Changes in caecal microbiota and mucosal morphology of weaned pigs

Marisol Castillo, Susana M. Martín-Orúe, Miquel Nofrarías, Edgar G. Manzanilla, Josep Gasa

Title: Changes in caecal microbiota and mucosal morphology of weaned pigs

Authors: Marisol Castillo, Susana M. Martín-Orúe, Miquel Nofrarías, Edgar G. Manzanilla, Josep Gasa

PII: S0378-1135(07)00208-8
DOI: doi:10.1016/j.vetmic.2007.04.026
Reference: VETMIC 3668

To appear in: *VETMIC*

Received date: 15-12-2006
Revised date: 4-4-2007
Accepted date: 17-4-2007

Please cite this article as: Castillo, M., Martín-Orúe, S.M., Nofrarías, M., Manzanilla, E.G., Gasa, J., Changes in caecal microbiota and mucosal morphology of weaned pigs, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.04.026

Changes in caecal microbiota and mucosal morphology of weaned pigs

Marisol Castillo[a], Susana M. Martín-Orúe [a*], Miquel Nofrarías[b], Edgar G. Manzanilla[a] and Josep Gasa[a]

[a] Animal Nutrition, Management and Welfare Research Group, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

[b]Centre de Recerca en Sanitat Animal (CReSA)-Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

[*] Corresponding author. Present address: Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel.: +34 93 5811504; fax: +34 93 5811494. E-mail address: susana.martin@uab.es (S.M. Matín-Orúe).

Abstract

An experiment was designed to monitor the changes in caecal microbiota associated with early-weaning. Twelve piglets (20 ± 2 days) from six different litters were selected from a commercial source. For the two experimental groups, one animal from each litter was weaned onto a post-weaning diet (W) and the other remained with the sow (S). After 1 week, animals were sacrificed and caecal samples taken. Microbial counts for total bacteria, enterobacteria and lactobacilli populations were determined by quantitative PCR using SYBR Green® dye. Microbial profiles were assessed by terminal restriction fragment length polymorphism (t-RFLP). Weaning promoted an increase in the enterobacteria:lactobacilli ratio (0.27 vs. 1.67 log/log 16S rRNA gene copy number, $P = 0.05$). Total bacteria and richness of the caecal microbial ecosystem (number of peaks) were similar in both experimental groups (49.3 for S and 53.4 for W, respectively, $P = 0.22$), although the band patterns were clearly grouped in two different clusters by dendogram analysis. Weaning was also associated with a decrease in crypt density, an increase in mytotic index and a decrease in the number of goblet cells. A reduced immunological response was also observed and was manifested by an increase in intraepithelial lymphocytes and lymphocyte density in the lamina propria. Weaning appears to be critical in the establishment of the caecal microbiota in pigs with important changes, particularly in microbial groups and in caecal mucosal architecture.

Keywords: caecal histology, microbiota, pig, real-time PCR, t-RFLP, weaning.

1. Introduction

At early-weaning, the pig is affected by a high number of stressors that increase its susceptibility to disease. As a result, lower growth rates and post-weaning diarrhoea are significant problems in pig production after early-weaning.

During natural weaning, the piglet is progressively introduced to several external feed sources and its fermentative capacity is progressively developed for approximately 10 weeks, at which time the animal is completely weaned. In commercial weaning, this process happens in a couple of days and post-weaning diarrhoea is mainly related to intestinal disbiosis during this rapid adaptation to the new diet. It is generally recognised that the establishment of a diverse bacterial microbiota, characteristic and dynamic for each individual (Simpson et al., 2000), plays a key role in the maintenance of the gastrointestinal health by preventing colonization by pathogens (Van Kessel et al., 2004). This beneficial microbiota is especially important at periods such as weaning, when the animal still has an immature immune system and depends on certain compounds in the sow's milk to prevent the growth of opportunistic bacteria (Edwards and Parret, 2002). There are several studies suggesting that early weaning causes substantial changes in the intestinal bacterial community (Franklin et al., 2002, Konstantinov et al., 2006), but more research is needed regarding the specific changes of the microbial ecosystem during this critical phase.

The gastrointestinal tract also needs to adapt to this new situation along with the microbial population. It is known that adaptation of the small intestine epithelium at weaning is greatly conditioned by bacterial colonization (Pluske, 1997). However, information on changes in caecal epithelium at weaning is scarce.

The objective of this study was to assess microbial shifts in the caecum of commercially weaned pigs using t-RFLP and real-time PCR, and to monitor changes in caecal mucosa after weaning.

