Nutritional strategy affects gut wall integrity in weaned piglets

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Nutritional strategy affects gut wall integrity in weaned piglets

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Abstract

Weaning is a stressful event for pigs and induces changes in the gut integrity of pigs. Feed intake is a very important determinant for gut integrity. In this thesis the effect of nutritional strategies (with regard to feed intake level and physical structure of the feed) on changes in gut morphology, barrier function and inflammatory response were investigated. In the experiments individually housed piglets were fed diets at different intake levels with different physical forms and compositions during 4 to 14 days post weaning. The working hypotheses were that high feed intake minimizes the degeneration of the gut integrity and stimulates the regeneration of a compromised integrity.

Weaning induced changes in gut integrity. Overall, feed intake level modulated parameters related to gut integrity like morphology, in vitro barrier function and inflammatory response. The effects found depended on sampling site in the gut and sampling day post weaning.

Only the supply of reconstituted milk resulted in voluntary feed intake level sufficiently high to maintain gut integrity. In vitro paracellular permeability for small molecules was transiently increased after weaning, while permeability for large molecules was decreased. High intake feed (milk) resulted in lower permeability values compared to a low intake level.

Changes in gut morphology and permeability were related to feed intake level. In most experiments, individual feed intake was also correlated with body weight gain and in some experiments with inflammatory response parameters. At a low feed intake level, gut barrier function was already affected at day 1 post weaning, followed by changes in gut morphology.

Variation in parameters related to gut integrity between piglets within trials and between experiments was high making it very difficult to compare data of different experiments.

Voorwoord

Het boekje is af en natuurlijk heb ik niet alles zelf kunnen en hoeven doen. Veel personen hebben ieder op hun eigen manier op bepaalde momenten een bijdrage geleverd.

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Contents

Chapter 1

General introduction

In the Netherlands, piglets are weaned abruptly at an age of 24-28 days. The weaning process is generally regarded as a stressful event (Worsaae & Schmidt, 1980) and involves complex social, environmental and dietary changes that interfere with gut development and adaptation (Pluske et al., 1997; Lallès et al., 2004). Normally, the weaning stress is associated with an immediate but transient drop in feed intake (Le Dividich & Herpin, 1994; Makkink et al., 1994; McCracken et al., 1995; Pluske et al., 1997; Bruininx, 2002; Spreeuwenberg, 2002).

Stress has been reported to affect gut mucosal structure and barrier function ((Perdue, 1999; Fioramonti, 2003). Stress may be an initiating factor for weaning-associated alterations in the stomach and gut resulting in changed gastric motility and emptying, mucus secretion, permeability and water absorption. At least the increase in intestinal permeability involves the hypothalamo-pituitary-adrenal axis and endogenous glucocoticoids. In rats, acute and chronic stressors alone or in combination with starvation have been shown to induce intestinal mucosal injury and increased permeability (Saunders *et al.*, 1994; Spitz *et al.*, 1996; Kiliaan *et al.*, 1998; Bagchi *et al.*, 1999; Wirén *et al.*, 1999; Santos *et al.*, 2000; Saunders *et al.*, 2002).

Recently, review articles have focussed on morphological and functional changes (Pluske, 2001), physiological changes (Lallès *et al.*, 2004), host-microbe interactions (Gaskins, 2003) and on the effects of dietary ingredients on the gut integrity and performance in weaned pigs ((Vente-Spreeuwenberg & Beynen, 2003; Verdonk *et al.*, 2005). Therefore, we will only focus on a few points from these reviews relevant to our study.

Feed intake is an important determinant of performance and gut morphology (Makkink, 1993; Pluske *et al.*, 1997; Spreeuwenberg, 2002) in weaned pigs. The drop in feed intake and the change in composition affect the intestinal micro flora and the small intestinal architecture (Pluske *et al.*, 1997; Konstantinov *et al.*, 2003). Combinations of functional dietary ingredients may support post-weaning feed intake and alleviate or prevent the compromise of the gut integrity caused by abrupt weaning at 24-28 days of age (Spreeuwenberg & Beynen, 2002). The quantity and composition of the digesta in the lumen which becomes available to the micro flora for fermentation will determine the number and type of micro organisms present and also their metabolic activity. The host and his commensal bacteria dialogue, establish and maintain a relationship (Kaiserlian *et al.*, 2005). Intestinal cells can recognize pathogens via so called pathogen associated molecular patterns through the expression of Toll like receptors (TLR). A variety of receptors recognize conserved motifs on pathogens that are

not found in higher eukaryotes. The expression of TLRs is modulated by a variety of factors such as microbial invasion, microbial components and cytokines. Recognition of microbial components by TLRs triggers activation of not only innate immunity but also adaptive immunity. Total parenteral nutrition (TPN) or starvation has been shown to shift the microbial population towards mucolytic bacteria (Deplancke *et al.*, 2002) indicating an increased adherence of Clostridium perfringens in the mucosa of the small intestine.

Intestinal permeability comprises a passage of molecules between epithelial cells (paracellular permeability) and through epithelial cells (transcellular permeability) (Duizer, 1999). Increased transepithelial permeation of macromolecules is found to occur in neonates and might be important for acquiring passive and active immunity. Stress, bacterial micro flora, viruses and epithelial cell damage have been reported to affect gut integrity and intestinal permeability (Santos & Perdue, 2000).

Cellular stressors and environmental insults can induce intestinal epithelial cells to express and produce a large number of chemokines and cytokines but the repertoire of the cells nonetheless is limited. The fact that (human) intestinal epithelial cells do not express mRNA for cytokines regulating antigen-specific immune responses combined with their capacity to secrete multiple cytokines with chemotactic and proinflammatory functions for divers leukocyte populations, suggests that intestinal epithelial cells primarily function as initiators of inflammation and immune response (Eckmann *et al.*, 1993; Jung *et al.*, 1995), rather than in sustaining ongoing antigen-specific adaptive immune response. When stimulated by enteric microbes, intestinal epithelial cells also produce a range of products and inflammatory mediators other than cytokines, such as cyclooxygenase (COX) 2 and nitric oxide (NO). Expression of cyclooxygenase (COX) 2 and increased production of prostaglandin E2 in intestinal epithelial cells leads to increased chloride secretion. Increased production of NO by upregulated expression of inducible nitric oxide synthase (iNOS) has been suggested as a cytoprotective mechanism mediating epithelial innate responses to i.e. minimally and non-invasive pathogens. NO may itself also be a toxic mediator enhancing cell death (Jones & Gores, 1997; Eckmann & Kagnoff, 2005). Most findings implicate pro inflammatory cytokines as agents which reduce barrier integrity but also anti inflammatory actions by cytokines are reported (McKay & Baird, 1999).

So consequently the reaction of the host on stressors like weaning depends on which reaction dominates. Some of the studies in this thesis have focused on that.

Weaning is associated with villous atrophy caused by an increased rate of cell loss and a decreased rate of cell renewal (Pluske *et al.*, 1997). An increased rate of cell loss can be due to apoptosis or programmed cell death. The small intestine is a sensitive organ for the induction of apoptosis following pathophysiological and physiological conditions. Apoptosis of differentiated enterocytes on the villous is rare under normal conditions but increases in response to a number of pathological and stressful conditions including lack of luminal nutrition, ischemia/reperfusion, burn trauma, treatment with anti cancer agents, zinc deficiency, aging (Fukuyama *et al.*, 2001).

The period of immediate anorexia after weaning is followed by a regenerative phase during which feed intake resumes. The regenerative phase is characterized by down regulating of many intestinal disorders most probably stimulated by the increased feed intake.

Studies in weaned piglets have been investigating the changes in the small intestine related to morphology (villous height, crypt depth, brush border bound enzymes), histology (number and type of goblet cells, number and type of immune cells) and micro flora (counts, fermentation products) in relation with feed intake and animal performance. Recently, early signs of local inflammatory response like immune cell infiltration, expression of pro inflammatory cytokines and cytoprotection molecules (McCracken et al., 1995; McCracken et al., 1999; Pié et al., 2004) as well as changes in barrier function like absorption, secretion and permeability (Spreeuwenberg et al., 2001; Boudry et al., 2004) have been reported in response to weaning. Data on the impact of weaning and feed intake on gut inflammatory response and barrier function in pigs however are scarce.

A concept of the weaning process and its consequences is shown in figure 1.

The research described in this thesis was conducted within the framework of a research programme investigating the effect of dietary strategy immediately after weaning on gut integrity. Results on the effect of dietary composition on gut integrity in weaned piglets have been described by Spreeuwenberg (2002). In this thesis the effect of feed intake and dietary physical form in the de- and regenerative phase on the inflammatory response and barrier function and their relationship with morphological indices was assessed. In the experiments several markers for different types of transepithelial transport (*in vitro*) and for inflammatory response were studied. In the experiments we compared animals with high voluntary feed intake (VFI) with pigs fed restricted.

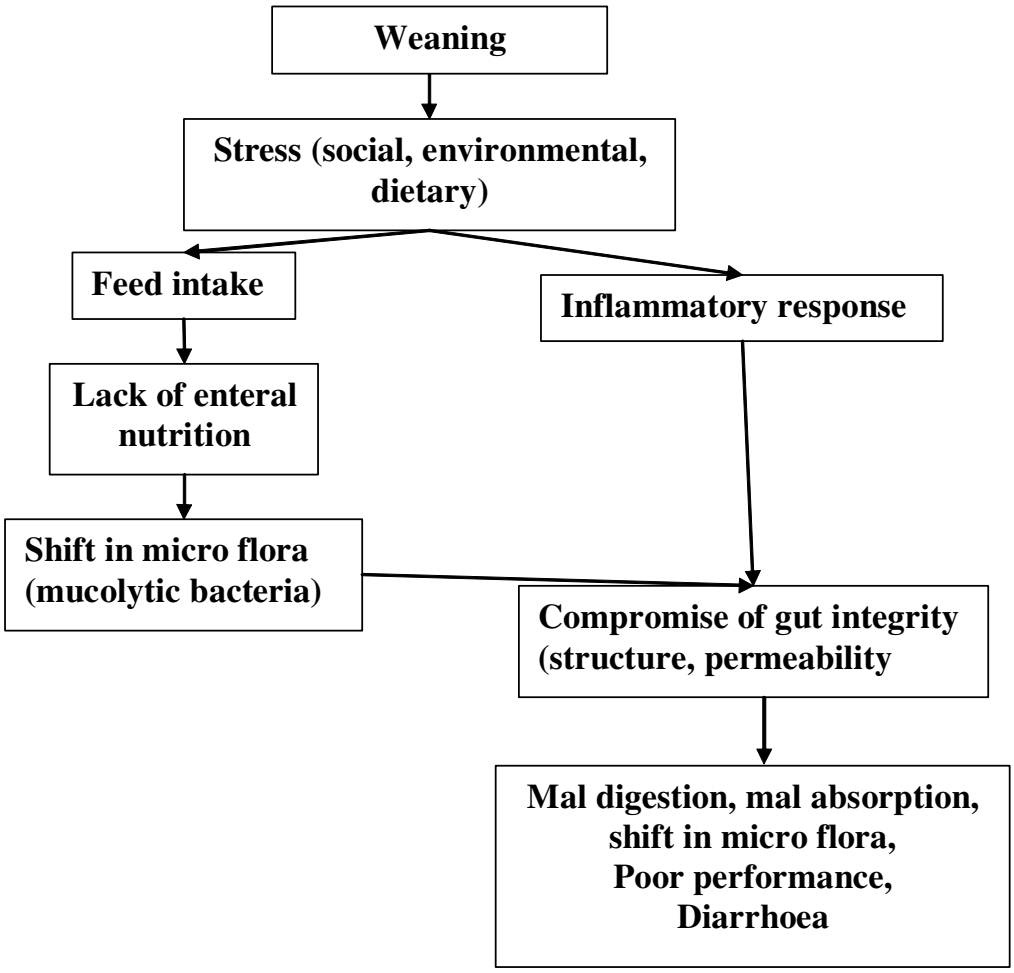

Figure 1. The weaning process and its consequences (adapted from Makkink (1993) and Spreeuwenberg 2002).

High intake of cows or sows milk can maintain the gut morphology post weaning as reported by Pluske *et al.* (1996) and Van Beers-Schreurs *et al.* (1998).

In chapter 2 we describe the effect of intake level of milk on gut integrity parameters like barrier function, inflammatory response and histo-morphological characteristics in pigs during a 4 day period post weaning. We tested the hypothesis that high feed intake in the post-weaning period can maintain gut morphology and that high feed intake is also positive for barrier function.

Milk protein is highly digestible. However, in weaner diets also protein sources with lower digestibility are sometimes being used. We hypothesized that even a small amount of easily

digestible protein will beneficially affect the physico-chemical conditions and micro flora in the lumen and will support mucosal morphology. A study on the effect of protein digestibility and feed intake level (restricted, high) on micro flora (stomach and jejunum) and small intestinal morphology during a 14 day period is described in chapter 3.

We also tested whether or not maintenance of gut morphology and body weight gain is related to sufficiently high feed intake. Chapter 4 describes the effect of feed intake level (restricted, high) during the degenerative phase and feed intake level (restricted, increasing and high) during the regenerative phase on inflammatory response, morphology and digestive capacity of weaned pigs fed a crumbled feed during a 7 day period. We hypothesized that a high intake prevents or limits the deterioration of the gut integrity in the degenerative phase and also supports the recovery of gut integrity during the regenerative phase.

Ad libitum milk intake maintains gut morphology after weaning. In chapter 5 we described the effect of feed intake level of a dry pelleted feed during the degenerative and regenerative phase on morphology, inflammatory response and barrier function using 4 marker molecules.

We tested the hypothesis that feed intake affects gut permeability in such a way that high feed intake prevents increased permeability.

Feeding *ad libitum* milk or liquid diets maintains or supports the gut morphology in weaned pigs. In chapter 6, the effect of physical form of a feed (dry versus slurry) when fed at a low level during the degenerative phase and an increasing level in the regenerative phase on gut barrier function, inflammatory response and morphology is described. We hypothesized that a diet in liquid form supports the gut integrity more than the same diet when fed as dry pellet.

In summary, the scope of this thesis was to:

i) investigate the effect intake feed level and or physical form of the diet on changes in gut integrity during the degenerative and regenerative phase;

ii) investigate the effect of protein digestibility on gut micro flora and morphology;

iii) assess the effect of physical form of the diet across and within experiments on gut integrity;

iv) assess the temporal and spatial sequence of changes in gut characteristics.

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Chapter 2

High level of milk intake maintains the epithelial barrier function in the pig small intestine after weaning*

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Abstract

Compromising alterations in gastrointestinal architecture are common during the weaning transition of pigs. The relation between villous atrophy and changes in epithelial barrier function at weaning is not fully understood. *In vitro* transepithelial transport, local inflammatory responses, and villous architecture in relation to energy intake level were studied. Fourty-eight 26-day-old pigs were weaned and fed a liquid milk replacer diet at a high or low energy intake level. The pigs at the high intake level were fed a liquid milk replacer (dry matter content: 186 g/kg) according to estimated (voluntary) feed intake based on body weight. The pigs at the low intake level were fed one third of this estimated amount of diet by reducing the dry matter content to 62 g/kg. Tissue samples of the small intestine were taken in six animals per intake level at d 0, 1, 2 and 4 post weaning to study intestinal integrity, which was measured by structure (at the proximal, mid and distal site), barrier function (mid site) and inflammatory response (mid site).

High feed intake level maintained integrity of the small intestine compared to that at weaning. Low feed intake however resulted in villous atrophy as well as increased gut wall permeability and inflammatory response. Piglets fed at a low intake level showed higher mRNA expression level of IL-1β and similar number of T cell subsets in mid jejunal tissue compared to that in animals fed at a high feed intake level.

Dry matter intake was related positively with villous height and negatively with paracellular transport.

In conclusion: A high feed intake level maintained integrity of the small intestine but at a low feed intake level villous atrophy, increased permeability and an inflammatory response were found.

Introduction

Weaning is a stressful event for pigs. Under commercial conditions, weaning involves complex social changes as well as changes in diet composition. As a result abrupt weaning is typically accompanied by low feed intake. Weaning also causes morphological and histological changes of the small intestine of pigs (Miller *et al.*, 1986; McCracken *et al.*, 1995; Pluske *et al.*, 1996b; Pluske *et al.*, 1996a; McCracken *et al.*, 1999). These changes include a reduction in villous height and an increased crypt depth. The magnitude of the changes is

related to feed intake of the piglets (Kelly *et al.*, 1991; Nabuurs, 1991; Pluske *et al.*, 1996b), independent of diet composition (McCracken *et al.*, 1995; McCracken *et al.*, 1999).Van Beers-Schreurs *et al.* (1998) found that the weaning transition itself explained part of the reduction in villous height and increased crypt depth. Villous height decreased and crypt depth increased significantly ($P<0.05$), when comparing unweaned piglets with piglets given sow's milk at a high energy-intake at 5 days after weaning. Similar to Pluske *et al.* (1996b) the reduction in villous height was even more pronounced when the piglets received a weanling diet or sow's milk at a low energy-intake ($P<0.05$). Dietary restriction induced lower villous height and lower number of goblet cells, deeper crypts and higher number of intra epithelial leucocytes in the small intestine of nursing pigs Núñez *et al.* (1996). Starvation decreased jejunal villous height and increased paracellular permeability in the ileum and jejunum of adult rats (Wirén *et al.*, 1999). A significant negative relation was found between the ATP levels in jejunal mucosa and the permeability (Yang *et al.*, 1999), indicating that at a low intra cellular energy level the permeability is increased.

Spreeuwenberg *et al.* (2001) investigated the effect of lactose to dairy protein ratio in a liquid milk replacer diet on recovery of structure and integrity of the small intestine of weaned piglets for 1 to 4 days after weaning. They showed that at a low intake level there was a tendency that a diet having high lactose to protein ratio resulted in higher villous length and lower paracellular permeability compared to diets having a low lactose to protein ratio. Inflammatory response parameters were not altered by diet composition and it was suggested that gut integrity was affected mainly by stress and by diminished enteral stimulation. Zijlstra *et al.* (1997) showed that protein-energy malnutrition prolonged diarrhoea and delayed small intestinal recovery from rotaviral enteritis in pigs. McCracken *et al.* (1999) concluded that weaning anorexia may contribute to local inflammation in the piglet small intestine. This inflammation was reflected by altered expression of the matrix metalloproteinase stromelysin and MHC class 1 genes as well as changes in the crypt-villous morphometry. Pié *et al.* (2004) showed an early and transient response in gene expression of inflammatory cytokines in the gut of weaned pigs. The expression of genes encoding for pro-inflammatory cytokines and enzymes related to inflammatory responses in the small intestine of pigs at weaning in combination with feed intake level has not been investigated. The relationship between epithelial barrier function, villous atrophy, mucus secretion and inflammation in the small intestine at weaning is not understood. The hypothesis tested in this experiment was that enteral stimulation after weaning maintains the integrity of the small intestinal epithelium. For

that purpose, piglets were fed at a high and low feed intake level. We investigated mucosal parameters and structure, inflammation and barrier function over time in response to diminished energy intake level. T-lymphocyte cellularity and the mRNA expression of cytokines and cytokine related enzymes were measured as indicator of inflammation. *In vitro* transepithelial permeability was measured in Ussing chamber as a functional indicator of mucosal integrity.

Materials and Methods

Animals and weaning.

Fourty-eight barrows, procured from a commercial farm (Great York × (Dutch Landrace × Finnish Landrace)) were used. The piglets were weaned at 25.9 (SD: 2.0) days of age. Creep feed was not provided during the suckling period. At weaning, pigs were removed from the sow and transported for 10 km to the TNO Nutrition research facility in Wageningen (the Netherlands). Upon arrival at the facility, pigs were weighed and housed individually in 50 × 90 cm^2 floor pens. The walls of the pens were transparent plastic enabling visual contact among the piglets. Each pen was equipped with a plastic trough. Water was supplied via the liquid milk replacer diet. Environmental temperature was kept constant at 24 °C. Lights were on continuously. The experimental protocol was approved by the Animal Care and Ethics Committee of the TNO research institute.

Feeds, feeding, and experimental design.

The experiment was carried out in 2 consecutive batches of 24 piglets each. On the day of weaning, dissection was performed on twelve randomly chosen animals to collect reference values. Additionally, the remaining 36 animals were randomly assigned to 2 groups based upon body weight (BW). The experimental groups were given a milk diet at either high or low energy intake level. The ingredient and nutrient composition of the diet is given in Table 1.

