technology Vietnam for financial support. V.P. was financed by the thematic research program MicroDrivE-Microbially Derived Energy (http://microdrive.slu.se/) at the Faculty for Natural Resources and Agriculture at the Swedish University of Agricultural Sciences. We also thank staff of laboratory of Faculty of Animal Science for skillful technical assistance with extraction DNA genomic and Dr. Sara Hallin, Anna-Ida J. Holmberg, Cecilia Petrén and Nina-Sofia Atanasova at the Department of Microbiology at SLU for the kind help with performing the DGGE-analysis.

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