2. Material and methods

2.1. Animals and housing

The experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval from the Animal Protocol Review Committee of the institution. Management, housing and slaughtering conditions conformed to the European Union Guidelines. A total of 12 piglets (4.4 ± 0.36 kg; 20 ± 2 days, mixed males and females) were selected from six commercial litters, taking initial body weight into account. One piglet from each litter was weaned and fed a commercial post-weaning diet (weaned group, W) for 1 week (Table 1); the other piglet remained on the original commercial farm with the sow and littermates (control group, S).

2.2. Sacrifice and sampling

On day 28, the animals were weighed and sacrificed with an intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain; 200 mg/kg BW). Animals were bled and the abdomen immediately opened from sternum to pubis. The whole gastrointestinal tract was removed, weighed and sampled. pH in four segments of the gastrointestinal tract was measured by insertion of a unipolar electrode through a small incision made in the wall (penetration pH meter CRISON 507, electrode Crison 52-32, Net Interlab S.A.L., Madrid, Spain). The pH measurements were performed in the middle of the caudal portion of the stomach, 15 cm proximal to the ileocecal valve, in the lowest part of the caecum and in the colon, 20 cm distal to the caecum. Samples

(1 g) of the caecal contents were taken and kept in tubes with 3 ml of ethanol (96%) as a preservative. Stomach, small intestine and large intestine were emptied and weighted. For histological study, samples from the middle caecum were opened longitudinally and fixed by immersion in 10% (v/v) buffered formalin immediately after slaughter.

2.3. DNA extraction

Samples of equivalent volume to 400 mg of digesta were preserved in ethanol and precipitated by centrifugation (13 000 *g* for 5 min). DNA in the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90 °C and an incubation step with lysozyme was added afterwards (10 mg/ml, 37 °C, 30 min) to improve bacterial cell rupture. The DNA was stored at –80 °C until analysis.

2.4. Real-time PCR (qPCR)

Total bacteria, lactobacilli and enterobacteria were quantified using real-time PCR following procedures and using primers described by Castillo et al. (2006). The oligonucleotides used were based on regions of identity within the 16S rRNA gene and were adapted from published specific primers or probes using the Primer Express Software (Applied Biosystems, CA, USA). Primers used for total bacteria were: F-tot (forward) 5'GCAGGCCTAACACATGCAAGTC3' and R-tot (reverse) 5'CTGCTGCCTCCCGTAGGAGT3'. For lactobacilli: F-lac 5'GCAGCAGTAGGGAATCTTCCA3' and R-lac 5'GCATTYCACCGCTACACATG3', and for enterobacteria F-ent 5'ATGGCTGTCGTCAGCTCGT3' and R-ent 5'CCTACTTCTTTTGCAACCCACTC3'. Amplification and detection of DNA by quantitative real-time PCR was performed with the ABI 7900 HT Sequence Detection

System using optical-grade 96-well plates and SYBR Green dye (PE Biosystems, Warrington, UK). For absolute quantification, PCR products obtained from the amplification of the whole 16S rRNA gene of *Escherichia coli* (CECT 515NT) and *Lactobacillus acidophilus* (CECT 903NT) were used to construct the standard curves. The PCR conditions corresponded to those published by Leser et al. (2002). The amplified gene from *E. coli* was used for absolute quantification of the total bacteria and enterobacteria and the amplified gene from *L. acidophilus* for quantification of the lactobacilli. Quantitative values were expressed as log of 16S rRNA gene copies/g fresh matter (FM). Enterobacteria:lactobacilli ratio were expressed as the difference of logarithms.

2.5. Terminal-Restriction Fragment Length Polymorphism (t-RFLP)

t-RFLP analysis of bacterial community was performed following the procedure described by Höjberg et al. (2005). Briefly, a 1497-bp fragment of the 16S rRNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5'-AAGGAGGTGATCCAGCCGCA-3'). Duplicate PCR analyses were perform for each sample. Fluorescent-labeled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µl of Milli-Q water. Then, the resultant PCR product was subjected to a restriction with *HhaI* (20 000 U/µl) (Biolabs Inc. New England, USA). Fluorescent-labeled terminal restriction fragments (TRF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with a 25-U detection threshold. Determination of the TRFs sizes in the range 50–

700 base pairs (bp) were performed with the size standard GS-1000-ROX (PE Biosystems).

2.5.1. Analysis of t-RFLP data

Sample data consisted of size (base pairs) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all TRF peak areas in the pattern was used to normalize the peak detection threshold in each sample. Following the method of Kitts (2001), a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by 323 area units (the area of the smallest peak at the 25 detection threshold in the sample with the smallest total peak area). For each sample, peaks with a lower area were deleted from the data set. New total area was obtained by the sum of all the remaining peak areas in each pattern.

Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated and dendograms were constructed using Fingerprinting II (Informatix, Bio-Rad, CA, USA) software and an unweighted pair-group method with averaging algorithm (UPGMA).

To deduce the potential bacterial composition of the samples, *in silico* restrictions for the major pig gut bacteria with the primers and the enzyme used were obtained using the analysis function TAP-tRFLP from the Ribosomal Database Project II software (Table 2).

2.6. Morphometric analysis

Tissue samples for histological study were dehydrated and embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Morphometric measurements were performed with a light microscope (BHS, Olympus, Spain). Crypt

depth (CD), intraepithelial lymphocytes (IEL), the index of mitosis, lamina propria cell density and goblet cell numbers in crypts were measured (Nofrarías et al., 2006). Measurements were performed in 10 well-oriented crypts from each animal. CD was measured using a linear ocular micrometer (Olympus, REF.209-35040, Microplanet, Barcelona, Spain). The same crypt columns were used to determine the number of IEL, goblet cells and index of mitosis (meta- and anaphases); these variables were expressed per 100 enterocytes. On the basis of the cellular morphology, differences between the nuclei of enterocytes, mitotic figures, goblet cells and lymphocytes were clearly distinguishable at 400× magnification. Lamina propria cell density was determined by counting total visibly stained nuclei and total lymphocytes in 10 fields (total area of 4000 μm^2) from each section using an ocular grid (Olympus, REF. 209-35046, Microplanet, Barcelona, Spain). Cell density was expressed as the number of total stained cells and the number of lymphocyte-like cells per 1000 μm^2 area. The number of lymphocytes in relation to the number of total cells was also calculated. Crypt density was also determined (Brunsgaard, 1997). All morphometric analyses were done by the same person, who was blind to treatment modality.

2.7. Statistical Analysis

The effect of weaning on body weights, pH, total bacteria, lactobacilli, enterobacteria, richness and histological measurements was tested with ANOVA using the GLM procedure of SAS statistic package (SAS Inst., Inc. 8.1, Cary, NC, USA). The pig was used as the experimental unit. Statistical significance was accepted at $P \leq 0.05$.

3. Results and discussion

Diarrhoea was not detected in the pigs and there was only one case of liquid faeces (W group). Initial body weight (BW) was similar for both groups, at 4.4 ± 0.16 kg for S and 4.4 ± 0.15 kg for W. As expected, at the end of the experimental period, BW was higher for piglets that remained with the sow than for weaned pigs (6.1 ± 0.25 versus 5.05 ± 0.27 kg for S and W, respectively, $P < 0.001$). Growth rate, expressed as average daily gain (ADG), was higher for S than for W pigs (0.25 ± 0.02 versus 0.10 ± 0.02 kg for S and W, respectively, $P < 0.001$).

3.1. Bacterial quantitative change measured by real-time PCR

The total microbial population, lactobacilli and enterobacteria were quantified in caecum digesta using qPCR (Figure 1).

The total bacteria counts, expressed as log 16S rRNA gene copies/g fresh matter (FM), did not differ between groups (12.84 and 12.81 log gene copy number/g FM for S and W, respectively, $P > 0.05$). Similar total faecal anaerobic counts after weaning were found by Franklin et al. (2002) in piglets weaned at 24 days.

Lactobacilli and enterobacteria have been traditionally selected as microbial groups with a particular significance for gut health. The ratio between these two bacterial groups has been routinely used as a gut-health indicator and it is desirable that lactobacilli outnumber enterobacteria to improve robustness against opportunistic pathogens. In our case, this ratio was higher in W than in S pigs, reflecting the negative effect of weaning on lactobacilli and enterobacteria populations (0.27 versus 1.76 for S and W group, respectively, $P = 0.05$)). An inverse correlation between lactobacilli and enterobacteria during the first week post-weaning has been reported previously (Risley et al., 1992; Jensen, 1998; Franklin et al., 2002).

3.2. Ecological bacterial changes, t-RFLP results.

198 To evaluate global changes in the microbial ecosystem, the t-RFLP method was
199 employed; a technique recently used to characterize the effect of different dietary
200 treatments on pig gut microbiota (Højberg et al. 2005).