Table 1 Diet composition of milk replacer diet

Item		
Ingredient (g/kg)		
Calcium caseinate	175.0	
Whey protein concentrate[1]	175.0	
Fat filled whey powder [2]	340.0	
Lactose	240.5	
Soy oil	13.0	
Vitamin + Mineral mix[3]	24.0	
Calcium carbonate	13.0	
Potassium bicarbohydrate	6.0	
Potassium phosphate dihydrate	13.5	
Digestible Energy (MJ/kg)	23.6	
Nutrients (g/kg)	calculated	analysed
Dry matter	978	
Crude protein	299	300
Crude fat	300	287
Ash	55	63
Carbohydrates [4]	324	

1 Esprion 580, DMV International, Veghel, The Netherlands. Crude protein, 780 g/kg. Fat, 75 g/kg

2 VanaGrasa 80 C, De Kieviet bv, Meppel, The Netherlands. Butter oil, 800 g/kg

3 Vitamin and mineral inclusion supplies (mg/kg milk replacer): retinol acetate, 6.9; cholicalciferol, 0.1; tocopherol, 50; thiamine, 6; riboflavin, 10; pyrdoxine, 4; cyanocobalamin, 0.25; d-pantothenic acid, 25; niacin amide, 40; l-ascorbic acid, 80; menadione, 4; folic acid, 1; biotin, 0.5; choline chloride, 1000; zinc oxide, 100; potassium iodate, 0.65; disodium selenium oxide pentahydrate, 0.5; copper sulfate pentahydrate, 80; ferrous sulfate heptahydrate, 400; manganous sulfate tetrahydrate, 60; cobalt sulphate heptahydrate, 10; magnesium oxide, 2,500; dicalcium phosphate, 7,500; sodium chloride, 5,000;

4 Carbohydrates = dry matter - crude protein- crude fat - ash - crude fiber (=0)

Piglets fed at the high intake level were offered feed according to formula 1. This formula describes the voluntary DE intake of weaned piglets from 5 to 15 kg based on BW (National Research Council, 1998). Piglets fed at the low intake level were fed one third of the calculated digestible energy (DE). Formula 1 is:

$$DE = ((455.5 \times BW) - (9.46 \times BW^2) - 1531) \times 4{,}184 \quad [1]$$

Where: DE = digestible energy intake (kJ/day); BW = body weight (kg).
The amount of milk replacer offered to the piglets was calculated daily. Body weight at specific days was calculated based on body weight upon arrival and the expected growth of 300 g / day and 60 g / day for the high and low intake group respectively. This was based on findings of Pluske *et al.* (1996b). The milk replacer at the high and low intake level was fed at a concentration of 186 g and 62 g per liter of water respectively. The pigs were fed 4 times a day: at 9.00, 12.30, 17.00, and 21.30 h. Feed refusals were collected, weighed and substracted from the amount of milk offered to calculate actual daily feed intake.

Growth and health.
Animals were weighed upon arrival and on the day of dissection to determine individual growth curves. Faeces were scored twice a day from 0 to 3: 0 = normal faeces, 1 = shapeless loose faeces, 2 = thick, liquid faeces, and 3 = thin, liquid faeces.

Sampling of gut tissue for morphology, permeability and gene expression measurements.
At the day of weaning and at the first, second, and fourth day post weaning, the piglets chosen for dissection were weighed and anaesthetized by inhalation of a mixture of N_2O / O_2 (ratio 2/1) and isoflurane. The concentration of isoflorane was adjusted to the depth of the narcosis (Guedel, stadium III, phase 2). A midline laparotomy was performed. At 3 different segments of the small intestine, tissue samples were taken: 0.5 m distal of the ligament of Treitz (proximal small intestine), 3.5 m distal of the ligament of Treitz (mid small intestine), and 0.5 m proximal to the ileo-caecal ligament (distal small intestine). After sampling, piglets were euthanized by an intra-cardiac injection (2 mL) of T61 (a watery solution containing a combination of embutramide, mebezoniumiodide and tetracainehydrchloride; Hoechst Holland, Amsterdam, the Netherlands).

Reverse Transcription (RT) and Polymerase Chain Reactions (PCR).

The mRNA expression level for interleukin (IL)1_β and tumor necrosis factor alfa (TNF_α) and mRNA expression level for the enzymes inducible nitric oxide synthase (iNOS) and cyclo oxygenase 2 (COX-2) all relative to the level of glyceraldehyde phosphate dehydrogenase (GAPDH) was measured as a marker for inflammation. Tissue samples of the mid jejunum were cut open and rinsed with phosphate buffer solution, deep frozen in liquid nitrogen for approximately 30 min and stored frozen at –80°C until analysis. Total RNA was extracted from samples (±30mg) using a QIA shredder and RNeasy kits (Qiagen, Crawley, UK) according to the manufacturer's protocol and quantified determining the relative optical densities (OD) at 260 nm by spectrofotometry using Spectramax plus . Per sample X µL (750 ng) RNA was used for RT reaction, using 1.5 µL 10 U/µL AMV reverse transcriptase (Promega Corporation, Madison, WI), 2.5 µL 100 µM T_{15} VN-primer, 1.5 µL 10 U/µL recombinant RNasin ® Ribonuclease, 3 µL 10 mM dTNP and 6 µL 25 mM $MgCl_2$ and 6 µL 5x Avian Myeloblastoma Virus (AMV) buffer (Promega) in 9.5-X µL RNase free water. The RT and PCR reactions were performed using a GeneAmp PCR system 9700 (Perkin Elmer, Foster City, CA) to from cDNA at 94°C for 30 sec, 55°C for 1 min, and 72°C for 45 sec. The PCR reactions were performed in a total volume of 25 µL containing 2.5 µL cDNA from the RT reaction, 2.5 µL 25 mM $MgCl_2$, 1.5 µL 10 mM dNTP, 0.25 µL 10 U/µL Taq-polymerase (Promega), 2.5 µL 10 x PCR buffer (Promega) and 0.5 µL 25 µM primer solution.

The PCR sequences for pigs used were:

GAPDH	upper:	5'-AAT-CCC-ATC-ACC-ATC-TTC-CA-3'
	lower:	5'-CCC-TGT-TGC-TGT-AGC-CAA-AT-3'
TNFα	upper:	5'-CCT-CAG-CCT-CTT-CTC-CTT-CC-3'
	lower:	5'-AGG-AGG-TTG-ACC-TTG-GTC-TG-3'
IL-1β	upper:	5'-GGC-CGC-CAA-GAT-ATA-ACT-GA-3'
	lower:	5'-CCC-CAA-AGA-AAT-TGA-CTC-CA-3'
COX-2	upper:	5'-GCA-TGA-GGT-CTT-TGG-TCT-GG-3'
	lower:	5'-GAT-TCC-TAC-CAC-CAG-CAA-CC-3'
iNOS	upper:	5'-CAT-CCT-TCT-GCC-CAC-TTC-C-3'
	lower:	5'-CTT-CGA-AAT-CCC-TCC-TGA-CC-3'.

Before the start of the first cycle all PCR were preceded by a denaturing step of 5 min at 94°C, and the last cycle step was followed by a final elongation step of 10 min at 72°C. The number of cycles was set at 25 to prevent the amount of PCR product from exceeding the

upward slope of a typical cycle-number-PCR product curve. The PCR products were separated on a 1.5% agarose gel. The gels were analyzed using FluorS (Bio-rad Laboratories, Hercules, CA). Data were expressed as the ratio between the mass of the cytokine/enzyme mRNA and the mass of GAPDH mRNA. GAPDH mRNA served as an internal control for the initial amount of RNA.

Transepithelial transport.

Trans and paracllular transport were determined *in vitro* using Ussing chambers. Samples (5 cm) of mid small intestinal tissue were taken, rinsed with an ice-cold buffer solution and cut open longitudinally. The mucosal layer was carefully stripped off the muscle layer. Flat sheets, in which isolated intestinal segments (0.2 cm^2) separate a 1.5 mL mucosal and a 1.5 mL serosal compartment, were placed in the Ussing chambers. Medium containing the radio-labeled GlySar (10 μM) and mannitol (10 μM) were added to the donor compartment (mucosal side). [^{3}H]mannitol and [^{14}C]GlySar radioactivity was determined in the samples and the tissue (at the end of the experiment) by Liquid Scintillation Counting (LSC) using DOT-DPM^{TM} (Digital Overlay Technique using the Spectrum Library and the External Standard Spectrum) for quench correction. Permeability coefficients (P_{app}) were determined based on the appearance of the probe at the serosal side.

Transepithelial transport of 2 compounds was measured in TNO transport chambers: [^{14}C]GlySar (Cambridge Research Biochemicals, Northwich, UK) and [2-^{3}H]mannitol (ICN Biomedicals, Zoetermeer, NL). GlySar is a small hydrophilic compound with a molecular weight of 146 dalton (D). GlySar is transported mainly via a transcellular route with a H^+-coupled di/tri-peptide carrier (Duizer, 1999). Mannitol has a molecular weight of 182 D and is transported mainly via a paracellular route (Duizer, 1999). Samples (5 cm) of mid small intestinal tissue were taken, rinsed with an ice-cold buffer solution of HEPES-buffered phenol red free Dulbecco's Modified Eagles Medium (DMEM) and cut open longitudinally. The tissue was placed with the mucosa on the upper side on a flat underground, and the mucosal layer was carefully stripped off the muscle layer - in order to preserve mucosal integrity - using a blunt razor blade. Samples of the mucosal layer were taken using a nine-mm steel punch. Flat sheets, in which isolated intestinal segments (0.2 cm^2) separate a 1.5 mL mucosal and a 1.5 mL serosal compartment, were placed in the Ussing chambers. The effective exposed area in the Ussing chamber was 0.196 cm^2. The radio-labeled GlySar and mannitol were mixed with unlabelled compounds to yield final concentrations of 10 μM. The donor

compartment (mucosal side) was filled with 1.25 mL HEPES DMEM medium containing radio-labeled GlySar (10 µM) and mannitol (10 µM). The receptor compartment (serosal side) was filled with 1.25 mL HEPES DMEM medium. Both compartments were aerated (O_2 / CO_2, 95 / 5) at a temperature of 37°C and stirred by gas lift. At indicated time points (15, 30, 45, 75, and 105 min), 0.5 mL samples were taken from the serosal side and the volume was reconstituted with DMEM without phenol red. ^{3}H and ^{14}C radioactivity was determined in the samples and the tissue (at the end of the experiment) by Liquid Scintillation Counting (LSC) using DOT-DPM^{TM} (Digital Overlay Technique using the Spectrum Library and the External Standard Spectrum) for quench correction. Apparent permeability coefficients (P_{app}) were determined based on the appearance of the probe at the serosal side according to the following equation:

$$P_{app} = R / (A * C_0) \quad [2]$$

Where: P_{app} = permeability coefficient from mucosal to serosal side (cm/sec); R = permeability rate (mol/sec); A = exposed intestinal area (cm^2); C_0 = initial concentration of test substance (mol/mL) at the mucosal side.

Morphology.

Tissue samples of the proximal, mid, and distal small intestine were taken after the intestine was cut open longitudinally at the anti-mesenteric attachment, prepared on dental wax with the villi on the upper side, and fixed in 0.1 M-phosphate-buffered formalin solution (40 mL/L). A three-mm wide zone from the mesenteric site was cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections were cut (5 µm) and stained with either the periodic acid Schiff method (PAS staining) or a combination of the basophilic dyes high iron diamine (HID staining) and alcian blue (AB staining). From the HE and PAS stained sections, crypt depth (µm), villus height (µm), and the number of goblet cells (per 100 µm crypt) were determined. One slide per piglet was used and the average value taken for a minimum of 5 villi and crypts. From the HE and the HID / AB stained sections, goblet cells of 5 crypts were classified as either sialomucin-containing (blue) or sulfomucin-containing (brown). The percentage of goblet cells containing sulfomucin was calculated.

T lymphocyte subsets.

To measure the number of $CD4^+$ and $CD8^+$ T lymphocytes, mid small intestinal tissues (3 cm) were deep frozen in liquid nitrogen for approximately 30 min, stored frozen at –80°C until cryo-sectioning at 5 μm thickness, and fixed in acetone for 7 min at room temperature (CD or cluster of differentiation molecules are cell surface markers of various leukocyte subsets). Cell labeling was performed by incubating the preparations overnight with murine antibodies directed against either porcine CD4 (clone number MIL-17 # MCA 1749, Serotec, Oxford, UK) or CD8 surface antigens (clone number MIL-12 # MCA 1223, Serotec, Oxford, UK). Subsequently, the samples were incubated with horse anti-mouse antibodies for 30 min followed by Universal peroxidase 3-amino-9-ethyl carbazole (AEC) substrate solution for 25 min. Isotonic phosphate buffer saline (PBS) was used to repeatedly wash the preparations. The tissue sections were counter-stained using hematoxylin, washed with tap water, and mounted. The number of $CD4^+$ and $CD8^+$ cells was determined per μm^2 of the crypts using light microscopy.

Statistical analysis.

A GLM procedure (SAS version 8.1, SAS Institute, Cary NC) was used to estimate the least square means of the treatments. Parameters were evaluated using the following statistical model:

$$y_{ijkl} = \mu + B_i + D_j + L_k + (BxD)_{ij} + (BxL)_{ik} + (DxL)_{jk} + e_{ijkl} \quad [3]$$

Where y_{ijkl} = independent variables; μ = overall mean; B_i = fixed effect of batch (i =1, 2); D_j = fixed effect of day of dissection (j = 1, 2, 3); L_k = fixed effect of energy intake level (i = 1, 2); $(BxD)_{ij}$ = interaction between batch (B) and day of dissection (D); $(BxL)_{ik}$ = interaction between batch (B) and feed intake level (L); $(DxL)_{jk}$ = interaction between day of dissection (D) and feed intake level (L) ; e_{ijkl} = error term.

The combinations of feed intake level and day post weaning were defined as treatments.

Differences between treatments were tested using Students t-test and to compare the value of treatments with the reference value at the day of weaning.

Pearson correlation analysis was performed to evaluate functional correlation between energy intake, morphology parameters and trans-epithelial transport. Significance was assigned at $P<0.05$, tendencies were assigned at $P<0.10$

Results

General.

Body weight at weaning was 7.9 kg (SD: 0.13). Average daily weight gain (g / day/ piglet) through the 4 day treatment period was 150 (SD: 40) for the high intake level and -65 (SD: 30) for the low intake level. Piglets produced normally shaped faeces (score 0). At the low intake level, one animal developed thick liquid faeces (score 2) and two animals had shapeless (loose) faeces (score 1). None of the pigs received medical treatment during the experimental period.

Energy intake.

Average DE intake in kJ per pig at the high intake level was 2801 (SD: 223), 5243 (SD: 405), 5867 (SD: 494) and 6005 (SD: 602) for day 1, 2 and 4 respectively. Average DE intake in kJ per pig at the low intake level was 575 (SD: 223) kJ, 1502 (SD: 405), 1885 (SD: 494) and 1741 kJ (SD: 602) for day 1, 2 and 4, respectively. The feed intake of all animals was lower than the amount offered. The factual feed intake as a percentage of the amount offered was 65% at day 1, 86% at day 2, 93% at day 3, and 89% at day 4 for the piglets fed the high level. The factual feed intake of the piglets fed the low level was 41% at day 1, 88% at day 2, 89% at day 3, and 80% at day 4 respectively. Over time, feed intake increased ($P<0.01$) for pigs fed each intake level.

Permeability of the intestinal mucosa.

Table 2 presents the effect of days post-weaning and feed intake level on trans-epithelial transport of GlySar (transcellular transport, $P_{appGlySar}$) and mannitol (paracellular transport, $P_{appMannitol}$). An interaction between intake level and day post-weaning did not occur for gut barrier properties. The day post-weaning and the feed intake level did not affect transcellular transport. However, the low feed intake level resulted in a significant increase in paracellular transport (12.07) when compared with high intake level (8.18).

A significant positive correlation was observed between paracellular and transcellular transport ($R_{Pearson}=0.43$; $P<0.05$, table 4). Paracellular transport was correlated negatively with villous height ($R_{Pearson}= -0.31$; $P<0.10$), dry matter intake ($R_{Pearson}= -0.46$; $P<0.05$) and the ratio $CD4^+$ / $CD8^+$ T cells ($R_{Pearson}= -0.35$; $P<0.10$).

Both paracellular and transcellular transport were positively correlated with the number of

$CD8^+$ T cells ($R_{Pearson}$=0.57 (P<0.01) and 0.44, (P<0.05), respectively). Transcellular transport was negatively correlated with villous height, villous to crypt ratio and dry matter intake.

Table 2 CD4+ and CD8+ T lymphocytes (10^{-6} μm^2 crypt lamina propria), permeability coefficients (paracellular: Papp Mannitol and transcellular: Papp Glysar) (10^{-6} cm/s) and IL-1β mRNA expression levels in mid jejunal tissue of pigs fed a liquid milk replacer at high or low intake level (L) at 0 to 4d (D) post weaning.

Intake level		High			Low			Statistical effect[1]		
Day post-weaning	0	1	2	4	1	2	4	SEM	L	D
Permeability coefficients										
Papp Mannitol	6.63	6.91	8.98	8.66	10.15	12.06[a]	14.00[a]	0.83	*	ns
Papp GlySar	16.62	10.79[a]	16.40	11.88	14.43	16.37	18.10	1.00	ns	ns
mRNA expression level										
IL-1β/gapdh	nd	4.6	0.6	1.5	11.7	3.0	7.8	1.24	ns	**
T lymphocytes										
CD4+	215	129	150	124	176	166	182	21.7	ns	ns
CD8+	117	150	72	109	122	100	178	20.1	ns	ns
CD4+/CD8+	2.2	1.3	2.7	1.4	1.2	1.8	2.3	0.23	ns	ns

[1] ns = not significant, t = p<0.10; * p< 0.05; ** p<0.01,

[a] values in a row with a superscript differ significantly (P<0.05) from the value at weaning (day 0)

nd = not determined

mRNA expression of IL-1β, TNFα, iNOS and COX-2 in intestinal tissue cells.

Expression levels of mRNA were not detectable for the enzymes iNOS and COX-2 as well as for the proinflammatory cytokine TNF_α. The values for IL-1β mRNA expression level at the mid small intestine for pigs fed the low and high intake level at 1, 2, or 4 days post weaning are shown in Table 2. An interaction between intake level and day post-weaning did not occur. Day post weaning tended (P<0.10) to affect IL-1β mRNA expression. Mean value for IL-1β mRNA expression was highest (8.1) at day 1 post weaning, followed by a sharp decrease at

day 2 (1.8) and increased values at day 4 (4.7) post weaning. Low feed intake level resulted in a significant threefold increase in IL-1β mRNA expression level compared with high feed intake level. At day 1 the low feed intake level tended to result in higher IL-1β mRNA expression level than the high feed intake level. The increase was significant at day 4. After correction for the effect of feed intake, IL-1β levels tended to be negatively correlated with paracellular transport and (log) number of sulfomucin-containing goblet cells ($R_{Pearson}$= -0.35 and -0.27; P<0.10 and P=0.12 respectively).

T-lymphocytes.

The numbers of $CD4^+$ and $CD8^+$ T cells (per 10^6 μm^2 crypt) at the mid small intestine from d 0 to d 4 post weaning are shown in Table 2. The number of $CD4^+$ T cells decreased numerically (not significant) after weaning. The decrease was most pronounced for the high intake level (42%) but it was not significantly different from the decrease at the low intake level (7%). The number of $CD8^+$ T cells was lower (not significant) at the day of weaning (110 x 10^{-6} μm^2) than at days 1 (143 x 10^{-6} μm^2), 2 (115 x 10^{-6} μm^2) and 4 (157 x 10^{-6} μm^2) post weaning. It was negatively related to feed intake ($R_{Pearson}$ = -0.38; P<0.10; Table 4). The mean increase was most pronounced for the low feed intake level (+34%) but this was not significantly different from the high intake level (+17%). The ratio $CD4^+$ cells to $CD8^+$ T cells was reduced directly after weaning, with the lowest ratio on day 1. The ratio was not affected by feed intake level. No significant correlation was found between the number of $CD4^+$ and $CD8^+$ T cells per crypt (Table 4). The number of $CD8^+$ T cells per crypt was negatively related with the villous height ($R_{Pearson}$ = -0.28; P<0.10) and villous to crypt ratio ($R_{Pearson}$ = -0.35; P<0.05. The number of $CD8^+$ T cells was positively related with crypt depth ($R_{Pearson}$ = 0.29; P<0.10).

Crypt goblet cells.

The number of goblet cells per 100 µm crypt was numerically higher for the low intake level (6.5) compared to the high intake level (6.1) but this was not significant (Table 3). The mean number of goblet cells crypt was slightly decreased at day 2 (6.3) and day 4 (6.1) when compared to day 1 (mean = 6.5). The effect of time post weaning on number of goblet cells was only significant ($P<0.05$) at the proximal site of the small intestine.

The percentage of sulfomucin containing cells in intestinal crypts was significantly ($P<0.01$) reduced by time post weaning at the mid small intestine. High feed intake level resulted in a numerically lower percentage sulfomucin-containing goblet cells compared to low feed intake level (not significant).

Villous height, crypt depth and small intestinal weight.

The weight of the small intestine per kg body weight was not affected by intake level. There was a tendency ($P<0.10$) however that time post weaning affected the length of the small intestine per kg body weight. The mean value at day 4 was 14% higher compared to day 1 (data not shown).

Morphology parameters of the small intestine at 0, 1, 2, and 4 days post weaning are shown in Table 3. An interaction between intake level and day post-weaning did not occur for morphology parameters. Feed intake level significantly affected the villous height at the proximal small intestine. The high feed intake level resulted in higher villous length compared to the low intake level. At the high feed intake level villous height was maintained. Mean villous height across the three sites was 96% that of the mean villous height at the day of weaning. Thus was not affected by time post weaning. Low feed intake however decreased villous height. Mean villous height at low intake was 82% that of the mean villous height at weaning. The reduction in villous height for low feed intake level was most pronounced (36%) at the proximal site at day 2 post weaning. Mean villous height at the low feed intake level at day 2 post weaning was significantly ($P<0.05$) lower compared with villous height at weaning. Mean villous height at day 1, 2 and 4 post weaning were 96%, 85% and 87% respectively of the value at weaning (not significantly different).

In contrast to its effect on villous height, feed intake level did not affect crypt depth, whereas time post weaning did (Table 3). Mean values for crypt depth were 91%, 96% and 116% that of the value at weaning for day 1, 2 and 4 post-weaning respectively. The reduction in crypt depth at day 1 was most pronounced for the low intake level and the increase in crypt depth at

day 4 was most pronounced for the high intake level. At the high intake level mean crypt depth at day 4 was significantly increased ($P<0.01$) compared with weaning.

Feed intake level did not affect the villous length to crypt depth ratio. In contrast, day post weaning significantly affected this ratio of the three sampled sites. The values at day 2 (1.9) and day 4 (1.7) were significantly lower compared to day 1 (2.3). At the low intake level the mean ratio across the three sites of the small intestine at day 2 and day 4 post weaning was significantly lower compared to weaning. Villous height was positively correlated with dry matter intake ($R_{Pearson}= 0.38$; $P<0.05$) and tended to be negatively correlated with $CD8^+$ T cells ($R_{Pearson}= -0.28$; $P<0.10$) (Table 4).

Table 3 Villous height (µm), crypt depth (µm), villous height to crypt depth ratio, goblet cells (number/100µm crypt, % sulfomucin containing cells) at the proximal (prox), mid and distal (dist) small intestine of weaned pigs fed a liquid milk replacer at high or low intake level (L) from 0 to 4d (D) post weaning

Intake level (L)	-	High			Low			Statistical effect[1]		
Day post-weaning (D)	0	1	2	4	1	2	4	SEM	L	D
Villous height										
Prox	502	453	469	474	425	322[a]	378[a]	49.7	*	ns
Mid	351	401	387	323	407	281	290	44.7	ns	t
Dist	255	222	226	235	211	200	223	23.0	ns	ns
Crypt depth										
Prox	178	169	188	223[a]	157	183	207	13.9	ns	**
Mid	176	165	158	208[a]	156	168	188	11.4	ns	**
Dist	157	142	136	189	135	148	172	14.0	ns	*
Villous to crypt ratio										
Prox	2.8	2.7	2.6	2.2	2.8	1.8[a]	2.0[a]	0.32	ns	ns
Mid	2.0	2.4	2.5	1.6	2.7[a]	1.7	1.6	0.26	ns	**
Dist	1.7	1.6	1.7	1.3	1.7	1.4	1.4	0.23	ns	ns
Goblet cells:										
Number	6.0	5.6	5.3	4.4[a]	5.8	6.4	5.0	0.45	ns	*
Prox	5.6	5.8	5.6	5.1	5.7	5.9	6.3	0.49	ns	ns
Mid	8.0	8.6	7.2	7.3	7.7	7.4	8.3	0.66	ns	ns
Dist										
% Sulfomucin containing cells										
Prox	29	35	34	35	24	43	46	8.4	ns	ns
Mid	30	11	20	45	19	29	50	11.7	ns	*
Dist	31	24	35	33	24	37	61[a]	12.6	ns	ns

[1] ns = not significant; t = p<0.10; * p< 0.05; ** p<0.01,

[a] Values in a row with a superscript differ significantly (P<0.05) from the value at weaning (day 0)

Table 4 Pearson correlation coefficients between the morphology parameters, T cell subsets, transcellular transport at the mid small intestine and dry matter intake of piglets fed a liquid milk replacer at high or low intake level from 0 to 4 d post weaning.

	Glysar [1]	$CD4^+$ T cells [2]	$CD8^+$ T cells [2]	$CD4^+$ / $CD8^+$ T cell ratio	Villous height [3]	Crypt depth [3]	Villous / crypt ratio	IL1/GAPDH ratio	Dry matter intake [4]		
									Day 1	Day 2	Day 1 to 4
Mannitol [1]	0.43 * [5]	ns	0.57 **	-0.35 t	-0.31 t	ns	ns	ns	-0.52 **	-0.52 **	-0.46 *
Glysar		ns	0.44 *	ns	-0.40 *	ns	-0.36 *	ns	-0.48 *	-0.48 *	-0.48 *
$CD4^+$			ns	0.45 **	ns	ns	ns	ns	ns	ns	ns
$CD8^+$				-0.50 **	-0.28 t	0.29 t	-0.35 *	ns	ns	ns	-0.38 t
$CD4^+$ / $CD8^+$					ns	ns	ns	ns	ns	ns	ns
Villous height						ns	nr[6]	ns	0.42 *	0.42 *	0.38 *
Crypt depth							nr	ns	ns	ns	ns
Villous / Crypt Ratio								ns	0.33 *	0.33 *	ns
Il1/GAPDH									ns	ns	ns

1 10^{-6} cm/s

2 10^{-6} μm^2 crypt

3 µm

4 g / piglet / day

5 P-value of the model: ns = not significant; t = P<0.10; * P< 0.05; ** P<0.01

6 nr = not relevant

Discussion

The present data demonstrate that villous architecture (villous height, crypt depth), gut wall permeability (paracellular transport) and inflammatory response (Il-1_β mRNA expression level) was affected by level of feed intake. The piglets were weaned abruptly at 26 days of age and fed a liquid milk replacer at a high or low intake level. At the high feed intake level, the piglets consumed on average 2801 kJ at day 1 post weaning. This was 65% of the amount offered. At the low feed intake level, the piglets only consumed an average of only 648 kJ during the first day post-weaning. Feed refusals in both groups might be related to the weaning process and for the pigs fed at the low intake level also to the low DM content of the milk, which might have negatively affected the taste. Compared to the pre-weaning milk consumption figures of piglets as reported by Harrell *et al.* (1993) our data on intake suggest that the small intestine was subject to a substantial decrease in enteral stimulation at weaning during 1 (high feed intake level) to 4 days (low feed intake level). The importance of enteral stimulation for mucosal homeostasis is well documented (Kelly *et al.*, 1991; McCracken *et al.*, 1995; Pluske *et al.*, 1997; Park *et al.*, 1998; Gannesunker *et al.*, 1999; McCracken *et al.*, 1999). Weaning can be considered as a stressful event (Funderburke & Seerley, 1990). Chronic or acute stressors alone (Bagchi *et al.*, 1999) or in combination with starvation (Spitz *et al.*, 1996) induce increased intestinal permeability to macromolecules and a decrease in transepithelial resistance (Wirén *et al.*, 1999; Santos *et al.*, 2000). The present data corroborate these results as a significant effect of feeding level was found on paracellular transport (P_{app}Mannitol), demonstrating a temporal relationship between low feed intake, an inflammatory response, increased transepithelial transport and compromised epithelial architecture. A lack of luminal nutrients might lead to mucolytic bacteria affecting epithelial cells. Deplancke *et al.* (2002) showed that the percentage mucus associated bacteria which could grow on mucin was increased and also *Clostridium perfringens* was enriched in the ileum of 7-d-old piglets fed by total parenteral nutrition compared to enteral nutrition. As a result of the attachment and invasion by bacteria, epithelial cells might start the synthesis of pro-inflammatory cytokines (Eckmann *et al.*, 1993; Shirkey *et al.*, 2003; Johnson *et al.*, 2005), which can recruit and activate immune cells to the challenged site and increase paracellular permeability of the gut epithelium. In this experiment, a numeric increase in paracellular transport was noted (not significant) comparing day 0 and 1 especially for the low intake group (53%). The positive relation between paracellular transport and the $CD8^+$ T cell

subset for piglets fed at the low feed intake level suggests the direct consequences of acute inflammation in the increase of paracellular transport.

Alterations in the number of $CD4^+$ and $CD8^+$ T cell lymphocytes cells likely result in a different cytokine repertoire (Winter *et al.*, 1999). Expansion of $CD8^+$ T cells results in the secretion of pro-inflammatory cytokines, which further compromise the epithelial barrier function (Madara, 1989; Taylor *et al.*, 1997). This compromise in epithelial barrier function is correlated with altered expression of tight junction genes (Tavalali *et al.*, 2002).

Our data demonstrate that a high feed intake level maintained villous height post weaning and increased crypt depth from day 2 post-weaning compared to the values at the day of weaning. At the low feed intake level, data of day 4 post-weaning showed the onset of repair for villous height. In contrast, McCracken and co-workers (1995) reported that in pigs weaned at 21 days the lowest villous to crypt ratio was reached at day 5 post- weaning. They compared the sequential effect on the villous to crypt ratio of a liquid milk replacer on days 0, 1, 2, 5, and 7 post weaning. Our data indicate that at a low intake level of milk the paracellular permeability is still increased at 4 days post-weaning compared with the day of weaning. Corrected for the effect of feed intake level, the IL-1β expression level tended to be negatively related with paracellular transport and number of sulfomucin-containing goblet cells ($R_{Pearson}$= -0.35 and -0.27; $P<0.10$ and $P=0.12$ respectively). Pié *et al.* (2004) studied the effect of weaning on the intestinal cytokine expression in pigs. They also found that cytokine expression was regulated during the weaning period according to spatial and temporal patterns. Marked upregulation of IL-1β of mRNA occurred in most parts of the intestine, whereas TNF_α mRNA increased only at specific sites. They concluded that weaning was associated with an early and acute inflammatory response characterized by an early upregulation of TNF_α and IL-1β expression during day 0 and day 2. McCracken *et al.* (1995) found even transiently increased plasma IL-1 levels after weaning.

The enzymes cyclo oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are involved in the synthesis of prostaglandins and nitric oxide respectively. Both enzymes can be upregulated during inflammatory responses by f.e. microbial products, proinflammatory cytokines and during reperfusion ischemia. The mRNA expression of COX-2 and iNOS however was not detected suggesting that (excessive) release of prostaglandins and nitric oxide is not involved in the weaning induced inflammatory response.

From this experiment it can be concluded that feed intake level affects structure, functionality and inflammatory response of the small intestine of weaned pigs. Low feed intake resulted in

a compromised gut integrity related to an IL-1β but not TNFα mediated inflammatory response.

Involvement of (excessive) release of nitric oxide and prostaglandins as a result of increased gene expression of the enzymes iNOS and COX-2 could not be shown in our study.

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Chapter 3

Feed intake level and dietary protein source affect gut integrity of weaned piglets*

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Abstract

An experiment had been designed to study the effect of the digestibility of dietary protein sources in combination with feed intake level on gut micro flora, blood leukocytes and mucosal architecture of the small intestine. It was hypothesised that high feed intake and high protein digestibility prevent gut deterioration and changes in micro flora during day 1 to 4 post weaning and enhances recovery during day 5 to 14 post weaning compared to low intake and low protein digestibility. 156 piglets of 7.2 kg (SD: 0.92) were weaned at 27 d of age (d0) compared to low intake and low protein digestibility. On the day of weaning, piglets were allotted to one of the 2 experimental feeding levels and to one of the experimental diets feeding levels. At d 0, 4, 7, and 14 post weaning piglets were euthanized and samples of digesta and tissue from the gastrointestinal tract were taken for evaluation of the morphology and micro flora. Results showed that the feed intake level affected the morphology of the small intestine as well as the pH and micro flora (especially in the stomach). The dietary protein source affected the number of leukocytes in the blood and the BWG till d7 and d14 post weaning. The dietary protein source did neither affect the pH and micro flora nor the morphology in the small intestine except for the villous height at the mid small intestine.

Introduction

Weaning in pigs is associated with adverse effects on the animal. There is mostly a growth stasis and a compromised integrity of the epithelium of the small intestine. These adverse effects can be both diet-dependent and diet-independent (McCracken *et al.*, 1995). Feed composition together with the digestive capacity of the animal and feed intake level determines the amount and type of nutrients available in the intestine for digestion and further in the gastro intestinal tract for fermentation. In the first days after weaning, there is a large variation in intake between animals (Makkink *et al.*, 1994; Bruininx *et al.*, 2004). McCracken and Kelly (1984) already recognised that low food intake in the period immediately after weaning is responsible for changes in gut structure and function. Kelly *et al.* (1991) found that piglets fed by stomach tube at a low feeding level showed villous atrophy and decreased crypt depth in the small intestine compared to pigs, fed at a high level of food by stomach tube. Pluske *et al.* (1996) found a linear relationship between total dry matter intake and

villous height in the small intestine at day 5 post weaning for piglets fed cows' milk on different levels of energy intake. Failure to maintain an optimal pH in the digestive tract will result into suboptimal digestion and micro flora proliferation. High fermentation activity in the gut lumen may cause an inflammatory response and villous atrophy in the small intestine and hence affect nutrient digestion and absorption. Milk protein is highly digestible and requires less HCl and/or pepsin to be digested compared to non-milk proteins. It is not well established how the protein digestibility affects the micro flora and morphology of the small intestine. In addition it is not known how it affects the leukocyte (the effector cells of the immune system) concentration in blood. The aim of this study was to evaluate the effect of feeding level of dry pelleted feed (restricted or *ad libitum*) and protein digestibility (high and low) on the integrity of the small intestine and the composition of the micro flora in the stomach and jejunum in pigs at weaning.

Material and methods

Animals, Weaning and Housing procedure.

In total 156 newly weaned barrows (age ±27 days, Great York × (Dutch Landrace × Finnish Landrace)) were used. Creep feed was not provided during the suckling period. On the day of weaning piglets were transported for 10 km to the research facility. Piglets were weighed and individually housed in 50*90 cm^2 floor pens with transparent plastic walls. Each pen was equipped with a plastic feeder with water supplied *ad libitum* via a nipple drinker. Temperature was set at 24 °C.

Feeds, Feeding and Experimental design.

Two experimental diets with a crude protein content of 195 g/kg were fed at *ad libitum* and restricted level. Part (80 g/kg) of the dietary protein either originated from highly digestible protein from skimmed milk (SMP) or from poorly digestible protein from hydrolysed feather meal (FM). The protein digestibility of SMP and FM at the terminal ileum was estimated to be 88 % and 65 %, respectively (Centraal Veevoederbureau, 2002). SMP or FM accounted for 41% of the CP in the diet, the other nutrients in both diets being similar (Table 1). The diets were balanced for ileal digestible, indispensable amino acids and lactose content. Before pelleting, the feed was milled at 4 mm. The experiment was carried out in 2 consecutive

batches. On day 0 (= day of weaning), the experiment started with 78 piglets per batch. Gut tissue samples were taken from six animals chosen at random as reference value on the day of weaning. The remaining 72 piglets were weighed and assigned to the four experimental groups based on body weight (BW). Littermates were equally divided across dietary treatments. Two groups of 9 piglets each were fed restricted the SMP diet (Group RSMP) or the FM diet (Group RFM). Two groups of 27 piglets each were fed *ad libitum* the SMP or the FM diet (Group ASMP and Group AFM respectively). Feed intake of each piglet was measured for days 1, 2 and 3. At day 3 post weaning, per batch 9 piglets of each group fed *ad libitum* were selected for their high voluntary feed intake (VFI) since weaning. The mean VFI of the selected piglets on day 3 after weaning was 98 g higher (mean: 267 g/day/piglet) than that of the remaining piglets fed *ad libitum* (169 g/day/piglet). The selected piglets had similar body weights. The remaining 18 piglets from each *ad libitum* fed group were excluded from the experiment. On days 4, 7, and 14, 3 piglets per treatment per batch were dissected and tissue samples of the small intestine were taken for histological analysis. The piglets were weighed upon arrival and on the day of dissection to determine individual growth rates. The restricted feeding level was 40 g per day during the first three days post weaning; from day four onwards the restricted feeding level was 33% that of the mean VFI of the *ad libitum* fed piglets on the previous day. Feed was given four times per day at 8:00 h, 12:00 h, 16:00 h and 20:00 h. At the day of dissection the restricted piglets were offered a meal 1 hour before dissection. Any abnormal faeces consistency and health problems were noted.

Sampling of the gut.

A midline laparotomy was performed under complete anaesthesia by inhalation of a mixture of N_2O / O_2 (ratio 2/1) and isoflurane. The concentration of isoflurane was adjusted to the depth of the anaesthesia (Guedel, stadium III, phase 2).

Digesta was collected from the stomach and from the first two meters of the small intestine. Tissue samples were taken from three jejunal sites: 0.5 m and 3.5 m distal from the ligament of Treitz and 0.5 m proximal to the ileo caecal ligament referred to as prox, mid and dist respectively. Pigs were euthanised by an intra-cardiac injection (2mL) of T61 (a watery solution containing a combination of embutramide, mebezoniumiodide and tetracainehydrochloride; Hoechst Holland N.V., Amsterdam, the Netherlands).

Table 1 Diet composition (as fed basis) of the diets containing skimmed milk powder (SMP) or feather meal (FM) as main protein source.

Item	SMP	FM
Ingredient (g/kg)		
Feather meal	-	100.0
Skimmed milk powder	230.0	-
Maize, pre-gelatinized	332.1	305.5
Barley	350.0	350.0
Lactose	-	117.5
Potato protein	30.0	30.0
Fish meal (70% crude protein)	30.0	30.0
Soybean oil	3.0	9.0
Premix [a]	10.0	10.0
Calcium carbonate	7.5	9.5
Monocalcium phosphate	3.0	14.0
Sodium chloride	2.0	4.0
Potassium carbonate	2.0	10.5
Sodium carbonate	-	2.0
L-lysine. HCl	-	6.5
DL-methionine	0.2	0.2
L-threonine	-	0.4
L-tryptophan	0.2	0.9
Calculated nutrient contents (g/kg)		
DM	892.7 (888)[b]	899.0 (900)
CP	192.4 (193)	199.4 (201)
Ash	42.2	37.9
Fat	32.7	40.0
CF	23.0	22.5
ME pigs (MJ/kg)	14.2	14.0
NE pigs (MJ/kg)	10.0	10.0
Ca	7.4	7.4
P	5.9	6.2
Total essential AA	88.7	88.4
Total non-essential AA	104.3	107.3
Ileal digestible Lys	10.4	10.4
Ileal digestible Met	4.3	2.5
Ileal digestible Met + Cys	6.3	6.5
Ileal digestible Thr	6.5	6.2
Ileal digestible Trp	2.0	2.0
Buffering capacity[c]	4.0	3.5

[a] Lactose based premix supplied (mg/kg dry feed): retinol acetate, 6.9 (i.e. 20000 IU); cholecalciferol, 0.1 (i.e. 4000 IU); tocopherol, 50; thiamin, 6; riboflavin, 10; pyridoxine, 4; cyanocobalamin, 0.25; d-pantothenic acid, 25; niacin amide, 40; l-ascorbic acid, 80; menadione, 4; folic acid, 1; biotin, 0.5; choline chloride, 1000; zinc oxide, 100; potassium iodate, 0.65; di-sodium selenium oxide pentahydrate, 0.5; copper sulfate pentahydrate, 80; ferrous sulfate heptahydrate, 400; manganous sulfate tetrahydrate, 60; cobalt sulfate heptahydrate, 10; magnesium oxide, 1,000

[b] () = analysed contents

[c] [delta pH] per ml 0.1 HCl added to a suspension of 20 g feed in 200 ml demi water.