201 The similarity indexes of the t-RFLP profiles illustrated in the form of a dendogram
202 are shown in Figure 2. It only shows the microbial profiles of 11 pigs due to the fact that
203 one pig had no digesta present in the caecum at the time of sacrifice. The effect of
204 weaning on the ecological composition of microbiota is clear, compared to other
205 possible factors, such as litter or individual effects. This was reflected in two clearly
206 separate clusters, one for each experimental group. There was one exception: a weaned
207 piglet was grouped in the suckling branch of the dendogram and, interestingly, was the
208 animal that showed liquid faeces. Separation of this piglet in the dendogram might
209 reflect the beginning of some kind of enteric disbiosis in this animal and the failure of
210 its microbial ecosystem to adapt to solid, dry food.

211 Microbial profiles of S pigs showed a higher similarity to one another (54–78%) than
212 those of W pigs, which showed more heterogeneous microbial profiles (25–76%). The
213 higher variability in microbial profiles in the W group suggests that the pigs suffered
214 stress at weaning and that each individual responded differently to that stress.

215 Microbiota richness was measured as the number of similar bands between both
216 experimental groups (49.34 for S and 53.40 for W, $P = 0.22$). Various studies have
217 described a marked decrease in biodiversity just after piglet weaning (Katouli et al.,
218 1997; Jensen-Waern et al., 1998), showing that early weaning involves a obvious
219 disruption in normal pig microbiota evolution. Thereafter, there is a re-establishment
220 process which can vary with time depending on a number of factors. In this study, the

221 weaned pigs were probably in the process of re-establishing a new microbial
222 equilibrium.

223 *In silico* restriction, using Ribosomal Database Project II, was used to deduce
224 potential ecological changes in the samples. However, it should be noted that dispersed
225 phylogenetic groups of bacteria may produce terminal restriction fragments (TRFs) of
226 identical size and that a single TRF in a profile may represent more than one organism
227 in the sample. Results are, therefore, presented as potential compatible bacterial species.
228 Note also that direct attribution of species to individual peaks is not unequivocally
229 possible unless fingerprinting is complemented with sequence analysis of clone
230 libraries.

231 In this study, compatible bacteria species were assigned to recently described, major
232 pig-gut bacterial groups (Leser et al., 2002). Table 2 shows the frequency of detection
233 of compatible bacteria that were represented in at least three animals.

234 Analysis of electropherograms revealed compatible TRFs with different lactic acid
235 bacteria including *L. acidophilus, L. bifermentum, L. brevis, L. casei, L. rhamnosum, L.*
236 *vaginalis, Lactococcus lactis, L. delbruekii sp. lactis, L. delbruekii sp. delbruekii,* and
237 *L. fructivorans*. Interestingly, the fragment of 62 base pairs, compatible with both *L.*
238 *lactis* and *L. vaginalis,* was present in all the animals, with a mean contribution of 15–
239 20% of total area. Although mean area for total lactobacilli was similar in both groups
240 (23.1 and 21.5 % for S and W pigs, respectively), S pigs showed higher diversity in
241 compatible TRFs with different lactobacilli species than W pigs. In particular, *L.*
242 *delbruekii* sp. *lactis* was present in five animals from this group, representing nearly 5%
243 of total area, whereas no animal of the W group showed any fragment with compatible
244 size. Similarly, *L. delbruekii* sp. *delbruekii* was present in four S pigs but in only one W

pig. The presence of a higher diversity of lactobacilli in S pigs has been reported previously (Krause et al., 1995; Konstantinov et al., 2006).

In the case of *Enterococcus* sp., five of six pigs in the S group showed a peak compatible with this group (2.18%), but only appeared in two pigs from the W group (1.35%). This is in agreement with Jensen (1998), who found a decrease in enterococci in weaned piglets.

Different species from the *Bacteroidetes* phylum can be compatible with a series of TRFs of similar size, ranging 89–104 bp (Table 2). Summed areas of these peaks represent 4.0 and 5.2% of total peak area for suckling and weaned pigs, respectively, and are, therefore, the second most importance group after lactobacilli. Adami and Cavazzoni (1999) have also described the importance of this group of bacteria in young piglets.

In our study, a peak compatible with *Clostridium coccoides* only appeared in three suckling pigs, representing 1.67% of total peak area. Similarly, a peak compatible with *C. butyricum* (0.86 %) was only found in the S group. Other studies have described *Clostridium* as one of the main anaerobic bacteria during the suckling period, declining progressively in abundance with the age (Swords et al., 1993). The presence of *C. coccoides* may be considered beneficial for the piglets due to its production of short-chain fatty acids. In fact, it has been used as a probiotic both in animals and humans (Han et al., 1984: Seki et al., 2003). Other peaks, compatible with different species from the *Clostridium* clusters I, IV and XIVa and XVIII, were found in both groups of animals, representing a mean of 2.49 and 1.99% of total area for S and W pigs, respectively.