Microbial composition of the digesta.

Samples of fresh digesta (2 g) of the stomach and from the first two meters of the small intestine were collected and immediately put into pre-weighed bottles with anaerobic transport medium. The digesta was cultured in 10-fold dilution series for quantitative analysis of microbial count. For analysis, 10 µl of each dilution was cultured on Columbia Blood agar (for aerobes and anaerobes; Oxoid, Basingstoke, England), Bacteroides Bile Esculin agar (for bacteroides; (Sutter *et al.*, 1975)), Brucella agar (for Bifidobacteria; (Sutter *et al.*, 1975)), Reinforced Clostridial agar (for clostridia; Oxoid, Basingstoke, England), Levine emb agar (for enterobacteriaceae; Oxoid, Basingstoke, England) Man-Rogoasa-Sharpe agar (for lactobacilli; Oxoid, Basingstoke, England) and Kanamycine Aesculine agar (for Streptococci faecalis; Oxoid, Basingstoke, England). The micro organisms were identified using standard microbiological counting techniques after 48 h incubation under aerobic conditions and after 120 h incubation under anaerobic conditions at 37 °C. Bacterial growth was expressed as ^{10}log number Colony-Forming Units (CFU) / g digesta. If the bacterial count was below the detection level than the detection level was used in the statistical calculations.

Morphology of gut wall.

For macroscopic morphology the tissue samples were cut open longitudinally at the anti mesenteric attachment and fixed on dental wax with the villi on the upper side and fixed in 0.1 M-phosphate-buffered formalin solution (40mL/L). The shape of the villi was studied with a dissection microscope and characterized according to a previously described classification method (Mouwen, 1972) using the following grading system: grade 0, a normal villous pattern with almost all finger shaped, long villi; grade 0.5, mixed finger- and tongue-shaped villi; grade 1, predominantly long to short tongue-shaped villi with few long tongue- and ridge-shaped villi; grade 2, mixture of short tongue-, leaf and ridge-shaped and convoluted villi; grade 2.5, similar to grade 2, but with flat areas; grade 3, flat mucosa.

To measure crypt depth and villous height, a 3mm wide zone from the mesenteric site was cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections were cut (5 µm) and stained with haematoxylin and eosin (HE staining) and the periodic acid Schiff method as previously described (Kik *et al.*, 1990).

Statistical analyses.

A GLM procedure (SAS version 8.1) was used to estimate the least square means of the treatments. Batch, day of dissection, feeding level and dietary protein source with the respective two way interactions were the independent variables of the model to evaluate the parameters on small intestinal morphology, blood leukocytes, microbial composition and the pH of the gastric and jejunal digesta. Parameters were evaluated using the following statistical model:

$$y_{ijklm} = \mu + B_i + D_j + F_k + P_l + (BxD)_{ij} + (BxF)_{ik} + (BxP)_{il} + (DxF)_{jk} + (DxP)_{jl} + (FxP)_{kl} + e_{ijklm} \quad [1]$$

Where y_{ijklm} = dependent variable; μ = overall mean; B_i = fixed effect of batch (i =1, 2); D_j = fixed effect of day of dissection (j = 1, 2, 3); F_k = fixed effect of feeding level (k = 1, 2); P_l = fixed effect of dietary protein source (l = 1, 2); $(BxD)_{ij}$ = interaction between batch (B) and day of dissection (D); $(BxF)_{ik}$ = interaction between batch (B) and feeding level (L); $(BxP)_{ik}$ = interaction between batch (B) and dietary protein source (P); $(DxF)_{jk}$ = interaction between day of dissection (D) and feeding level (L); $(DxP)_{jl}$ = interaction between day of dissection (D) and dietary protein source (P); $(FxP)_{jk}$ = interaction between day of feeding level (F) and dietary protein source (P) ; e_{ijklm} = error term.

Significance was assigned at $P<0.05$, tendencies were assigned at $P<0.10$

Results

General.

Feed intake (FI) from days 0 – 4 and 0 – 7 were not affected by dietary protein source. Average FI of pigs fed restricted was 32 g/d and 62 g/d for day 0 – 4 and 0 – 7 compared to 169 g/d and 226 g/d for the pigs fed *ad libitum*. The body weight gain (BWG) during day 0 – 4 and 0 – 7 was -83 g/d and -24 g/d for the pigs fed restricted and 125 g/d and 149 g/d for the pigs fed *ad libitum*.

Voluntary FI during day 0 – 14 was significantly affected by dietary protein source. FI was 108, 107, 434 and 339 g/d for the RSMP, RFM, ASMP and AFM pigs respectively. There was also a significant interaction between feeding level and dietary protein source for BWG. BWG was 44, 39, 424 and 201 g/d for the RSMP, RFM, ASMP and AFM pigs respectively. Twelve animals showed diarrhoea/thin faeces but medical treatment was not given. Ten out of the twelve animals showing diarrhoea were fed the feather meal diet *ad libitum*.

Morphology.

Piglets fed *ad libitum* had higher villous length and crypt depth at all sites of the small intestine than pigs fed restricted (Table 2). Skim milk protein resulted in higher villous length at all days post weaning compared to feather meal (FM). However, this was only statistically significant ($P<0.05$) at the mid jejunum. The protein source did not affect crypt depth or the ratio villous length to crypt depth. An interaction occurred between dietary protein source and feeding level at the distal jejunum for villous length and at the proximal jejunum for crypt depth resulting in significantly higher villous length and deeper crypts for piglets fed the skimmed milk protein (SMP) diet *ad libitum* (Table 2).

Table 2 Histological characteristics of the small intestine (µm) for piglets at restricted or *ad libitum* feeding level (**F**) receiving diets with skimmed milk powder (SMP) or feather meal (FM) as main dietary protein source (**P**).

Feeding level (**F**)	Restricted		*Ad libitum*		Statistical significance[1]			
Protein source (**P**)	SMP	FM	SMP	FM	SEM	**P**	**F**	**P×F**
Villous height								
Prox	251[a]	241[a]	330[b]	298[b]	15.1	ns	***	ns
Mid	249[a]	242[a]	320[b]	261[a]	14.8	*	**	t
Dist	169[a]	184[a]	266[c]	224[b]	12.5	ns	***	*
Crypt depth								
Prox	188[a]	211[a]	281[c]	245[b]	8.7	ns	***	**
Mid	180[a]	184[a]	254[c]	231[b]	7.0	ns	***	t
Dist	148[a]	160[a]	194[b]	180[b]	6.7	ns	***	t
Villous height to								
Prox	1.37	1.16	1.19	1.23	0.08	ns	ns	ns
Mid	1.43[a]	1.32[ab]	1.25[ab]	1.13[b]	0.08	ns	*	ns
Dist	1.2	1.16	1.38	1.27	0.08	ns	t	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***; P<0.001

[abc] values in the same line without a common character in the superscript differ significantly (P<0.05)

Compared to the day of weaning, villous length was decreased to 62% and 73% at day 4 and 7 post weaning and recovered to 94% of pre-weaning values at day 14 (Table 3). The reduction of the villous length was more pronounced in the proximal jejunum than in the distal jejunum. Crypt depth was increased to 113%, 130% and 139% at day 4, 7 and 14 post-weaning respectively. Villous length to crypt depth ratio was decreased to 56%, 54% and 68% at day 4, 7 and 14 post-weaning respectively.

Table 3 Histological characteristics of the small intestine (µm) for piglets at restricted or *ad libitum* feeding level (**F**) at day 0, 4, 7 and 14 post weaning (**D**).

Feeding level (**F**)		Restricted			*Ad libitum*			Statiscical significance[1]			
Day postweaning (**D**)	0	4	7	14	4	7	14	SEM	**D**	**F**	**D×F**
Villous height											
Prox	449	220^{a}	231^{ab}	287^{c}	260^{abc}	278^{bc}	405^{d}	18.5	***	***	t
Mid	357	192^{a}	227^{a}	318^{b}	233^{a}	234^{a}	406^{c}	18.0	***	**	t
Dist	241	166^{a}	183^{ab}	181^{ab}	193^{ab}	217^{b}	325^{c}	15.3	***	***	***
Crypt depth											
Prox	174	167^{a}	203^{b}	229^{b}	216^{b}	262^{c}	309^{c}	10.5	***	***	ns
Mid	181	167^{a}	192^{ab}	187^{ab}	204^{b}	239^{c}	283^{d}	8.6	***	***	**
Dist	129	153^{a}	159^{a}	150^{a}	173^{ab}	194^{b}	194^{b}	8.2	ns	***	ns
Villous height to crypt											
Prox	2.59	1.37^{b}	1.14^{ab}	1.29^{ab}	1.23^{ab}	1.08^{a}	1.32^{b}	0.09	t	ns	ns
Mid	2.00	1.18^{ab}	1.24^{ab}	1.71^{c}	1.14^{a}	1.00^{a}	1.44^{b}	0.09	***	*	ns
Dist	1.90	1.12^{a}	1.20^{a}	1.21^{a}	1.16^{a}	1.15^{a}	1.67^{b}	0.10	**	t	*

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***:P<0.001

[abc] values in the same line without a common character in the superscript differ significantly (P<0.05)

In contrast to the villous height and crypt depth, the shape of the villi was not affected by protein source in the diet and feeding level (data not shown). However, dissection day post weaning showed significant effects on shape of the villi at the proximal and mid section of the small intestine. The villi were longer and more finger like at day 4 post weaning compared to day 7 and day 14 post weaning (Table 4).

Table 4 Morphological shape of the villi at the proximal (prox), middle (mid) and distal (dist) small intestine of piglets at restricted or *ad libitum* feeding level (**F**) at day 0, 4, 7 and 14 post weaning (**D**). The shape is expressed on a scale of 0 to 3 (0 = normal and finger shaped villi, 3 = flat mucosa).

Feeding level (**F**)		Restricted			*Ad libitum*			Statiscical significance[1]			
Day postweaning (**D**)	0	4	7	14	4	7	14	SEM	**D**	**F**	**D×F**
Site											
Prox	0.43	0.70^{a}	0.93^{abc}	1.04^{c}	0.75^{ab}	0.97^{bc}	0.86^{abc}	0.08	*	ns	ns
Mid	0.63	0.66^{ab}	1.06^{cd}	0.80^{b}	0.55^{a}	0.89^{bc}	1.16^{d}	0.08	***	ns	**
Dist	0.64	0.76	0.80	0.72	0.74	0.75	0.68	0.10	ns	ns	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***; P<0.001

[abcd] values in the same line without a common character in the superscript differ significantly (P<0.05)

Gastric and jejunal micro flora and pH.

Protein source did not affect pH of gastric and jejunal digesta. However, *ad libitum* feeding level resulted in significantly (P<0.05) lower pH values for gastric and jejunal digesta compared to restricted feeding level. There was an interaction (tendency) between protein source and feeding level in the stomach. This resulted in higher pH values in gastric digesta (P=0.07) for skim milk powder compared to feather meal at the *ad libitum* feeding level (Table 5). Compared to the pH at the day of weaning, the pH of gastric digesta was decreased at day 4 and 7 post weaning and increased at day 14 post weaning. The pH in jejunal digesta was lower at day 14 post weaning compared to the pH at the day of weaning (Table 6).

Table 5 Gastric and jejunal pH of digesta for piglets fed a diet with the skimmed milk powder (SMP) or feather meal (FM) diet at restricted or *ad libitum* feeding level (**F**).

Feeding level (**F**)	Restricted		*Ad libitum*		Statistical significance[1]			
Protein source (**P**)	SMP	FM	SMP	FM	SEM	**P**	**F**	**P×F**
Site								
Stomach	3.7[a]	4.0[a]	3.6[ab]	2.9[b]	0.24	ns	*	t
Jejunum	6.5[a]	6.4[ab]	6.0[b]	6.0[b]	0.16	ns	*	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***; P<0.001

[ab] values in the same line without a common character in the superscript differ significantly (P<0.05)

Table 6 Gastric and jejunal pH of digesta for piglets at day 0, 4, 7 and 14 post weaning (**D**).

	Day of dissection post weaning				Statistical significance[1]	
	0	4	7	14	SEM	**D**
Site						
Stomach	3.7	3.1[a]	3.5[ab]	4.1[b]	0.21	*
Jejunum	6.3	6.3[ab]	6.4[a]	6.0[b]	0.14	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***; P<0.001

[ab] values in the same line without a common character in the superscript differ significantly (P<0.05)

The *ad libitum* intake level resulted in significantly higher counts for most of the investigated microbial species in gastric digesta and tended to result in higher number for Bifido and Clostridia in jejunal digesta compared to the restricted intake level (Tables 7 and 8). In contrast, the *ad libitum* intake level resulted in lower numbers of Enterobacteriaceae in gastric digesta (P<0.01) and Streptococci (P<0.10) in jejunal digesta compared to the restricted intake level. The FM diet tended to result in higher number for Enterobacteriaceae (in gastric digesta) compared to the SMP diet (Table 7).

Table 7 The bacterial count (^{10}log (colony forming units/g digesta)) in the stomach of pigs fed at restricted (R) or *ad libitum* (A) feed intake level (**F**) a diet (**P**) based on skimmed milk powder (SMP) or feather meal (FM) from 0 – 14 days post weaning (**D**)

	Aerobes	Anaerobes	Bifidobacteria	Clostridia	Entero	Lactobacilli	Streptococci
Weaned	5.5	6.7	6.1	6.2	3.5	6.4	4.1
R	6.0	6.2	5.8	6.0	4.3	6.0	3.9
A	6.5	7.0	6.8	6.7	3.3	6.9	3.7
SMP	6.3	6.7	6.5	6.5	3.6	6.6	3.7
FM	6.2	6.6	6.1	6.2	4.0	6.3	3.9
Day 4	5.9	6.1	5.9	5.8	3.3	5.9	3.6
Day 7	6.5	7.0	6.7	6.5	4.1	6.8	3.9
Day 14	6.5	6.8	6.3	6.7	4.1	6.6	4.0
Statistical							
F	*	**	**	*	**	**	ns
P	ns	ns	ns	ns	t	ns	ns
D	t	**	t	*	*	*	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01

There were no significant differences between the two diets for the other bacteria measured. The diet did not affect the number of the bacteria in the jejunum (Table 8). The number of bacteria in jejunal digesta was smaller than in gastric digesta for all species except for Enterobacteriaceae.

Table 8 The bacterial count (^{10}log (colony forming units/g digesta)) in the first 2 meters of the small intestine of pigs at restricted (R) or *ad libitum* (A) feeding level (**F**) a diet (**P**) based on skimmed milk powder (SMP) or feather meal (FM) from 0 – 14 days post weaning (**D**).

	Aerobes	Anaerobes	Bifidobacteria	Clostridia	Entero	Lactobacilli	Streptococci
Weaned	4.6	6.1	5.7	5.7	3.2	6.3	3.6
Feeding level							
R	5.2	5.6	5.0	5.0	4.3	5.1	3.8
A	5.0	5.5	5.4	5.4	3.9	5.4	3.4
SEM	0.2	0.1	0.2	0.2	0.2	0.2	0.2
Diet							
SMP	5.2	5.5	5.1	5.0	4.0	5.3	3.6
FM	5.0	5.7	5.2	5.3	4.2	5.3	3.6
SEM	0.2	0.1	0.2	0.2	0.2	0.2	0.2
Day post							
4	5.2	5.7	5.2	5.1	4.1	5.2	3.5
7	5.2	5.6	5.5	5.6	4.3	5.5	3.7
14	4.9	5.4	4.8	4.8	3.9	5.0	3.6
SEM	0.2	0.2	0.2	0.2	0.3	0.2	0.2
P-value[1]							
F	ns	ns	t	t	ns	ns	t
P	ns	ns	ns	ns	ns	ns	ns
D	ns	ns	t	*	ns	ns	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05

Blood leukocytes.

Overall, SMP showed higher average number of blood leukocytes than FM but this difference already existed at the day of weaning. The SMP diet resulted in a higher percentage of lymphocytes (P<0.05) and basophils (P<0.10) and higher percentage of segmented cells (P<0.10) in the blood compared to FM (Table 9). Feed intake level did not affect the number of blood leukocytes. The number of leukocytes was significantly affected by the day post weaning.

Table 9 Total number (#, 10^9/L) and percentage (%) of leukocytes (SEM) in blood of piglets fed a diet (**P**) containing skimmed milk powder (SMP) or feather meal (FM) at restricted or ad libitum feeding level (**F**).

Feeding level (**F**)	Restricted		Ad libitum		Statistical		
Protein source (**P**)	SMP	FM	SMP	FM	SEM	**P**	**F**
Parameter							
Leukocytes (#)	14.3[a]	17.9[bc]	15.8[ab]	18.4[c]	0.82	***	ns
Lymphocytes (%)	51.5[ab]	45.0[a]	53.5[b]	49.3[ab]	2.45	*	ns
Segmented (%)	42.6[ab]	49.3[b]	41.9[a]	44.8[b]	2.44	t	ns
Bar (%)	1.4	1.1	1.1	1.6	0.30	ns	ns
Eosinophils (%)	1.5	1.5	1.0	1.4	0.24	ns	ns
Basophils (%)	0.6	0.5	0.6	0.2	0.16	t	ns
Monocytes (%)	2.4	2.6	1.9	2.7	0.35	ns	ns

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***: P<0.001

[abc] values in the same line without a common character in the superscript differ significantly (P<0.05)

At day 14 post weaning the number of leukocytes was increased significantly (Table 10) compared to the weaning values. The percentage of lymphocytes was transiently increased at day 4 post weaning but decreased to the weaning level at day 7 and 14. The percentage of segmented cells was decreased at day 4, 7 and 14 post weaning compared to the day of weaning. The percentage of eosinophils (d4, d7, d14) and monocytes (d14) was significantly increased post weaning.

Table 10 Total number (#, 10^9/L) and percentage (%) of leukocytes (SEM) in blood for piglets at different days post weaning (**D**).

	Day of dissection post weaning				Statistical	
	0	4	7	14	SEM	**D**[1]
Parameter						
Leukocytes (#)	14.9[a]	14.7[a]	16.5[a]	20.4[b]	0.82	***
Lymphocytes (%)	43.5[a]	57.7[b]	49.4[a]	48.7[a]	2.45	**
Segmented (%)	52.9[a]	36.9[c]	44.4[b]	44.5[b]	2.44	***
Bar (%)	1.8	0.7	1.5	1.2	0.30	t
Eosinophils (%)	0.0[a]	2.1[b]	1.6[b]	1.7[b]	0.24	***
Basophils (%)	0.1	0.6	0.6	0.6	0.16	ns
Monocytes (%)	1.7[a]	2.0[a]	2.5[ab]	3.4[b]	0.35	**

[1] ns = not significant; t: 0.05<P<0.10; *: P<0.05; **: P<0.01; ***: P<0.001

[abc] values in the same line without a common character in the superscript differ significantly (P<0.05)

Discussion

The aim of this study was to evaluate the effect of the digestibility of dietary protein sources (high and low) in combination with feed intake level (restricted or *ad libitum*) on the composition of the micro flora in the stomach and jejunum, mucosal architecture of the small intestine and blood leukocytes.

In the present study, *ad libitum* feed intake resulted in significant ($P<0.01$) higher villous length and crypt depth than restricted feed intake at all three sites in the jejunum. *Ad libitum* feed intake resulted in low ratio of villous length to crypt depth at the mid jejunum. This result corroborates with earlier results of Makkink *et al.* (1994), McCracken *et al.* (1995), Pluske *et al.* (1996), Van Beers-Schreurs *et al.* (1998) and McCracken *et al.*(1999). They all found a relationship between voluntary feed intake and mucosal architecture in weaned piglets.

Núñez *et al.* (1996) found higher villous length but shorter crypt dept for malnourished piglets at day 35 post weaning. In our trial *ad libitum* feed intake for the SMP group and the FM group was similar at day 4 and day 7 but was higher for the SMP group compared to the FM group at day 14 post weaning. Therefore differences in results can not be fully attributed to the protein source.

The low voluntary feed intake of piglets fed a commercial weaner diet compared to the group fed *ad libitum* sow milk Van Beers-Schreurs (1998) were in line with these results. Piglets fed the weaner diet had lower villous length but deeper crypts in contrast to our findings.

Stereomicroscopic observations of the tissue samples did not show any difference in shape and length of the villi between the protein sources or the feeding levels. At day 4 post weaning the villi at the proximal and mid jejunum were longer and shape than at day 7 and day 14 post weaning. Cera *et al.* (1988) found a decrease in villous height at day 3 post weaning but no denuded villous tips or signs of villous atrophy were seen at photographs. At day 14 post weaning the lengthened villi were also not finger-like but more longitudinally flattened and tongue-shaped. This technique of characterizing the shape of villi might be less sensitive and more qualitatively than the staining technique where length of villous and crypt is measured.

We found decreased villous length and increased crypt depth for the *ad libitum* feed intake. Pluske *et al.* (1997) indicated that villous atrophy after weaning is caused by either an

increased rate of cell loss or a reduced rate of cell renewal. If villous shortening occurs via an increased rate of cell loss, then it is associated with increased crypt-cell production and hence with increased crypt depth (e.g. microbial challenge, antigenic components of feed stuffs) in the time there after. Villous atrophy might also be due to decreased rate of cell renewal that is the result of reduced cell division in the crypts (e.g. fasting).

The feeding level influenced the pH in the stomach and jejunum. The feeding level also affected the number of bacteria especially in the stomach. In the stomach at the high feeding level the amount of aerobic bacteria, anaerobic bacteria, Bifidobacteria, Clostridia and Enterobacteriaceae were significantly increased and the number of Enterobacteriaceae was significantly decreased. In the jejunum, only the number of Bifidobacteria and Clostridia tended to be increased, while the number of Streptococci tended to be decreased.

Milk protein resulted in higher villous length compared with feather meal protein. This was significant ($P<0.05$) in the mid jejunum. Milk protein at the *ad libitum* feed intake level resulted in higher villous length and crypt depth compared to the feather meal protein. Li *et al.* (1991) also found superior gut structure and weight gains for milk protein based diets compared with several soy based type diets. In contrast, Makkink *et al.* (1994), Van Beers-Schreurs (1996), McCracken *et al.* (1999) and Spreeuwenberg (2002) did not find an effect of diet (protein) composition on mucosal architecture. The difference in villous height between proteins sources in our study was strangled with a difference in cumulative voluntary feed intake between pigs fed the SMP and FM diet *ad libitum* as revealed by analysis of variance using actual feed intake as co-variable.

Moughan *et al.* (1991) found no significant effect of protein source in milk formulas on gastric pH when fed to pigs of 37 days old. Wilson and Leibholz (1981) concluded that neither protein source nor the age of pigs (14d – 35d) influenced gastric pH (mean value 4.05). The pH values found in our experiment were similar to the values reported by them.

Supplementation of a corn-soy bean meal based diet with fumaric or citric acid did not affect gastric and jejunal pH and gastric Cl^- concentration (Risley *et al.*, 1992). They reported a significant decreased gastric pH after weaning whereas we found a transiently decreased pH followed by an increased gastric pH compared to the weaning value. Risley *et al.* (1992) also reported no effect of post-weaning age on pH in jejunum contents.

The dietary protein source did not affect the microbial counts in gastric and jejunal digesta except for the number of gastric Enterobacteriaceae. The number of leukocytes in blood was

significantly affected by the protein source. The number was lower for the SMP diet compared to the FM diet. Namkung *et al.* (2004) also reported no effect of supplementation of corn-soy diets with either antibiotic, herbal extract or blends of organic acid on white blood cell profile in pigs 14d after weaning. In contrast, Kreukniet *et al.* (1990) and Mroz *et al.* (2003) reported that climatic treatments (cold, draught, fluctuating temperatures) and oral challenge with pathogenic E. coli respectively significantly affected the number of blood leukocytes in weaned pigs.

In our study, the pigs fed ad libitum the FM diet more frequently showed diarrhoea than pigs fed the SMP diet. Most probably this has been caused by protein fermentation by the high amount of undigested protein and limited amount of easily fermentable dietary fibre (Jensen, 2001). Dong *et al.* (1996) also showed that an excess of protein reaching the large intestine led to increased diarrhoea in early weaned pigs. Smulders *et al.* (2003) reported negative effects of higher levels of indigestible protein in diets with equal levels of digestible amino acids on BWG and feed conversion ratio when fed to broilers.

The time post weaning significantly affected villous length and crypt depth. Villous length was reduced at day four post weaning with 37% and day seven post-weaning (PW) with 32% compared to the villous length at the day of weaning. Villous length at day 14 PW was increased back to 94% of the length at the day of weaning. Crypt depth was increased at day four, seven and 14 PW with 13%, 30% and 39% respectively compared to the crypt depth at the day of weaning.

Jensen (1998) reported pronounced microbial fermentation in the stomach and small intestine of pigs. In six-weeks-old pigs equal amounts of organic acids were produced in the stomach, small intestine and large intestine. Also Cranwell *et al.* (1976) reported high concentration of lactic acid in the stomach of suckling piglets indicating that a substantial part of the lactose in sow's milk underwent bacterial fermentation in the stomach of suckling pigs. The microbiota in the gastrointestinal tract is unstable during the first week post weaning and it takes 2 to 3 weeks after weaning before the fermentative capacity of the microbiota in the hind gut has developed. Sutton and Patterson (1996) and Jensen (1998) reported a reduction in the numbers of lactobacilli, which are often predominant in the stomach and small intestine of young pigs. In our study we also found a reduction of lactobacilli although this reduction was smaller. This was may be caused by the lower number of lactobacilli at the day of weaning in our study compared to their studies.

Jensen (1998) reported pH values in the stomach ranging from 2.5 to 3 in unweaned pigs and 3.5 in pigs one week after weaning. Jensen (1998) reported pH values in jejunum of 6.1 to 6.3.

In conclusion, the present study showed that feed intake level affected pH and microbial counts in the stomach and jejunum as well as the villous architecture in the small intestine. The dietary protein source did neither affect the pH and microbial counts nor the villous architecture in the stomach and small intestine. However, dietary protein source did affect the BWG, the immune response and voluntary feed intake (at d14). Piglets fed on the restricted level had more compromised epithelium than piglets which had high intake (ad libitum intake level). Based on these findings it was concluded that too low level of enteral stimulation by nutrients was the cause of this compromised gut epithelium. Morphology of the small intestine was already compromised at day 4 post weaning independent of the dietary protein digestibility.

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Chapter 4

Feed intake level modulates gut integrity but not the digestive capacity in weaned piglets*

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Abstract

Weaning of pigs induces changes in the mucosal integrity of the small intestine. The present study was designed to study the modulatory effect of different post weaning feed intake levels on the compromise and recovery of the mucosal integrity of the small intestine for one week after weaning. Parameters which represent structure and functionality of the intestinal mucosa were measured. It was hypothesised that high feed intake prevents gut deterioration during day 1 to 3 post weaning and enhances recovery during day 3 to 7 post weaning. 72 piglets were weaned at 26 d of age (d0) with body weight 8.4 kg (SD: 0.82). At weaning piglets were randomly allotted to one of the 3 experimental feeding levels and to one of the 3 days of dissection. Piglets were euthanized and samples of gut tissue and digesta were taken at d 0, 3, or 7 post weaning. Results showed that the feed intake level affected the villous architecture and weight of the small intestine. At d3 villous height and crypt depth were affected by feeding strategy. At d7 post weaning, differences in villous height between the 3 feed intake strategies (low, increasing, high) were not significant. The specific activities of amino peptidase and sucrase-isomaltase in the brush border and the mRNA gene expression level of interleukin (IL) 1□ and IL-8 in the epithelium were not affected by feed intake level. It was concluded that a feed intake level lower than maintenance level negatively affects gut integrity at d 3 post weaning. High intake of dry feed could not prevent the changes in the gut wall induced by weaning but resulted in less compromised gut architecture. We hypothesized that high feed intake attenuates the changes in gut integrity and stimulates the intestinal repair in newly weaned piglets compared to low feed intake level. Feed intake levels between 1 x (low) and 2.5 x (high) maintenance requirement for energy resulted in similar gut integrity at d7 post weaning. Furthermore, weaning induced changes in villous architecture start proximal and spread distal.

Introduction

Weaning is inevitably a stress for piglets. This is manifested particularly during the first few days post weaning in terms of low feed intake, independently of dietary composition (1995). A very low feed intake after weaning results in a insufficient supply of nutrients to the small intestine. This lack of nutrients leads to an increased number of mucolytic bacteria like *Clostridium perfringens* associated to the mucosa (Deplancke *et al.*, 2002). As a result of this epithelial cells will react by producing pro inflammatory cytokines (Eckmann & Kagnoff, 2001; Johnson *et al.*, 2005), which recruit and activate immune cells. Subsequently this may give early gut functional disorders (villous atrophy associated usually with diminished brush border enzyme activity, increased paracellular permeability and an inflammatory response (Kelly *et al.*, 1991; McCracken *et al.*, 1995; McCracken *et al.*, 1999; Spreeuwenberg *et al.*, 2001).

The magnitude of the changes seems to be related to the level of feed intake of the piglets (Kelly *et al.*, 1991; Núñez *et al.*, 1996; Pluske *et al.*, 1996; Van Beers-Schreurs *et al.*, 1998; Verdonk *et al.*, 2001), independent of diet composition (Makkink *et al.*, 1994; McCracken *et al.*, 1995; McCracken *et al.*, 1999). Similar to Pluske et al. (1996) and Van Beers-Schreurs et al. (1998) we showed that a high intake of milk immediately after weaning prevented the changes in villous architecture (Verdonk *et al.*, 2001). On the other hand a low intake of milk after weaning resulted in decreased villous height and increased paracellular permeability of the gut wall to small hydrophilic molecules. It also increased the mRNA expression of the proinflammatory cytokine interleukin (IL) 1β (Verdonk *et al.*, 2002b).

Inflammatory responses can be initiated by the release of IL-1β, which is a proinflammatory cytokine and IL-8 which is a chemokine (Eckmann & Kagnoff, 2001). IL-8 is a potent neutrophil attractant and is expressed and secreted by intestinal epithelial cells. Upregulation of IL-8 expression can be done by cytokines, such as IL-1β. Thus it seems that absence of sufficient nutrients for the gut mucosa is the reason for a compromised integrity of the gut.

In the present experiment it was tested whether high intake (2.5 x maintenance requirement for energy (NE_m)) of a non milk diet could prevent the onset of gut wall damage and stimulate the repair of a compromised integrity of the small intestine. Parameters related to gut integrity such as morphology, enzyme activity and the gene expression of proinflammatory cytokines were evaluated

Materials and Methods

An experiment with weaned piglets [(Duroc × Yorkshire synthetic) × (Yorkshire × Dutch landrace)] was performed, in two consecutive batches at the Swine Research Centre (SRC) of Nutreco (Boxmeer, The Netherlands). Piglets did not have access to creep feed during the suckling period to prevent adaptation to solid feed.

Piglets and weaning.

Barrows (n=72) were weaned at d 26 (SD: 1.4) of age and 8.4 kg body weight (BW) (SD: 0.82). At weaning (d0), piglets were removed from the sow, weighed and housed individually in 77×76×69 cm^3 pens. Each pen was equipped with a through and a drinking nipple. Environmental temperature was maintained at 27 °C. Lights were on from 06:00 h till 22:00 h. The experimental protocol was approved by the Animal Care and Ethics Committee of the University of Nijmegen (the Netherlands).

Feeds, feeding and experimental design.

On the day before weaning, piglets were weighed and randomly allotted to one of 6 experimental groups. Littermates were divided across experimental groups. The groups differed in feed intake level and / or day of dissection. The experimental diet was based on corn, corn starch (pre-gelatinised), barley, soya flour and wheat gluten (Table 1) and was fed as crumbs. The analysed nutrient content of the diet was according to expectation and therefore not shown. On the day of weaning (d 0), dissection was performed on a group of 8 piglets to collect reference values. The remaining 5 experimental groups were dissected at d 3 or 7 post weaning. 40 piglets were provided a high feeding level (2.5 x estimated maintenance (NE_m) requirement for energy) from d 0 onwards. At d 3, 16 animals with high voluntary feed intake and average BW were selected out of this group of 40 piglets. 8 piglets with high voluntary feed intake were dissected at d 3, the other 8 piglets at d 7. The remaining 24 piglets were excluded from the experiment. From d 0 to d 3, 24 piglets were fed restricted (0.25 to 1.0 NE_m respectively). At d 3, 8 piglets were dissected and the remaining 16 piglets were divided into two groups and fed at a low level (1.0 x NE_m) or at increasing level (1.5 x NE_m at

d 4 to 2.5 x NE_m at d 6). The feeding level of the low intake group (L) was 1.0 x NE_m from d3 onwards. Piglets were fed 4 times per day: 09:00, 11:30, 14:00, 17:00 h. Feed refusals were collected, weighed and subtracted from the amount of feed offered to calculate actual daily feed intake.

Technical results and health.

Piglets were weighed at d –1, 0, 3 and d 7 post weaning. Average daily gain (ADG) was calculated for the periods 0 to 3, 3 to 7 and 0 to 7. Faecal consistency was monitored twice a day and quantified using a scale ranging from 0 to 3 with 0 = normally shaped faeces, 1 = shapeless (loose) faeces, 2 = thick liquid (soft) faeces, and 3 = thin liquid faeces.

Sampling of small intestine.

At d 0, 3 and 7 postweaning, piglets to be sampled were weighed and euthanised with a 5 mL intra-cardiac injection of Euthestate® (pentobarbital sodium 200 mg/mL; Ceva Sante Animale B.V. Maasluis, The Netherlands). A midline laparotomy was performed. At two different segments of the small intestine, tissue samples were taken at the ligament of Treitz (proximal small intestine) and 3 m distal of the ligament of Treitz (mid small intestine).

Morphology.

For analysis of morphology parameters, tissue samples (2 cm) of the proximal and mid small intestine were cut open longitudinally at the anti-mesenteric attachment, prepared on dental wax with the villi on the upper side and fixed in 0.1 mol/L phosphate buffered formalin solution (40 mL/L). A 3-mm wide zone from the mesenteric site was cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections (5 µm) were cut and stained with the periodic acid / Schiff procedure (PA/S). From the stained sections, crypt depth (µm) and villous height (µm) were determined.

Table 1 Ingredient composition (g/kg) of the diet

Corn	250
Barley	150
Wheat gluten (CP= 81.5)	100
Soya flour (CP= 51.2)	160
Lactose	50
Pre-gelatinised corn starch	192.9
Limestone	10.8
Mono calcium phosphate	16.3
Salt	9.4
Soya oil	43.0
Choline-chloride 50 %	2.8
Vitamin and mineral mix [1]	2.0
Titanium oxyde	5.0
Lysine	5.6
Threonine	2.2
Tryptophan	0.1
Chemical composition [2]	
Moisture	88
Crude protein	208
Crude fat	64
Crude fibre	24
Ash	54
Starch	426
Sugar	22
Nett Energy (kcal)	2550
Ileal digestible amino acids:	
- Lys	11
- Met	2.8
- Met + cys	6.2
- Thr	7.6
- Try	2.0
- Glu + gln	45

[1] Vitamin and mineral inclusion supplies ($mg \cdot kg^{-1}$ feed): Vitamin A, 10000 IE; Vitamin D3, 2000 IE; Vitamin E, 65000 IE; Vitamin K3, 2.0 mg; Vitamin B1, 1.0 mg; Vitamin B2, 3.0 mg; Panthoteic acid, 10.0 mg; Niacin, 20.0 mg; Biotin, 30 mcg; Vitamin B12, 20 mcg; Folic acid, 0.2 mg; Vitamin B6, 4.0 mg; Fe, 160.0 mg; Cu, 160.0 mg; Zn, 100.0 mg; Mn, 30.0 mg; I, 10.0 mg; Se, 0.2 mg; Antioxidants (E130, E320, E321), 60.0 mg

[2] Vitamins and minerals: Ca, 7.5 g/kg; P, 6.1 g/kg; Na, 3.7 g/kg; K, 5.3 g/kg; Cl, 6.4 g/kg; Cu, 162 mg/kg; Fe, 241 mg/kg; Zn, 117 mg/kg; Mn, 39 mg/kg; Vit E, 40 IE.

Specific brush border enzyme activity.

To measure the specific enzyme activity of isomaltase-sucrase and aminopeptidase in the brush border of the small intestine, a proximal small intestinal tissue sample (15 cm) was taken and rinsed with 50 ml 0.01 M phosphate-buffered saline at 4^0C (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.9% (w/v) NaCl). The tissue was placed on a flat surface with the mucosa facing upwards. The mucosal layer was carefully stripped off the muscle layer and deep frozen in liquid nitrogen, stored frozen at –80 °C until analysis. The enzyme activity was measured as described by (Pusztai *et al.*, 1996). The mucosal layer was homogenised in ice-cold twice distilled water using a Virtis blender (The Virtis Company, Gardiner, NY, USA) at full speed for 10 min at 0 °C to give a final concentration of 5 % (v/w). Subsequently, the homogenates were sonicated twice at 0 °C for 15 sec, separated by a 30 sec interval, at an amplitude of 24 µm with an MSE Soniprep 150 (Beun de Ronde B.V., Abcoude, The Netherlands). The protein content of the resulting sonicates was determined (Smith *et al.*, 1985), adjusted to approximately 350 µg protein mL and used to measure enzyme activities. The activities were tested under conditions of linearity with respect to the enzyme concentration and incubation time. The analyses were done in triplicate on each intestinal segment per piglet. The activity of sucrase-isomaltase (EC 3.2.1.48) was measured with saccharose (Messer & Dahlqvist, 1966) as substrate (1 unit = 1 µM disaccharide hydrolysed min^{-1}), and the activity of aminopeptidase (EC 3.4.11.2) using L-alanine-p-nitroanilide (Marouz *et al.*, 1973) as substrate (1 unit = 1 nM substrate hydrolysed per min) and expressed as enzyme units per g protein.

Gene expression.

The mRNA expression level for interleukin 1β (IL-1□) and the interleukin 8 (IL-8) relative to the level of glyceraldehyde phosphate dehydrogenase (GAPDH) was quantified by Real Time reverse transcription Polymerase Chain Reaction procedures (RT-PCR) and used as a marker for inflammation. Tissue samples of the proximal jejunum were cut open at the anti mesenteric side and rinsed with phosphate buffer solution. The epithelium was scraped off, deep frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from the epithelial samples (±25mg) using a QIA shredder and RNeasy kits (Qiagen, Crawley, UK) according to the manufacturer's protocol and quantified determining the relative optical densities (OD) at 260nm by spectrofotometry using a Spectramax plus spectrophotometer

(Molecular Devices). Per sample 750 ng RNA was used for RT reaction, using 1.5 µL 10 U/µL AMV reverse transcriptase (Promega Corporation, Madison, WI), 2.5 µL 100 µM T_{15} VN-primer, 1.5 µL 10 U/µL recombinant RNasin ® Ribonuclease, 3 µL 10 mM dTNP and 6 µL 25 mM $MgCl_2$ and 6 µL 5x AMV buffer (Promega). The volume of the reaction mixture was adjusted to 30 µL by addition of RNase free water. The RT-PCR was performed using the Taqman Universal Mastermix (PE Applied Biosystems). Amplification and detection of specific products were performed using the I-cycler PCR apparatus (BioRad) Each reaction mixture contained 13.2 µL PE mastermix (Perkin-Elmer, Applied Biosystems) 1.13 µL forward primer (20 µM stock), 1.13 µL reverse primers (20 µM stock) and 1.0 µL Taqman probe (5 µM stock). During the PCR, the following temperature profile was used: an initial enzyme activation step of 95°C for 10 min followed by 50 cycles each of 60°C for 1 min followed by 95°C for 15 sec. Cytokine and GAPDH mRNA-specific amplification primers and probes were designed using the Primer Express software program (PE Applied Biosystems). The primer-probe sets used were:

- for GAPDH:

forward primer: AGC CTC AAG ATC ATC AGC AAT G

reverse primer: ACT GTG GTC ATG AGT CCT TCC A

taqman probe: CAC CCC TGG CCA AGG TCA TCC A

- for IL-1β:

forward primer: TCT GCC CTG TAC CCC AAC TG

reverse primer: CTC TGG CGG CCT TTG GA

taqman probe: CTC TCA AGC AGA ACA AAA GCC CGT CTT CC

- for IL-8:

forward primer: CTG TTG CCT TCT TGG CAG TTT

reverse primer: CAT CGA AGT TCT GCA CTT ACT CTT G

taqman probe: CCT GCT TTC TGC AGC TCT CTG TGA GGC

All probes were labelled with the fluorescent reporter dye 5-decarboxyfluorescein (FAM) at the 5' end and the quencher N, N, N, N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. The values for IL-8 RNA were not normally distributed and therefore ^{10}log transformed.