Peaks compatible with *Fibrobacter succinogenes* and *Fibrobacter intestinalis* were found in both groups (5.3% of total peak area for suckling and 3.4% for weaned pigs). Bacteria belonging to this genus, which show high cellulolytic and hemicellulolytic enzyme activities, have been reported previously from the porcine gastrointestinal tract (Varel and Yen, 1997). Although fibre was absent in the milk diet and at a very low level in the dry feed, the presence this genus could indicate the ability of the pigs to select a particular indigenous microbiota, allowing effective digestion of fibre with growth and change in diet.

Potential compatible peaks with species from the *Enterobacteriaceae* family, such as *E. coli*, were found in only four pigs, even though enterobacteria counts were determined by qPCR in all animals. A bias in the amplification of particular sequences caused by preferential annealing of particular primer pairs to certain templates and the difficulty of amplifying bacteria in lower proportions in complex samples, such as digesta, might explain the absence of compatible TRFs.

3.3. Intestinal morphology

Results of empty weight, pH in different gastrointestinal compartments and histological measurements in caecal samples are shown in Table 3.

Weight of stomach and small intestine did not differ between groups; however, the empty weight of the large intestine was significantly higher in the W than in the S group. This increase in large intestine weight could correspond to the beginning of fermentative activity in this compartment due to an increase in the amount of dietary fermentable material arriving in the caecum. Moreover, pH values confirm this increase in fermentation, with significant decreases in caecum and colon pH. The relationship between the beginning of fermentative activity and the development of the large

292 intestine has been described previously and could be mediated by a trophic effect of an
293 increasing amount of fermentation products, such as SCFA (Frankel et al., 1994).

294 Regarding caecal histology, differences in crypt depth were not detected, but the
295 crypts were further apart and showed a significant increase in mitotic index in W
296 animals. Brunsgaard (1997) also found increases in the proliferative activity of crypts
297 in the hindgut of pigs at weaning, with a corresponding increase in crypt size. In the
298 current study, increased mitotic activity was observed without crypt depth variation,
299 suggesting both increased renewal and apical epithelial loss in the crypt.

300 Concerning the intestinal barrier, both cells producing the mucous layer and immune
301 cells were studied. A decrease in the number of goblet cells was found in the W group
302 compared to the S group. Mucus production and breakdown, as well as the number of
303 goblet cells, are regulated by both microbial and host-related factors (Deplancke and
304 Gaskins, 2001). However, little information is available on the effect of weaning and
305 diet on mucin secretion. Pestova et al. (2000) found increased concentrations of
306 intestinal mucins in ileal digesta after weaning, but no measurements were taken in the
307 hindgut.

308 Immune cells, another important component of the intestinal barrier, also showed
309 differences between groups. Both intraepithelial and lamina propria lymphocytes were
310 higher in weaned pigs, suggesting an increase in activity of the immune system.

311 An increase in intraepithelial lymphocytes, the first cell line of immune defence,
312 (Cheroutre 2004) is related to changes in intestinal microbiota rather than changes in
313 nutrition (Rothkötter et al., 1999). The changes in intestinal microbiota could also be
314 responsible for the variations observed in the caecum, but the effect of fresh nutrients
315 coming from the solid feed cannot be ruled out.

316

317 **4. Conclusions**

318 The caecum undergoes significant microbiological and morphological changes at
319 weaning to develop fermentative activity. Commercial weaning produced marked
320 changes in pig caecum microbiota, with an increase in the enterobacteria:lactobacilli
321 ratio. t-RFLP showed differences in bacterial profiles between groups. Though only
322 assumptions can be made, suckling pigs showed a higher diversity in lactic acid bacteria
323 species than the weaned group and showed peaks, compatible with *C. coccoides* and *C.*
324 *butyricum* species, that were absent in weaned pigs. These changes in microbiota are
325 accompanied by significant changes in caecal histology, with increased proliferative
326 activity in crypts and increased activity of the mucosal immune system.

327

328 **Acknowledgements**

329

330 This work was financial supported by a Ministerio de Ciencia y Tecnología project
331 (AGL2005-0738-C02-01) and a Generalitat de Catanunya FPI grant. The authors thank
332 I. Cornax and L. Flatow from UCDavis (CA) for the assistance with language
333 manuscript corrections.

334

335 **References**

336 Adami, A., Cavazzoni, V., 1999. Occurrence of selected bacterial groups in faeces of
337 piglets fed with *Bacillus coagulans* as probiotic. J. Basic Microbiol. 39, 3-9.

338 Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of
339 whole cells for determinative, phylogenetic and environmental studies in
340 microbiology. J. Bacteriol. 172, 767-770.
341 Brunsgaard, G., 1997. Morphological characteristics, epithelial cell proliferation, and
342 crypt fission in cecum and colon of growing pigs. Digest. Dis. Sci. 42, 2384-
343 2393.
344 Castillo, M., Martín-Orúe, S.M., Manzanilla, E.G., Badiola, I., Martín, M., Gasa J.,
345 2006. Quantification of total bacteria, enterobacteria and lactobacilli populations
346 in pig digesta by real-time PCR. Vet. Microbiol. 114, 165-170.
347 Cheroutre, H., 2004. Starting at the beginning: new perspectives on the biology of
348 mucosal T cells. Annu. Rev. Immunol. 22, 217–246.
349 Deplancke, B., Gaskins, H.R., 2001. Microbial modulation of innate defence: goblet
350 cells and the intestinal mucus layer. Am. J. Clin. Nutr. 73, 1131S-1141S.
351 Edwards C.A., Parret, A.M., 2002. Intestinal flora during the first months of life: new
352 prospective. Br. J. Nutr. 88 Suppl. 1, S11-S18.
353 Frankel, W.L., Zhang, W., Singh, A., Klurfeld, D.M., Don, S., Sakata, T., Modlin, I.,
354 Rombeau, J.L. 1994. Mediation on the trophic effects of short-chain fatty acids
355 on the rat jejunum and colon. Gastroenterology. 106, 375-380.
356 Franklin, M.A., Mathew, A.G., Vickers, J.R., Clift, R.A., 2002. Characterization of
357 microbial populations and volatile fatty acid concentrations in the jejunum,
358 ileum, and cecum of pigs weaned at 17 vs. 24 days of age. J. Anim. Sci. 80,
359 2904-2910.
360 Han, I.K., Lee, S.C., Lee, J.H., Kim, J.D., Jung, P.K., Lee, J.C. 1984. Studies on the
361 growth promoting effects of probiotics II. The effect of *Clostridium butyricum*

362 ID on the performance and changes in the microbial flora of the faeces and
363 intestinal contents of the broiler chicks. Korean J. Anim. Sci. 26, 159-165.
364 Hojberg O., Canibe, N., Poulsen, H.D., Hedemann, M.S., Jensen, B.B., 2005. Influence
365 of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in
366 newly weaned piglets. Appl. Environ. Microbiol. 71, 2267-77.
367 Jensen-Waern, M., Melin, L., Lindberg, R., Johannisson, A., Petersson, L., Wallgren,
368 P., 1998. Dietary zinc oxide in weaned pigs-effects on performance, tissue
369 concentrations, morphology, neutrophil functions and faecal microflora. Res.
370 Vet. Sci. 64, 225-231.
371 Katouli, M., Lund, A., Wallgren, A.P., Kuhn, I., Soderlind, O., Mollby. R., 1995.
372 Phenotypic characterization of intestinal Escherichia coli of pigs during
373 suckling, postweaning, and fattening periods. Appl. Environ. Microbiol. 61,
374 778-783.
375 Katouli, M., Lund, A., Wallgren, P., Kuhn, I., Soderlind, O., Mollby, R., 1997.
376 Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs
377 during pre- and post-weaning periods. J. Appl. Microbiol. 83, 147-154.
378 Katouli, M., Melin, L., Jensen-Waern, M., Wallgren, P., and Möllby, R., 1999. The
379 effect of zinc oxide suppplementation on the stability of the intestinal flora with
380 special reference to composition in coliforms in weaned pigs. J. Appl.
381 Microbiol. 87, 564-573.
382 Kitts, C.L., 2001. Terminal Restriction Fragment Patterns: a tool for comparing
383 microbial commun ities and assessing community dynamics. Curr. Issues Intest.
384 Microbiol. 2, 17-25.