Mucin.

The content of mucin in ileal digesta was analysed in samples of d 0 and d 3. Digesta (±0.5g) was diluted with 5 ml phosphate buffer solution, centrifuged and split into 3 batches for the analysis of mucin type 1 (MUC1), mucin type 2 (MUC2) and mucin type 3 (MUC3). Each batch was incubated with mouse monoclonal IgG1 antibody against either human MUC1 protein tandem repeat (clone VU4H5) or human MUC2 protein tandem repeat (clone Ccp58) or with mouse monoclonal IgG2a antibody against human MUC3 protein tandem repeat (clone M3.1). All monoclonal antibodies were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, USA). The relative quantity of mucin was determined by western blotting.

Stastistical analysis.

A combination of sampling day and feed intake level was considered as a treatment. A GLM procedure (SAS version 6.12, SAS Institute, Cary, NC) was used to estimate the least-square means of the different treatments. The two-way interaction of batch × treatment was not significant and therefore not included in the final model:

$$y_{ijk} = \mu + B_i + T_j + e_{ijk} \qquad [1]$$

where y_{ijk} = dependent variable; μ = overall mean; B_j = fixed effect of batch (i=1,2); T_j = fixed effect of treatment (sampling day x feed intake level) (j=1,2,…,6); e_{ijk} = error term.

An effect of day post weaning or feed intake level was tested using the contrast statement.

The values for faeces score were analysed using the Kruskal-Wallis χ^2 test of the Npar1way procedure.

Results

Health.

The health of the piglets was good. Medical treatment was not given. The feed intake level did not significantly affect the faeces score. The average incidence of faeces inconsistency (% of days with faeces score was ≥ 1) at day 3 post weaning was 8% (SD: 15.4) and 4 % (SD: 11.9) for the low and high intake group respectively. The average incidence of faeces inconsistency at day 7 was 25 % (SD: 23.8), 34 % (32.3) and 32 % (32.2) for the low, increasing and high intake group respectively.

Morphology, weight and length of the small intestine.

The results on villous architecture are shown in Table 2. The feed intake level significantly affected the villous height and crypt depth at the proximal site and the villous height at the mid site. The high intake level resulted in significantly higher villi and deeper crypts compared to the low intake level. This effect was most pronounced at the proximal site. At d7 post weaning, the increasing feed intake level resulted in similar villous height and crypt depth compared to both the low and high intake level.

The day post weaning tended to affect ($P<0.10$) the villous height but the effect was site dependent. At the proximal site, the villous height at d3 was 50% of the value at d0 and partially recovered at d7 to 61% of the value at d0. In contrast, villous height on d3 at the mid small intestine was 64% of the value at d0 but further decreased till 50% at d7.

The day post weaning significantly affected the crypt depth resulting in deeper crypts at d3 (+10%) and d7 (+43%) compared to d0. The crypts at d7 were significantly deeper compared to d3 at both sites of the small intestine. The increase in crypt depth at d7 was most pronounced at the proximal site.

The high intake level resulted in higher weight of the small intestine at d7 compared to the low intake level. The increasing intake level resulted in intermediate values. The weight of the small intestine was significantly decreased at d3 compared to the day of weaning for the low intake level. At d7, the weight of the small intestine was increased significantly compared to d0. This increase was most pronounced for the high intake level (data not shown).

The length of the small intestine was increased by age. The small intestine at d7 was significantly longer compared to d3 and tended to be longer compared to d0. The feed intake level did not affect the length of the small intestine (data not shown).

Table 2 Villous height (µm), crypt depth (µm) and villous height to crypt depth ratio at the proximal and mid small intestine, mucosal enzyme activity (aminopeptidase and sucrase-isomaltase, units per g protein) and mRNA expression level of interleukin 8 (IL-8, relative to the expression of glyceraldehyde phosphate dehydrogenase) at the proximal small intestine and mucin content of ileal digesta of weaned pigs fed a pelleted diet at low (**L**), increasing (**I**) or high (**H**) intake level from 0 to 7d post weaning.

Treatment		Weaned	**L**	**H**	**L**	**I**	**H**	Statistical significance						
Post weaning day			3			7		SEM	**H** vs. **L**	**H** vs. **I**	**I** vs. **L**	d0 vs. d3	d0 vs. d7	d3 vs. d7
Villous height	Prox	570^{a}	221^{c}	354^{b}	325^{b}	338^{b}	374^{b}	33.3	**	ns	ns	***	***	*
	Mid	511^{a}	302^{bc}	356^{b}	223^{c}	256^{c}	282^{bc}	31.8	t	ns	ns	***	***	*
Crypt depth	Prox	226^{c}	241^{bc}	268^{bc}	285^{b}	301^{ab}	348^{a}	16.5	**	t	ns	ns	***	***
	Mid	177^{b}	181^{b}	198^{b}	266^{a}	274^{a}	256^{a}	16.0	ns	ns	ns	ns	***	***
Villous height to	Prox	2.70^{a}	0.94^{c}	1.38^{b}	1.17^{bc}	1.13^{bc}	1.09^{bc}	0.15	ns	ns	ns	***	***	ns
Crypt depth ratio	Mid	3.12^{a}	1.67^{b}	1.82^{b}	0.92^{c}	0.94^{c}	1.11^{c}	0.18	ns	ns	ns	***	***	***
Aminopeptidase	Prox	584^{a}	329^{bc}	313^{c}	351^{bc}	377^{bc}	421^{b}	37.2	ns	ns	ns	***	***	t
Sucrase-Isomaltase	Prox	65^{a}	23^{b}	13^{b}	18^{b}	18^{b}	18^{b}	4.0	ns	ns	ns	***	***	ns
10 log mRNA IL8	Prox	-2.76	-2.96	-2.62	-2.95	-2.95	-2.85	0.21	ns	ns	ns	ns	ns	ns
Mucin														
Type 1	Ileal	22.3^{a}	6.4^{b}	9.3^{b}	nd	nd	nd	3.2	ns	nd	nd	**	nd	nd
Type 2	Ileal	45.6^{a}	1.3^{b}	1.5^{b}	nd	nd	nd	5.8	ns	nd	nd	***	nd	nd
Type 3	Ileal	0.9^{a}	0.2^{b}	0.5^{ab}	nd	nd	nd	0.23	ns	nd	nd	t	nd	nd

ns = not significant; t = p<0.10; * p< 0.05; ** p<0.01; *** p<0.001

^{ab} = values at a small intestinal site without a common character in the superscript differ significantly

nd = not determined

Enzyme activity and gene expression of proximal jejunal mucosa and mucin content of ileal digesta.

The mucosal specific enzyme activity for aminopeptidase (AMP) and sucrase- isomaltase (SIM) was expressed as units per g mucosa protein. The results for AMP and SIM are shown in Table 2. The feed intake level did not affect the specific activity of AMP and SIM. In contrast, the day of dissection significantly affected the specific activity of AMP and SIM resulting in decreased values at d3 and d7 compared to d0. The specific activity of AMP was 54% at d3 and recovered numerically at d7 to 66% of the value at weaning. The recovery of the specific AMP activity was most pronounced for the high intake level. The specific SIM activity was also decreased at d3 compared to the day of weaning but did not show any recovery at d7.

Expression level of and interleukin 8 mRNA (IL-8) relative to the mRNA expression level of GAPDH at the proximal small intestine at 0, 3 and 7 days post weaning are shown in Table 2. Feed intake level and day post weaning did not affect the IL-8 expression. The IL-8 mRNA expression tended to be positively correlated ($P<0.10$, $r_{pearson} = 0.28$) with feed intake till d 3. Only in 9 out of 48 samples, the quantity of IL-1 mRNA was higher than the detection limit. The IL-1 mRNA expression level was positively correlated ($P<0.05$, $r_{pearson} = 0.69$) with crypt depth.

The content of mucin type 1 (MUC1), type 2 (MUC2) and type 3 (MUC3) in ileal digesta are presented in Table 2. Feed intake level did not affect the mucin content in ileal diegsta. The MUC2 content was positively correlated with villous height at the proximal ($P<0.10$, $r_{pearson} = 0.40$) and mid SI ($P<0.01$, $r_{pearson} = 0.69$) and negatively correlated with crypt depth at the proximal SI ($P<0.01$, $r_{pearson} = -0.61$). MUC2 was also positively correlated with the specific activity of AMP and SIM. Day post weaning significantly affected the content of mucin types in the ileal digesta. Values for mucin content were decreased at day 3 post weaning compared to the day of weaning.

Performance.

Pigs fed at the high intake level had a clearly higher feed intake and body weight gain during the 7d period compared to pigs fed at the low intake level. Pigs fed at the increasing intake level had intermediate values, between the high and low feeding level. Average feed intake per pig for the low, increasing and high intake level was 110, 135 and 202 g/d for day 0 – 7

respectively. The body weight gain per pig during the 7d period for the low, increasing and high intake level was 45, 66 and 168 g/d respectively.

Discussion

In this study we compared the effect of a low, increasing and high feed intake level on gut integrity of piglets at weaning. We hypothesized that a high feed intake level supplies sufficient nutrients to the gut preventing degradation of the intestinal mucosa by mucolytic bacteria and increased synthesis of pro inflammatory cytokines by epithelial cells. Commonly used parameters to study gut morphology and digestive capacity like villous height and crypt depth and specific brush border enzyme activity were measured. These parameters have been established rather well (Pluske *et al.*, 1997; Lallès *et al.*, 2004). In addition, we established whether the feed intake level interfered with the mucin content of ileal digesta as well as with the mRNA expression level of inflammatory cytokines in the small intestinal epithelium.

The feed intake level significantly affected the weight of the small intestine similar to that reported by Kelly *et al.* (1991; Núñez *et al.*, 1996) and Núñez *et al*. (1996).

Feed intake level clearly affected the changes in the villous architecture at d3 post weaning. A high feed intake level resulted in higher villi and deeper crypts compared to an intake level at maintenance level (low). The observed changes were similar to the changes as reported by Van Beers-Schreurs *et al.* (1998), Pluske *et al.* (1996) and Núñez *et al*. (1996). The villous atrophy was most pronounced for the low feed intake level at the proximal and the mid small intestine at d3 and d7 respectively. This is in line with results of Marion *et al.* (2002). They also concluded that the change in villous architecture started proximal and spread distally in the small intestine. Vente-Spreeuwenberg *et al.* (2004) showed that there may be even recovery of the mucosa at the proximal small intestine while distally there may still be further compromise. The imposed feed intake level did not affect the mucosal specific enzyme activity. However, voluntary feed intake was positively correlated with amino peptidase. These results corroborate with the results of Hampson (1986) and Kelly *et al.* (1991) but are in contrast with the results of Núñez *et al*. (1996). Nuñez *et al*. (1996) reported higher specific activities of disaccharidases and leucine aminopeptidase in the duodenum and first part of the jejunum in pigs fed restricted for 30d and starved overnight. The increased levels of specific enzyme activity might be due to longer adaptation period in their study compared to our

study. Boudry et al. (2002) and Vente-Spreeuwenberg *et al*. (2004) showed that diet induced changes occur in weaned pigs for sucrase-isomaltase but not for amino peptidase.

The epithelial cells lining the intestinal epithelial tract provide both a physical barrier and have evolved inducible innate strategies that offer rapid responses to corrosive and pathogenic challenges. One of these strategies is the mucus layer. High molecular weight glycoproteins termed mucins are the main determinants of the physical and functional properties of the mucus. Mucins can be categorized as secreted mucins (i.e. mucin type 2 (MUC2)) or membrane associated mucins (i.e. MUC1 and MUC3).

The high feed intake level resulted in numerically higher content of mucin in ileal digesta at d3 despite a higher diluting factor by the high intake resulting probably in more digesta passing through the gut. In the literature several factors are mentioned to affect the synthesis and secretion of mucins like stress (Castagliuolo *et al.*, 1996), route of feeding (parenteral versus enteral, Counour *et al.(Conour et al., 2002)*), dietary composition (Sharma *et al.*, 1997) and presence of probiotic microbes (Mack *et al.*, 2003). Data on gut mucin biology in pigs are scarce and rather conflicting (review by Lallès *et al*. 2004). Weaning in pigs induced an early drop, followed by an increase between day 3 and day 15 post weaning in goblet cell density in the villi (Dunsford *et al.*, 1991). We found no effect of time post weaning and diet composition in pigs fed liquid milk replacer during 4 days post weaning at low intake levels on crypt goblet cell numbers (Spreeuwenberg *et al.*, 2001). Also intake level did not significantly affect crypt goblet cell number during 4 days post weaning in pigs fed liquid milk replacer (Verdonk, unpublished). Gu *et al.* (2002) suggested a decrease in goblet cell number post weaning and higher levels in pigs weaned at an earlier age. Malnutrition during 30 days lowered the number of goblet cells in the villi Núñez *et al*. (1996) and the mucin level in goblet cells (Lopez-Pedrosa *et al.*, 1998). Pestova *et al.* (2000) found increased density of high molecular-weight glycoprotein (mucin) in ileal digesta at day 7 post weaning compared to the day of weaning. Conour *et al.* (2002) found increased goblet cell numbers in new born piglets after 3 days total parenteral nutrition or a combination of enteral and parenteral nutrition not related to gene expression levels of proinflammatory cytokines. *In vitro* we did not find an effect of several nutrients (crystalline amino acids, monosaccharides, fatty acids) on the secretion of MUC2 in HT29-MTX mucus producing cells under non-cytotoxic exposure conditions, using relatively poor (i.e. not serum-containing) culture medium (data unpublished).

The effect of the feed intake level on inflammation of the proximal intestine was not clear. Only in limited number of samples we found values for IL1 mRNA expression level above the detection limit. This is in line with results of another study in our lab (Verdonk *et al.*, 2002a) in weaned pigs evaluating the effect of dietary physical form (slurry, dry pellet) on the onset and recovery of compromised gut integrity. Stimulated or infected human intestinal cells also express and secrete IL-1β generally at a much lower level compared to IL-8 (Kagnoff & Eckmann, 1997). In another study, we qualitatively assessed the expression levels of genes encoding for mediators of inflammation in jejunal tissue of weaned piglets fed a milk diet (Verdonk *et al.*, 2002b). We found significantly higher IL-1β mRNAexpression levels at the low intake level compared to the high intake level but no mRNA expression for the proinflammatory cytokine tumor necrosis factor α and the enzymes inducible nitric oxide synthase and cyclo oxygenase 2. This indicated that (excessive) release of the nitric oxide and prostaglandins in the gut epithelium are not involved in weaning anorexia. Pié *et al.* (2004) reported a transiently increased IL-1β mRNA expression in weaned pigs at d1 at the mid small intestine and at d2 at the distal small intestine and proximal colon. In our study, values of mRNA expression levels of IL-8 in the proximal small intestine at d3 and d7 were similar to the values at weaning. This is similar to results of Pié *et al.* (2004) who found that mRNA expression levels of IL-8 were only increased at d1 at the mid small intestine and returned to levels similar or lower than those recorded on the day of weaning.

In conclusion: At d7 post weaning the only significant difference between pigs fed at the high and pigs fed at the low level was the crypt depth. Values for all other parameters were similar for both groups. This implicates that a feed intake level in the range between 1 to 2.5 times maintenance requirement for energy does not differentially affect gut integrity at d7 post weaning.

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Chapter 5

Feed intake level modulates gut morphology but not gut permeability in weaned piglets

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Abstract

This experiment was designed to test the hypothesis that a compromised small intestinal integrity in newly weaned piglets is caused by a limited mucosal nutrient supply. Therefore we studied the modulating effects of feed intake level on the onset and recovery of changes in the small intestine of pigs post weaning. Parameters evaluating structure and barrier function of the intestinal were measured. 48 piglets of 8.0 kg (SD: 0.82) were weaned at 26d of age (d0). Piglets with a high voluntary feed intake directly after weaning were compared with piglets fed restrictedly. Piglets were euthanized and samples of gut tissue were taken at d 0, 4, or 7 post weaning. Results showed that weaning induced acute and long lasting changes in gut morphology and permeability. Intake level of dry pelleted feed affects villous architecture but intake level of dry feed did not affect mucosal permeability to small or large molecules. A restricted feed intake level, below maintenance level, negatively affected gut morphology at d 4 post weaning. This effect could be alleviated but not completely prevented by a high voluntary feed intake of dry pelleted feed directly after weaning.

Introduction

The weaning transition of piglets compromises small intestinal mucosal integrity, as was concluded from a decreased villous height, a decreased brush border enzyme activity, a increased paracellular permeability and an inflammatory response which occurred directly after weaning (Kelly *et al.*, 1991b; McCracken *et al.*, 1995; McCracken *et al.*, 1999; Spreeuwenberg *et al.*, 2001). The magnitude of these changes seems to be related to the level of feed intake of the piglets, but not to diet composition (Makkink, 1993; McCracken *et al.*, 1995; McCracken *et al.*, 1999; Boudry *et al.*, 2004).

In accordance with Pluske *et al.* (1996b) and Van Beers-Schreurs *et al.* (1998), we showed that a high intake of milk immediately after weaning prevented changes in villous architecture and maintained gut integrity. In contrast, a low intake of milk after weaning resulted in decreased villous height and increased paracellular permeability of mannitol (Verdonk *et al.*, 2001). Boudry *et al.* (2004) showed that weaning induced transient and long-lasting modifications of paracellular barrier properties of the piglet intestine independent of the diet composition.

We hypothesised that a high feed intake level attenuates the deterioration of the gut integrity and stimulates intestinal repair in newly weaned piglets compared to low feed intake level.

The aim of this experiment was to study the effect of feed intake level on the onset and repair of changes the small intestinal integrity, investigating both structural and functional parameters.

Materials and Methods

Animals

This experiment with weaned piglets [(Yorkshire x Pietrain) × Dalland] was performed at the experimental research facilities of the Animal Sciences Group (Lelystad, The Netherlands). Creep feed was not provided during the suckling period to prevent adaptation to solid feed.

Barrows (n=48) were weaned at 26 (SD: 1.4) days of age and 8.0 (SD: 0.82) kg body weight (BW). At weaning, piglets were removed from the sow, weighed and housed individually in 77×76×69 cm pens. Each pen was equipped with a feeding through and a drinking nipple. Ambient temperature was maintained at 27°C. Lights were on from 05:00 h till 23:00 h. The experimental pelleted diet (Table 1) that was fed after weaning was based on barley, corn, wheat gluten and soy flakes.

On the day of weaning (d0), piglets were weighed and randomly allotted to one of three experimental groups, containing a total of 12, 24 and 12 piglets for groups 1 to 3, respectively. Directly after allocation piglets from Group 1 were dissected to collect reference values. The other two experimental groups differed in feed intake level. 24 piglets (Group 2) were fed a high feeding level (2.5 x the maintenance requirement for net energy (NE_m)) directly after weaning. At d3, 12 animals with high voluntary feed intake and average BW were selected: 6 of those piglets were dissected at d4 and 6 piglets at d7. The remaining 12 piglets from Group 2 were excluded from the experiment at d3. Piglets from Group 3 were fed restricted (0.5 x NE_m to 1.0 x NE_m respectively) from d0 to d3. At d4, 6 piglets were dissected. The remaining 6 piglets from Group 3 were fed at a restricted increasing level (1.5 x NE_m at d4 increasing to 2.0 x NE_m at d6) and dissected at d7.