385 Konstantinov, S. R., Awati, A. A., B. A. Williams, B. G. Miller, P. Jones, C. R. Stojes,
386 A. D. L. Akkermans, H. Smidt, and W. M. de Vos., 2006. Post-natal
387 development of the porcine microbiota composition and activities. Environ.
388 Microbiol. 8, 1191-1199.
389 Krause, D.O., Easter, R.A., White, B.A., Mackie, R.I., 1995. Effect of weaning diet on
390 the ecology of adherent lactobacilli in the gastrointestinal tract of the pig. J.
391 Anim. Sci. 73, 2347-2354.
392 Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M., Moller, K.,
393 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract
394 microbiota revisited. Appl. Environ. Microbiol. 68, 673-690.
395 National Research Council, 1998. Nutrient requirements of swine. National Academy
396 Press, Washington, DC.
397 Nofrarias, M., Manzanilla, E. G., Pujols, J., Gibert, X., Majó, N. Segales, J., and Gasa,
398 J. 2006. Effects of spray dried porcine plasma and plant extracts on intestinal
399 morphology and on leukocyte cell subsets of weaned pigs. J. Anim. Sci. 84,
400 2735-2742.
401 Pestova, M.I., Clift, R.E., Vickers, R.J., Franklin M.A., Mathew, A.G., 2000. Effect of
402 weaning and dietary galactose supplementation on digesta glycoproteins in pigs.
403 J. Sci. Food Agri. 80, 1918-1924.
404 Pluske, J.R., Hampson, D.J., Williams, I.H., 1997. Factors influencing the structure and
405 function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51,
406 215-236.

Pluske, J.R., Pethick, D.W., Hopwood, D.E., Hampson, D.J., 2002. Nutritional influences on some major enteric bacterial diseases of pigs. Nutr. Res. Rev. 15, 333-371.

Risley, C.R., Kornegay, E.T., Lindemann, M.D., Wood, C.M., Eigel, W.N., 1992. Effect of feeding organic acids on selected intestinal content measurements at various times postweaning in pigs. J. Anim.Sci. 70, 196-206.

Rothkotter, H.J., Mollhoff, S., Pabst, R., 1999. The influence of age and breeding conditions on the number and proliferation of intraepithelial lymphocytes in pigs. Scand. J. Immunol. 50, 31-38.

Seki, H., Shiohara, M., Matsumura, T., Miyagawa, N., Tanaka, M., Komiyama, A., Kurata, S. 2003. Prevention of antibiotic-associated diarrhea in children by *Clostridium butyricum* MIYAIRI. Pediatr. Int. 45, 86-90.

Simpson, J.M., McCrcken, V.J., Gaskins, H.R., Mackie, R.I., 2000. Denaturing gradient gel electrophoresis analysis of 16S Ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. Appl. Environ. Microbiol. 66, 4705-4714.

Stewart, C.S. 1999. Microorganisms in hindgut fermentors. In: Mackie R.I., White, B.A. (Eds), Gastrointestinal Microbiology, Chapman and Hall Microbiology Series. New York.

Swords, W.E., Wu, C., Champlin, F.R., Buddington, R.K., 1993. Postnatal changes in selected bacterial groups of the pig colonic microflora. Biol. Neonate. 63, 191-200.

Van Kessel, A., Shirkey, T.W., Siggers, R.H., Drew, M.D., Laarveld, B., 2004. Commensal bacteria and intestinal development. Studies using gnotobiotic pigs.

In: Tucker L.A., Taylor-Pickard, J.A. (Eds), Interfacing immunity, gut health and performance. Nottingham University Press, Nottingham, UK.

Varel, V.H., Yen, J.T., 1997. Microbial perspective on fiber utilization by swine. J. Anim. Sci. 75, 2715-2722.

435 **Tables**

436 Table 1. Control diet composition (as fed basis).

Ingredient	g/kg
Corn	46.7
Full fat extruded soybeans	17.0
Lactose	15.0
Soybean meal	10.0
Potato protein	3.77
Whey powder	1.52
L-Lysine HCl	0.17
DL-Methionine	0.098
L-Threonine	0.009
Bicalcium phosphate	3.04
Salt	0.79
Calcium carbonate	0.44
Vit-Mineral premix	0.25
Sepiolite	1.26

437

438

439 Table 2. Theoretical restriction 5'-fragment length predicted for the major pig gut bacteria. Results were obtained from the TAP-RFLP tool
440 of the Ribosomal Database II Project software.