Table 1 Ingredient composition and nutrient content of the experimental diet (g/kg)

Ingredient	
Barley	478.1
Corn	200.0
Soy flakes (crude protein >467g/kg)	65.0
Corn starch	14.0
Hydrolyzed wheat gluten	75.0
Potato protein	10.0
Fish meal (crude protein >680g/kg)	10.0
Whey powder	50.0
Fat/oil	15.0
Molasse	30.0
Premix[1]	10.0
Calcium carbonate	18.0
Monocalcium phosphate	12.5
Salt	2.0
$NaHCO_3$	1.0
$KHCO_3$	2.0
L-lysine HCl	5.5
DL-methionin	0.4
L-threonin	0.9
L-tryptophane	0.4
Nutrient	Content[2]
Moisture	88
Crude protein (N x 6.25)	188 (188)
Ash	61 (56)
Fat	37.6
Crude fiber	30.1
Nett Energy pigs (MJ/kg)	9.5
Ca	10.2 (10.0)
P total	6.2 (6.3)
P digestible	3.6
Amino acids:	
Lys, total	11.2 (10.4)
Met+Cys, ileal dig.	6.0
Thr, total	7.1 (7.4)
Ile, total	7.0 (7.0)
Glutamic acid	44 (44)

[1] Vitamin and mineral inclusion supplies ($mg \cdot kg^{-1}$ feed): Vitamin A, 10000 IE; Vitamin D3, 2000 IE; Vitamin E, 65000 IE; Vitamin K3, 2.0 mg; Vitamin B1, 1.0 mg; Vitamin B2, 3.0 mg; Panthoteic acid, 10.0 mg; Niacin, 20.0 mg; Biotin, 30 mcg; Vitamin B12, 20 mcg; Folic acid, 0.2 mg; Vitamin B6, 4.0 mg; Fe, 160.0 mg; Cu, 160.0 mg; Zn, 100.0 mg; Mn, 30.0 mg; I, 10.0 mg; Se, 0.2 mg; Antioxidants (E130, E320, E321), 60.0 mg

[2] Calculated with analysed content between brackets

Formula 1 describes the amount of net energy for maintenance (NE_m) of the piglets according to their metabolic weight at the day of weaning (National Research Council, 1998).

$$NEm(kJ / day) = 326.4 * BW_0^{0.75} \quad [1]$$

where NE_m is the net energy intake at maintenance level and BW_0 is BW at d0 (kg). Piglets were fed three times per day at 06:00, 10:30 and 15:00 h. Feed refusals were collected, weighed and subtracted from the amount of feed offered to calculate actual daily feed intake (g/kg $BW^{0.75}$).

Technical results and health.

Piglets were weighed at d0 and at the day of dissection. Average daily gain (ADG) was calculated for the periods 0 to 4 and 0 to 7 days post-weaning. Faecal consistency was monitored daily and quantified using a scale ranging from 0 to 3 with 0 = normally shaped faeces, 1 = shapeless (loose) faeces, 2 = thick liquid (soft) faeces, and 3 = thin liquid faeces.The experimental protocol was approved by the Animal Care and Ethics Committee of the Animal Sciences Group (Lelystad, the Netherlands).

Sampling of small intestine.

At 0, 4 and 7 days post-weaning, piglets used for dissection were weighed and euthanised with a 5 mL intra-cardiac injection of Euthestate® (pentobarbital sodium 200 mg/mL; Ceva Sante Animale B.V. Maasluis, The Netherlands). A midline laparotomy was performed. Tissue samples were taken at the ligament of Treitz (proximal small intestine) and 3 m distal of the ligament of Treitz (mid small intestine).

For analysis of morphological parameters, tissue samples (2 cm) of the proximal and mid small intestine were cut open longitudinally at the mesenteric attachment, prepared on dental wax with the villi on the upper side and fixed in 0.1 mol/L phosphate buffered formalin solution (40 ml/L). A 3-mm wide zone from the mesenteric site was cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections (5 µm) were cut and stained with the periodic acid / Schiff procedure (PA/S). From the stained sections, crypt depth (µm) and villous height (µm) were determined.

For the measurement of transepithelial transport two adjacent samples (5 cm) of proximal small intestinal tissue were taken and rinsed with an ice-cold buffer solution of HEPES-buffered phenol red free Dulbecco's Modified Eagles Medium (DMEM) and cut open longitudinally. The tissue was placed with the mucosa facing up on a flat underground, and the mucosal layer was carefully stripped off the muscle layer - in order to preserve mucosal integrity - using a blunt razor blade. Flat sheets were placed in the Ussing chambers for measuring the transepithelial transport of 4 compounds: Glycyl-L-Sarcosine (GlySar); mannitol; sodium fluorescein (Na-Flu) and horse radish peroxidase (HRP). The effective areas exposed in the Ussing chamber were 0.196 cm^2 and 0.7 cm^2 respectively. [^{14}C]GlySar (Cambridge Research Biochemicals, Northwich, UK, Mw 146 Da) is a small hydrophilic molecule transported mainly via a transcellular route with a H^+-coupled di/tri-peptide carrier (Duizer, 1999). Na-Flu (F6377, Sigma St. Louis, MO, Mw 376 Da) is a small hydrophilic molecule. [2-^{3}H]mannitol (ICN Biomedicals, Zoetermeer, NL, Mw 182 Da) is a small hydrophilic globular shaped molecule and is transported passively mainly via the paracellular route (Duizer, 1999). Horseradish perioxidase (HRP, 40 kD; type VI, Sigma, St. Louis, MO) transported intact (presumed paracellularly) or degraded (presumed transcellularly), is a marker to measure transepithelial macromolecular transport (Bland, 1988). The radiolabeled GlySar and mannitol were mixed with unlabelled compounds to yield final concentrations of 10 µM. The mucosal side was filled with 1.25 ml HEPES DMEM medium containing radiolabeled GlySar (10 µM) and mannitol (10 µM). The serosal side was filled with 1.25 ml HEPES DMEM medium. Both compartments were aerated (O_2 / CO_2, 95 / 5) at a temperature of 37°C and stirred by gaslift. At indicated time points (15, 30, 45, 75, and 105 min), 0.5 ml samples were taken from the serosal side and the volume was reconstituted with DMEM without phenol red. ^{3}H and ^{14}C radioactivity was determined in the serosal samples and the tissue (at the end of the experiment) by Liquid Scintillation Counting (LSC) using DOT-DPM™ (Digital Overlay Technique using the Spectrum Library and the External Standard Spectrum) for quench correction. HRP and Na-Flu dissolved in Ringer solution were added at the mucosal side in a final concentration of 10^{-5} M and 10^{-3} M respectively. Serosal samples of 400 µL were taken at 30, 60, 90, 120 and 180 minutes and were replaced by 400 µL fresh Ringer to keep the volume constant. Intact HRP in the serosal samples was measured enzymatically (Bijlsma *et al.*, 1996) as an indication of paracellular transport of large molecules. In short 200 µL phosphate buffer (0.1 M, pH 6.0) containing 0.003% H_2O_2 and 0.008% *o*-dianisidine dihydrochloric acid was added to a 30 µL sample of test solution and

mixed. The linear, HRP concentration-dependent rate of increase in optical absorption at 460 nm was determined with a Biorad Microplate Reader Model 550. Na-Flu in the serosal samples was determined by measuring the fluorescence in 50 µL samples in a 96-wells microplate with a Wallac VICTOR ^{TM} 1420 Multilabel Counter.

Apparent permeability coefficients (P_{app}) were determined based on the appearance of the compound at the serosal side according to the following equation:

$$P_{app} = R / (A * C_0) \quad [2]$$

Where: P_{app} = permeability coefficient from mucosal to serosal side (cm/s); R = permeability rate (mol/s); A = exposed intestinal area (cm^2); C_0 = initial concentration of test substance (mol/ml) at the mucosal side.

Stastistical analysis.

A combination of sampling day and feed intake level was considered as a treatment. A GLM procedure (SAS version 8.1, SAS Institute, Cary, NC) was used to estimate the least-square means of the different treatments. The final model used was:

$$y_{ij} = \mu + T_i + e_{ij} \quad [3]$$

where y_{ij} = dependent variable; µ = overall mean; T_i = fixed effect of treatment (sampling day x feed intake level) (i=1,2,..,5) which corresponds to d0, d4-high, d4-low, d7-high, d7-low, respectively); e_{ij} = error term . An effect of day post weaning or feed intake level was tested using the contrast statement.

Pearson correlation analysis for data at day 4 and day 7 post-weaning was performed to evaluate the correlation between feed intake, morphology parameters and trans-epithelial transport traits of the proximal small intestine.

Results

Performance.

The feed intake at d7 post-weaning of one piglet of the d7-high and two piglets of the d7-low group was less than 50% of the amount of feed offered. These piglets were excluded from the data set, prior to calculation of the average feed intake and body weight gain. The average feed intake per piglet in the 7-day period was 268 and 185 g/d for the high and restricted feed intake level respectively, whereas the mean body weight gain of the piglets in the 7-day period was 234 (SD:71) and 107 (SD:18) g/d for the high and restricted feed intake group respectively. Thin faeces were not observed.

Morphology and permeability.

Results in Table 2 show the effects of age (4 and 7 days post-weaning) and feeding level on morphology and permeability. The transepithelial permeability of the small molecules GlySar (transported mainly transcellular) and Na-Flu (transported both trans- and paracellular) were not affected by the feed inatke level. The P_{app}GlySar tended to be higher at d4 (+41%) compared to the intial value at weaning and decreased at d7 to values not different from the weaning value. The P_{app}Na-Flu was significantly lower at d4 (-46%) and d7 (-36%) compared to weaning. Paracellular transport to small and large molecules was measured as P_{app}Mannitol and P_{app}HRP values respectively. The Mannitol permeability was increased numerically (+20%, $P>0.10$) at d4 post weaning and decreased (41%, $P<0.05$) at d7 post weaning compared to the day of weaning and this effect was most pronounced at the high feed intake level. The P_{app}HRP was not affected by feed intake. Independent of the post-weaning feed intake level, the permeability of HRP was significantly decreased at d4 (-61%) and at d7 (-48%). Villous height at the mid small intestine tended to lower values at d4 and d7 in piglets fed the low feeding level compared to the high feeding level. Crypt depth increased after weaning at both the low and high feeding level, although its magnitude was significantly smaller at the low feeding level. Across experimental groups, age as days post-weaning significantly affected the villous height, crypt depth, villous height to crypt depth ratio and permeability of the small intestine. Villous height at d4 and d7 post weaning was significantly lower compared to the villous height at the day of weaning ($p< 0.01$). Mean villous height at d4 was significantly lower (-19%) compared to d7. This effect was most pronounced at the mid-small intestine and occurred in all groups. Mean crypt depth at d4 was significantly increased (+20%; $P<0.05$) compared to crypt depth at the day of weaning. Mean crypt depth

at d7 was significantly higher (+21%, P<0.01) compared to d4 irrespective of the dietary feed intake.

Table 2 Villous height (µm), crypt depth (µm), villous height to crypt depth ratio at the proximal (prox) and mid small intestine and permeability coefficients (P_{app}) of mannitol (10^{-6} cm/s), HRP (10^{-8} cm/s), GlySar (10^{-6} cm/s) and Na-Flu (10^{-4}cm/s) at the proximal small intestine of weaned pigs fed a pelleted diet at a high (H) and restricted (R) feed intake level (FI) from 0 to 7d post weaning.

Feed intake level (FI)		-	**H**igh		**R**estricted		Level of significance				
Day post weaning		0	4	7	4	7		FI	Day post weaning		
Site							SEM	**H**vs**R**	0vs4	0vs7	4vs7
Villous architecture											
Villous height	prox	562 ^{a}	375 ^{b}	428 ^{ab}	417 ^{b}	353 ^{b}	54.9	ns	**	**	ns
	mid	491 ^{a}	397 ^{b}	438 ^{ab}	343 ^{b}	354 ^{b}	36.0	#	**	*	ns
Crypt depth	prox	232 ^{c}	336 ^{a}	360 ^{a}	262 ^{bc}	313 ^{ab}	18.7	**	**	***	#
	mid	181 ^{c}	233 ^{ab}	272 ^{a}	210 ^{bc}	215 ^{bc}	19.1	*	*	**	ns
Villous to crypt ratio	prox	2.5 ^{a}	1.2 ^{b}	1.2 ^{b}	1.7 ^{b}	1.1 ^{b}	0.24	ns	***	***	ns
	mid	2.8 ^{a}	1.7 ^{b}	1.7 ^{b}	1.7 ^{b}	1.7 ^{b}	0.25	ns	***	***	ns
Permeability coefficients											
P_{app}Mannitol	prox	8.6	11.1	4.2	9.6	6.7	2.13	ns	ns	ns	*
P_{app}HRP	prox	4.3 ^{a}	2.3 ^{ab}	2.3 ^{ab}	1.0^{b}	2.1^{b}	0.89	ns	**	*	ns
P_{app}GlySar	prox	13.9	21.2	15.8	18.0	18.5	3.37	ns	#	ns	ns
P_{app}Na-Flu	prox	3.3 ^{a}	1.8 ^{b}	2.3 ^{ab}	1.7 ^{b}	1.9 ^{b}	0.46	ns	**	*	ns

ns = not significant; # p<0.10; * p< 0.05; ** p<0.01, *** p<0.001,

^{abc} Values in a row without a common letter in the superscript differ significantly (P<0.05)

In Tables 3 and 4 correlations between morphology, transepithelial transport and feed intake on d4 and d7 respectively are given. No significant correlations between feed intake of day 1

to 4 and gut wall traits at day 4 were found, whereas feed intake of day 1 to 7 showed a positive correlation with morphology at day 7 (Table 4).

Table 3 Pearson correlation coefficients between morphological and epithelial transport parameters at the proximal small intestine and dry matter intake of piglets fed a pelleted feed on day 4 post weaning.

	Glysar	HRP	Na-Flu	Villous height	Crypt Depth	Villous to crypt Ratio	Metabolic Feed Intake d1-4
Mannitol	ns[1]	-0.72 0.106	ns	ns	ns	ns	ns
GlySar		ns	ns	ns	ns	ns	ns
HRP			0.59 #	ns	ns	ns	ns
Na-Flu				ns	ns	ns	ns
Villous height					-0.78 **	nr[2]	ns
Crypt depth						nr	ns
Villous to Crypt Ratio							ns

[1] P-value of the model: ns = not significant; # P<0.10; * P< 0.05; ** P<0.01

[2] nr = not relevant

On day 4 there was a clear negative relation between villous height and crypt depth while on d7 post-weaning this relation tended to be positive (P=0.10). With regard to transport traits across the gut wall the HRP and mannitol permeability values which represent paracellular transport of large and small molecules respectively tended to be related negatively at day 4 but were not related at day 7. HRP and Na-Flu showed a clear positive correlation coefficient, most obvious with increasing age (p<0.10 at d4 and p<0.001 at d7). On d7 feed intake was correlated to morphology. Crypt depth showed highest positive correlation to feed intake level during d1-4 whereas villous height showed highest positively correlation with feed intake during d5-7. At d7, initial feed intake post-weaning (d1-4) tended to have a negative effect on

mannitol and a positive effect on Na-Flu transport. At d7 villous height and crypt depth were positively correlated with P_{app}GlySar and P_{app}Na-Flu respectively. P_{app}HRP and P_{app}Na-Flu were also correlated positively. At d7 villous height and GlySar permeability and crypt depth and Na-Flu permeability were highly correlated ($p< 0.05$).

Table 4 Pearson correlation coefficients between morphological and epithelial transport parameters at the proximal small intestine and dry matter intake of piglets fed a pelleted feed on day 7 post weaning

	GlySar	HRP	Na-Flu	Villous height	Crypt Depth	Villous / crypt Ratio	Metabolic Feed Intake		
							d1-4	d5-7	d1-7
Mannitol	ns [4]	ns	ns	ns	ns	ns	-0.76 #	ns	ns
GlySar		ns	ns	0.85 *	ns	0.73 #	ns	ns	ns
HRP			0.89 ***	ns	ns	ns	ns	ns	ns
Na-Flu				ns	0.68 *	ns	0.58 t	ns	ns
Villous height					0.49 0.105	nr[2]	0.65 *	0.77 **	0.76 **
Crypt depth						nr	0.72 **	0.53 #	0.64 *
Villous to Crypt Ratio							ns	0.60 *	0.53 #

[1] P-value of the model: ns = not significant; # P<0.10; * P< 0.05; ** P<0.01; *** P<0.001

[2] nr = not relevant

Discussion

The present data demonstrate that villous architecture (villous height and especially crypt depth) but not gut wall permeability was affected by level of intake of dry pellets during the first days post-weaning. The sequential effect on mucosal barrier function in the pig small intestine was more pronounced than the effect of feeding level during the first seven days post weaning. The piglets were weaned abruptly at 3.5 weeks of age and offered a pelleted diet. The piglets fed at the high intake level consumed on average 12% up to 87% of the amount offered at d1 and d7 post weaning respectively. This suggests that the weaning-stress disrupted the body's internal cues for hunger and overpowered its ability to regulate feed intake. This resulted into a mean energy intake of 666 kJ and 4284 kJ ME per piglet per day at d1 and d7 post-weaning for the high-intake group. At the restricted feed intake level, the piglets consumed on average 8% up to 85% of the amount offered at d1 and d7 post weaning. The mean energy intake in the restricted intake group was 40 kJ and 2870 kJ ME per piglet per day at d1 and d7 post-weaning. Voluntary milk consumption before weaning averages 5000 kJ ME per piglet per day (Harrell *et al.*, 1993), which implies that the reduction in energy intake was at least 80% due to weaning. Thus, the small intestine was subject to a substantial decrease in enteral stimulation at weaning and the first days thereafter for all piglets in the restricted intake group and most piglets from the high intake group. High feed intake level resulted in less affected structure (villous height and especially crypt depth). The importance of enteral stimulation for mucosal homeostasis is well documented (Kelly *et al.*, 1991b; McCracken *et al.*, 1995; Pluske *et al.*, 1997; Park *et al.*, 1998; Lallès *et al.*, 2004). Only feeding piglets with either sow's or cow's milk at a high intake level was shown to prevent harmful effects on the small intestine of weaning (Pluske *et al.*, 1996b; Van Beers-Schreurs *et al.*, 1998; Verdonk *et al.*, 2001). Although no significant effects of feeding level on transepithelial transport were found, at d7 feed intake during d1 to 4 was correlated negatively with Mannitol and positively with Na-Flu transport.

Boudry *et al.* (2004) showed that para cellular permeability as assessed by transmucosal resistance decreased at d2 post-weaning in the proximal jejunum and showed a 160% increase at the ileal site from d5 onwards. Our results suggest an increased paracellular transport (P_{app}Mannitol) at d4 and a decreased level at d7 post-weaning. In this study we found a decreased transport of intact HRP at d4 and d7 irrespective of the feeding level. Boudry *et al.* (2004) found that the HRP flux at the jejunum transiently dropped at d2 and d5 to approximately 25% of pre-weaning values and reached values at d15 that were still lower than

the initial values at weaning. By contrast, in the ileum and colon the HRP flux was not affected by weaning. Van der Meulen *et al.* (2003) found that the HRP flux was higher at d4 and d7 post-weaning in pigs weaned at 4 and 7 weeks and irrespective of creep feed intake. The post-weaning transepithelial transport of Na-Flu was not affected.

We showed (Verdonk *et al.*, 2001) that the paracellular transport of mannitol was increased significantly already at d1 post-weaning at the low intake level whereas it was not increased at the high intake level in pigs fed a liquid milk replacer.

The data of this experiment demonstrate that high feed intake level resulted in numerically less decreased villous height post-weaning which corroborates with earlier findings of Kelly *et al.* (1991a) and Pluske *et al.* (1996a) providing continuous nutrition to weaned pigs. McCracken and co-workers (1995) reported the lowest villous / crypt ratio at day 5, when comparing the sequential effect of the villous / crypt ratio of a liquid milk replacer on days 0, 1, 2, 5, and 7 post-weaning.

We found that villous height at d7 post-weaning was positively correlated with feed intake. This correlation has been reported earlier (Kelly *et al.*, 1991b; McCracken *et al.*, 1995; Pluske *et al.*, 1996b; Van Beers-Schreurs *et al.*, 1998). A high feed intake level resulted in higher villi and deeper crypts compared to a restricted intake level (below maintenance requirement till d3 and increasing to 2 x maintenance requirement for energy at d6).

The data presented in this article show that weaning induces acute and long-lasting changes in gut morphology and permeability. Intake level of dry pelleted feed affects these changes positively. Voluntary intake of dry feed however was lower than that of milk pre-weaning and could not fully prevent the changes. Restricted intake (below maintenance till d3 and increasing till 2 x maintenance requirement at d6) resulted in values for gut morphology and permeability similar to values for the high intake level.