Bacteria groups	Compatible bacteria [a]	*In silico* restriction [b]	Real restriction [c]	Frequency [d]	
				Suckling	Weaned
Lactic Acid Bacteria	*L. acidophillus, L. brevis, L. bifermentum, rhamnosum, casei*	597, 598, 599	597, 599	1 (1.45)	2 (0.54)
	L. delbruekii sp. *delbruekii*	254	254	4 (1.96)	1(0.98)
	L. delbruekii sp. *lactis*	223	221-223	5 (4.59)	0
	L. fructivorans	68	68	3 (0.88)	3 (0.59)
	Lactococcus lactis, Lactobacillus vaginalis	61	62	6 (14.22)	5 (19.42)
	Enterococcus sp.	216, 218, 220	214	5 (2.18)	2(1.35)
Bacteroides and relatives	*Cytophaga*	92, 94, 96, 100	89-104	6 (4.06)	5 (5.17)
	Flexibacter	82, 84, 90, 94, 96, 97			
	Bacteroides	95, 96, 98, 101, 102, 104			
Fibrobacter	Fibrobacter succinogenes	139, 141, 145	138, 140, 142-145	6 (1.80)	4 (2.70)
	Fibrobacter intestinales	148, 152	148-152	6 (3.46)	5 (0.69)
Clostridium and relatives	*Clostridium coccoides*	66	66	3 (1.67)	0
	C. butyricum	544	544	5 (0.86)	0
	Eubacterium	188, 190, 192, 194, 203	188-193	2 (1.19)	4(0.63)
	Ruminococcus	189			
	Clostridium clostridiforme, C. symbiosum	190			
	Roseburia	192			
	Butyrivibrio	193			
	Clostridium spp.	229, 231, 233, 237	229-232, 237	6 (1.30)	5(1.36)
Proteobacteria	Escherichia sp	371, 372, 373, 374	376-377	3 (0.51)	1(0.55)
	Other enteric bacteria (Salmonella, Citrobacter, Klebsiella)	(367, 370, 372), 373, 371			

441 [a] Major pig gut bacteria, based on the work of Leser et al., 2002, with a potential compatible fragment found in at least three animals. Other peaks with 58, 59, 69, 111-
442 120, 123, 133, 162, 211, 278 and 279 did not correspond with any 16S rRNA sequences in the database from the Ribosomal Database Project 8.1 software.
443 [b] *In silico* restriction was performed using the tap-tRFLP tool from the Ribosomal Database project II.
444 [c] Terminal fragment length obtained after PCR product restriction with Hha I.
445 [d] Number of animals that showed the peak in each experimental group. The mean peak area (%) value in these animals with the peak is shown in brackets.

Table 3. Weights and pH of the stomach, small and large intestine, and caecal morphology of the early-weaned pigs receiving sow milk or dry feed[1].

	Suckling	Weaned	*P*-value
Empty weight, g			
Stomach	37.4 ± 7.54	38.7 ± 4.88	0.737
Small intestine	330 ± 56.4	370 ± 98.1	0.393
Large intestine	120 ± 31.2	195 ± 98.1	0.027
pH			
Stomach	3.2 ± 0.84	4.4 ± 0.67	0.019
Ileum	7.0 ± 0.44	6.7 ± 0.37	0.371
Cecum	6.3 ± 0.13	5.8 ± 0.12	<0.0001
Colon	6.9 ± 0.29	6.03 ± 0.18	<0.0001
Caecum histological study			
Crypt depth, µm	373 ± 48.6	359 ± 30.6	0.566
Crypt density[2]	12.4 ± 0.36	10.5 ± 0.25	<0.001
Mitoses / 100 cells	1.0 ± 0.47	1.8 ± 0.47	0.021
Goblet cells / 100 cells	25.5 ± 3.48	19.4 ± 3.30	0.011
Intraepithelial lymphocytes / 100 cells	1.4 ± 0.67	2.6 ± 0.91	0.024
Lymphocyte density[3]	1.7 ± 0.45	2.3 ± 0.40	0.045
Total cell density[3]	8.7 ± 0.90	8.9 ± 0.47	0.665
Lymphocyte / Total cells	0.2 ± 0.04	0.3 ± 0.07	0.109
Mucosal thickness, µm	470 ± 74.8	528 ± 70.8	0.243
Muscular layer thickness, µm	370 ± 49.5	328 ± 37.0	0.111
Total intestinal wall thickness, µm	848 ± 98.1	856 ± 95.1	0.899

[1] Values are means, n=6.
[2] Number of crypts appearing over a 1mm distance across the basal part of the mucosa.
[3] Number of lymphocytes like cells or total cells per 1000 μm^2 area in the lamina propria.

454 **Figure captions**

455

456 **Figure 1.** Bacterial loads in caecum measured by quantitative PCR (log 16S rRNA gene
457 copies/g FM) in early-weaned pigs receiving sow milk or dry feed

458

459 **Figure 2.** Dendogram illustrating the correlation between experimental diets in t-RFLP
460 banding patterns. The dendogram represents results from 11 piglets sacrificed on day
461 28. There was no digesta content in one of the weaned piglet's caecum. The dendogram
462 distances are in percentage of similarity