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Chapter 6

Gut integrity in weaned piglets fed wet or pelleted diets at equally restricted levels of energy intake*

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Abstract

In total 30 piglets were weaned at 28 days of age to study the gut integrity when feeding either wet or pelleted diets at a restricted energy level. On days 0, 2 and 6 post-weaning, 5-cm mucosal slices from the proximal and middle part of the small intestine were taken to assess: 1) the *in vitro* trans-cellular and para-cellular epithelial permeation routes, using_radio-labelled dipeptide glycyl-L-sarcosine ([^{14}C]GlySar) and [2-^{3}H]mannitol as markers, respectively; 2) gut morphological indices (villi/crypt sizes); 3) the mRNA expression levels of pro-inflammatory interleukin 1β (IL-1β) and IL-8 in the mucosa. In general, the results documented that the days post-weaning exerted more impact on the measurements than the physical form of the diet. The trans-cellular transport (P_{app}GlySar) was elevated ($P < 0.05$) in piglets fed the pelleted diet. The histo-immunological changes in the intestine such as villous heights, crypt depths and mRNA expression levels of interleukin 8 (IL-8) were not affected by the dietary physical form. Pearson's correlation coefficients (r) were found meaningful ($P<0.05$) only for P_{app}GlySar with crypt depth (r= -0.44), and for lg (mRNA IL-8) with villous height (r=0.42).

Introduction

In farming conditions the digestive system of newly weaned piglets is immediately deprived of mother's milk, and a new diet in dry (pellets or mesh) or liquid form is offered instead. This deprivation is part of the weaning stress and is manifested particularly during the first few days in terms of low feed intake, independently of dietary composition (McCracken *et al.*, 1995).The gut integrity in terms of villous height (VH) and crypt depth (CD) in the small intestine of weaned piglets may be maintained after weaning by a high intake of sow's milk (Van Beers-Schreurs *et al.*, 1998) or cow's milk (Pluske *et al.*, 1996; Verdonk *et al.*, 2001). A high intake of dry feed could not completely maintain gut integrity (Van Beers-Schreurs *et al.*, 1998). Low intake of milk or non-milk liquid diets prevented the gut atrophy (Núñez *et al.*, 1996; Pluske *et al.*, 1996; Van Beers-Schreurs *et al.*, 1998; McCracken *et al.*, 1999; Spreeuwenberg *et al.*, 2001). Feeding a non-milk liquid diet led to a higher feed intake and higher villi on days 8 and 11 post weaning compared to the same diet in pelleted form (Deprez *et al.*, 1987). The authors implied that pellets may be more abrasive to the gut brush border, possibly causing a decrease in the villous height by increasing the shedding/premature extrusion of enterocytes, although the higher villi in piglets fed the liquid diet could also be associated with a greater energy intake. To our knowledge, no comparative studies are available on the gut integrity in piglets fed liquid or dry pelleted, non-milk diets at equally restricted levels of energy intake.

The present study was designed to evaluate the effect of physical form of a non milk diet fed either or as liquid or dry pellets in newly weaned piglets fed at restricted intake level on the gut integrity. Morphology (VH, CD), pro-inflammatory response parameters (mRNA gene expression level of interleukin (IL)1_{β} and interleukin 8) and barrier function (*in vitro* transepithelial transport of nutrients) of the small intestine are used as indicators.

Materials and methods

Animals, housing and feeding.

A study was undertaken with 30 piglets (Yorkshire x [Dutch Landrace x Yorkshire]). Animals were weaned at 26 days of age at the average body weight of 7.3±0.9 kg. They were

transported for approximately 60 km and afterwards housed individually in 50*90 cm^2 floor pens with transparent plastic walls for a period of 6 days. Each pen was equipped with a plastic feeder trough and water was supplied via a nipple drinker. Six piglets were directly sacrificed and sampled at the day of weaning, whereas the remaining 24 animals were randomly allotted to two equal experimental groups of 12 piglets. Group 1 was fed the experimental diet in a pelleted form, whereas group 2 received the same diet in a wet form (dry pellets mixed with water 0.5 h before feeding; feed: water = 1:2.5). Animals were offered 3 meals daily. The feeding levels were gradually increased over the time, from 0.5 x maintenance requirement for energy (NE_m) on the day of weaning (day 0), until 2.0 x NE_m on day 6 (Table 1), as recommended/established by the Dutch Board for Feedstuff Evaluation (Centraal Veevoederbureau, 2002). The composition of the experimental diet is shown in Table 2. Feed refusals were collected and weighed daily. In case of wet feeding, they were dried in two batches (for days 1+2 and days 3+6).

Table 1 Feeding schedule

Physical Form	Feeding level (× maintenance level for energy requirement (NE_m))						
day	0	1	2	3	4	5	6
Dry	0.5	0.75	1	1.5	2	2	2
Liquid	0.5	0.75	1	1.5	2	2	2

Sampling of the gut for histology, permeability and inflammatory response measurements.

On days 0 (weaning), 2 and 6 post-weaning, six piglets were euthanized to collect 5-cm slices of mucosa from the proximal and mid part of the small intestine (at the ligament of Treitz and 3 m distal of the ligament of Treitz, respectively). These piglets were weighed and anaesthetised by injection of Nembutal and inhalation of a mixture of N_2O / O_2 (ratio 2/1) and isoflurane. The concentration of isoflurane was adjusted to the depth of the narcosis (Guedel, stadium III, phase 2). A midline laparotomy was performed.

Table 2 Ingredient and nutrient composition of the diet

Ingredients	(g/kg)
Barley	482.6
Maize	200.0
Soy flakes: crude protein 480 g/kg	65.0
Fish meal: crude protein > 680 g/kg	45.0
Potato protein	50.0
Sweet whey powder	50.0
Molasses (sugar cane)	30.0
Soy oil	15.0
Maize starch	21.5
Limestone	17.0
Mono calcium phosphate	9.0
Salt	2.0
Mineral-vitamin premix	10.0
L-lysine HCl	1.9
DL-methionine	0.7
L-tryptophan	0.3
Nutrients	(g/kg)
Dry Matter	889
Ash	54
Crude protein (N x 6.25)	181
Crude fibre	32
Crude fat (ether extract)	42
Sugar	55
Starch	345
Ca	9.8
Mg	1.5
Total P	6.2
Na	1.8
K	8.2
Cl	4.1
Cu (mg/kg)	31
Zn (mg/kg)	107
Fe (mg/kg)	216

Morphology.

For determination of villous height and crypt depth, mucosal slices of the proximal and mid small intestine were cut open longitudinally at the anti-mesenteric attachment, prepared on dental wax with the villi on the upper side, and fixed in 0.1 M-phosphate-buffered formalin solution (40 mL/L). A three-mm wide zone from the mesenteric site was cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections were cut (5 µm) and stained with the periodic acid Schiff method (PAS staining). From the PAS stained sections, the morphometric indices were determined.

Gene expression.

The mRNA expression level in epithelium for interleukin 1β (IL-1β) and the interleukin 8 (IL-8) relative to the level of glyceraldehyde phosphate dehydrogenase (GAPDH) was quantified by Real Time reverse transcription Polymerase Chain Reaction procedures (RT-PCR) and used as a marker for inflammatory response. IL-1β is a proinflammatory cytokine and IL-8 is a chemokine, a potent neutrophil attractant which is constitutively expressed by intestinal cells (Stadnyk, 1994). Upregulation of IL-8 can occur by cytokines, such as IL-1β but also by attachment and invasion of epithelial cells by bacteria (Kagnoff & Eckmann, 1997). In the intestine, many cell types can secrete chemokines; these include monocytes, macrophages and epithelial cells (Eckmann *et al.*, 1993). Tissue samples of the proximal jejunum were cut open at the anti mesenteric side and rinsed with phosphate buffer solution. The epithelium was scraped off, deep frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from tissue samples (±30mg) using a QIA shredder and RNeasy kits (Qiagen, Crawley, UK) according to the manufacturer's protocol and quantified determining the relative optical densities (OD) at 260 nm by spectrophotometry using a Spectramax plus spectrophotometer (Molecular Devices). Per sample 750 ng RNA was used for RT reaction, using 1.5 µL 10 U/µL Avian Myeloblastoma Virus (AMV) reverse transcriptase (Promega Corporation, Madison, WI), 2.5 µL 100 µM T_{15} VN-primer, 1.5 µL 10 U/µl recombinant RNasin ® Ribonuclease, 3 µL 10 mM dTNP and 6 µL 25 mM $MgCl_2$ and 6 µL 5x AMV buffer (Promega). The volume of the reaction mixture was adjusted to 30 µL by addition of RNase free water. The RT-PCR was performed using the Taqman Universal Mastermix (PE Applied Biosystems). Amplification and detection of specific products were performed using the I-cycler PCR apparatus (BioRad). Each reaction mixture contained 13.2 µL PE mastermix

(Perkin-Elmer, Applied Biosystems) 1.13 µL forward primer (20 µM stock), 1.13 µL reverse primers (20 µM stock) and 1.0 µL Taqman probe (5 µM stock). During the PCR, the following temperature profile was used: an initial enzyme activation step of 95°C for 10 min followed by 50 cycles each of 60°C for 1 min followed by 95°C for 15 sec. Cytokine and GAPDH mRNA-specific amplification primers and probes were designed using the Primer Express software programme (PE Applied Biosystems).

The primer-probe sets used were:

- for GAPDH:

forward primer: AGC CTC AAG ATC ATC AGC AAT G

reverse primer: ACT GTG GTC ATG AGT CCT TCC A

taqman probe: CAC CCC TGG CCA AGG TCA TCC A

- for IL-1β:

forward primer: TCT GCC CTG TAC CCC AAC TG

reverse primer: CTC TGG CGG CCT TTG GA

taqman probe: CTC TCA AGC AGA ACA AAA GCC CGT CTT CC

- for IL-8:

forward primer: CTG TTG CCT TCT TGG CAG TTT

reverse primer: CAT CGA AGT TCT GCA CTT ACT CTT G

taqman probe: CCT GCT TTC TGC AGC TCT CTG TGA GGC

All probes were labelled with the fluorescent reporter dye 5-decarboxyfluorescein (FAM) at the 5' end and the quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end.

Transepithelial transport.

Transepithelial transport was measured *in vitro* using 5-cm mucosal slices from the proximal small intestine. Each sample was placed in the TNO transport chamber and [^{14}C]GlySar (Cambridge Research Biochemicals, Northwich, UK) was used to quantify transcellular transport, whereas [2-^{3}H]mannitol (ICN Biomedicals, Zoetermeer, NL) was addressed for paracellular routes. GlySar is a small hydrophilic compound with a molecular weight of 146 Da. GlySar is transported mainly via a transcellular route with an H^+-coupled di/tri-peptide carrier (Duizer, 1999). Mannitol has a molecular weight of 182 D and is transported mainly via a paracellular route (Duizer, 1999). Intestinal tissues were rinsed with an ice-cold buffer solution of HEPES-buffered phenol red free Dulbecco's Modified Eagles Medium (DMEM) and cut open longitudinally. The tissue was placed with the mucosa on the upper side on a flat underground, and the mucosal layer was scraped of the muscle layer using a blunt razor blade. Samples of the mucosal layer were taken using a nine-mm steel punch. Flat sheets, in which isolated intestinal segments (0.2 cm^2) separate a 1.5 ml mucosal and a 1.5 ml serosal compartment, were placed in the Ussing chambers. The effective exposed area in the Ussing chamber was 0.196 cm^2. The radio-labeled GlySar and mannitol were mixed with unlabelled compounds to yield final concentrations of 10 μM. The donor compartment (mucosal side) was filled with 1.25 ml HEPES DMEM medium containing radio-labeled GlySar (10 μM) and mannitol (10 μM). The receptor compartment (serosal side) was filled with 1.25 ml HEPES DMEM medium. Both compartments were aerated (O_2 / CO_2, 95 / 5) at a temperature of 37°C and stirred by gaslift. At indicated time points (15, 30, 45, 75, and 105 min), 0.5 ml samples were taken from the serosal side and the volume was reconstituted with DMEM without phenol red. ^{3}H and ^{14}C radioactivity was determined in the samples and the tissue (at the end of the experiment) by liquid scintillation counting (LSC) using DOT-DPM™ (Digital Overlay Technique using the Spectrum Library and the External Standard Spectrum) for quench correction.

Permeability coefficients (P_{app}) were determined based on the appearance of the probe at the serosal side according to the following equation:

$$P_{app} = R / (A * C_0) \quad [1]$$

Where: P_{app} = permeability coefficient from mucosal to serosal side (cm/sec); R = permeability rate (mol/sec); A = exposed intestinal area (cm^2); C_0 = initial mucosal concentration of test substance (mol/mL).

Statistical analysis.

Data were analyzed using the GLM procedure of the SAS (SAS Inst., Inc. Cary, NC, version 8.1), according to the following model:

$$y_{ij} = \mu + T_i + e_{ij} \quad [2]$$

where:

y_{ij} = dependent variable; μ = overall mean; Ti = fixed effect of treatment (i=1…5); e_{ij} = error term. The combinations of physical form of the diet and day post weaning were defined as treatments. The effect of physical form of the diet and day of dissection were evaluated using the contrast statement. Pearson correlation analysis was performed to evaluate a possible statistical correlation between feed intake, histomorphometric indices and trans-epithelial transport. Significant differences between least squares means were tested at the probability $P<0.05$, whereas tendencies were assigned at $0.05 <P<0.10$.

Results

Clinical health.

Overall, no clinical (acute) health problems related to any specific dietary treatment were encountered by piglets used in this short-term experiment (weaning at 26 days of age to 6 days post-weaning). None of the piglets received medical treatment during the experimental period. The rate of factual feed consumption was lower than the quantity offered, irrespective of the treatment. In consequence, the average growth rate was also low, although it varied very substantially between animals. Piglets fed the pelleted and wet diets through the 6 day experimental period grew 40±84 and 50±69 g per day, respectively.

Feed intake.

The daily rations were formulated to be isocaloric and isonitrogenous, and addressed as dry pellets (without mixing with water prior to feeding) or as slurry (the pellets mixed with water prior to feeding) at equally restricted net energy intakes. However, irrespective of the treatment, all piglets consumed factually less feed than their daily allowances offered. By wet feeding, the average feed intakes (re-calculated to 90% DM content as in pellets) over days 1 to 6 post-weaning were 40±23, 81±45, 116±60, 139±72, 170±88 and 193±100 g/day/piglet, respectively. These amounts on days 2 and 6 were thus by 28 and 38% lower than predicted. By dry feeding, the factual amounts of consumed pellets (at 90% DM) over days 1 to 6 post-weaning were 3±1, 21±27, 81±83, 160±87, 195±43 and 174±65 g/day/piglet, respectively. These amounts on days 2 and 6 were equal to 11% and 55% of the daily allowances, respectively. This means that piglets which received wet daily rations consumed more (by 17%) on day 2 post-weaning, and less (by 17%) on day 6 post-weaning, compared to the piglets fed dry pellets. To avoid a risk of biasing the responses of gut histomorphology, permeability and pro-inflammatory cytokines due to the differences in feed consumption, we used actual feed intake as a co-variable in the statistical model.

Permeability and gene expression of proinflammatory cytokines.

The results on P_{app}GlySar, P_{app}Mannitol and gene expression levels for interleukin 8 (mRNA IL8) in animals on different dietary treatments are presented in Table 3. We decided to omit the values of mRNA expression level of IL1 since only 5 readings were above the detection

limit (1 pig at the day of weaning, 2 pigs fed the liquid diet at day 6, and 2 pigs fed the dry diet at day 2). The physical form of the diet did not significantly affect the mRNA IL8 expression level and P_{app}Mannitol, but significantly affected the P_{app}GlySar. Compared to feeding with pellets, the wet feeding resulted in lower P_{app}GlySar (-35%; $P<0.05$) compared to feeding with dry pellets. The days post-weaning of piglets affected ($P<0.05$) the mRNA IL8 expression level and the gut permeability. The lg (mRNA IL8) was 81% and 79% that of the value at the day of weaning for d2 and d6 respectively. The paracellular transport (P_{app}Mannitol) was three fold at day 2 ($P<0.001$) and two fold at day 6 ($P<0.05$) compared to the day of weaning. The transcellular transport (P_{app}GlySar) was increased at day 2 (+140%; $P<0.01$) and at day 6 (+164%; $P<0.001$) compared to the day of weaning. The values at day 2 and day 6 were similar.

Villous height and crypt depth.

Interrelationship between the physical form of daily rations for piglets and the histomorphological indices on days 0, 2 and 6 post-weaning is illustrated in Table 4.

No significant interaction between the dietary treatment and age of piglets on the intestinal histomorphology was found. The dietary physical from did not significantly affect the villous height, crypt depth and villous height to crypt depth ratio at the proximal and mid small intestine of piglets. Numerically higher villi (by 15%) and deeper crypts (by 7%) were found in piglets fed pellets. Wet feeding resulted in a progressive decrease of villous height until day 6 post-weaning, whereas feeding dry pellets led to a partial restoration of villous height at day 6 resulting in values similar to those recorded at weaning.

There was a clear age effect on the changes in villi/crypt dimensions. Crypts tended ($P<0.10$) to be more shallow at day 2 (by 16%) and deeper (by 15%) at day 6 compared to day 0 (weaning), irrespective of the dietary treatment. Besides, crypts were deeper at day 6 compared to day 2 ($P<0.001$). The decrease at day 2 was more for the liquid diet (by 22%) than for the pelleted. The mean villous length to crypt depth ratio was significantly decreased at day 2 (by 13%; $P>0.10$) and further decreased at day 6 (by 35%; $P<0.05$) compared to the day of weaning. This decrease was most clear for the liquid diet. Mean villous height was significantly decreased (by 25%) at day 2 and day 6 post-weaning in comparison to weaning (day 0).

Table 3 Permeability coefficients for mannitol (P_{app}Mannitol) and GlySar (P_{app}GlySar) (10^{-6} cm/s) and the gene expression level for interleukin 8 (mRNA IL8) in proximal jejunal tissue of pigs fed either liquid (**L**) or dry pelleted (**D**) diets at restricted intake levels during days 0 to 6 post weaning.

Physical form of the feed	No feed	Liquid (**L**)		Dry pellets (**D**)		Statistical significance				
Day post weaning	0	2	6	2	6		Physical form	Post weaning days		
						SEM	**L** vs. **D**	0 vs. 2	0 vs. 6	2 vs. 6
Gene expression level										
- lg mRNA IL8	-2.57 [b]	-1.98 [a]	-2.09 [ab]	-2.19 [ab]	-1.99 [a]	0.18	ns	*	*	ns
Permeability coefficients										
- P_{app}Mannitol	5.1 [a]	14.3 [c]	13.2 [bc]	16.1 [c]	7.7 [ab]	2.5	ns	***	*	*
- P_{app}GlySar	7.8 [a]	16.3 [b]	17.0 [bc]	21.0 [bc]	24.1 [c]	2.4	*	**	***	ns

ns = not significant; t = $p<0.10$; * $p< 0.05$; ** $p<0.01$; *** $p<0.001$,

[abc] Means in a row without a common superscript differ significantly at $p<0.05$

Table 4 Villous height (µm), crypt depth (µm) and villous height to crypt depth ratio at the proximal (prox) and middle (mid) small intestine of weaned pigs fed either liquid (**L**) or dry pelleted (**D**) diets at restricted intake levels during days 0 to 6 post weaning.

Physical form of the feed	No feed	Liquid (**L**)		Dry pellets (**D**)		Statistical significance				
Day post weaning	0	2	6	2	6		Physical form	Post weaning days		
						SEM	**L** vs. **D**	0 vs. 2	0 vs. 6	2 vs. 6
Villous height										
Prox	520 ^{b}	351 ^{a}	353 ^{a}	396 ^{ab}	434 ^{ab}	44.4	ns	*	*	ns
Mid	432 ^{b}	346 ^{ab}	278 ^{a}	337 ^{ab}	361 ^{ab}	45.1		ns	t	ns
Mean	476 ^{b}	349 ^{a}	315 ^{a}	367 ^{a}	398 ^{ab}	35.4	ns	**	*	ns
Crypt depth										
Prox	235 ^{ab}	182 ^{a}	289 ^{b}	209 ^{a}	286 ^{b}	21.4	ns	ns	t	***
Mid	201 ^{b}	155 ^{a}	218 ^{b}	186 ^{ab}	218 ^{b}	15.5	ns	ns	ns	**
Mean	218 ^{bc}	169 ^{a}	253 ^{c}	198 ^{ab}	252 ^{c}	16.4	ns	t	t	***
Villous to crypt ratio										
Prox	2.3 ^{b}	2.0 ^{ab}	1.4 ^{ab}	2.0 ^{ab}	1.6 ^{ab}	0.26	ns	ns	*	t
Mid	2.4 ^{b}	2.3 ^{b}	1.4 ^{a}	1.9 ^{ab}	1.7 ^{ab}	0.32	ns	ns	*	t
Mean	2.3 ^{c}	2.1 ^{bc}	1.4 ^{a}	1.9 ^{bc}	1.6 ^{ab}	0.25	ns	ns	*	*

ns = not significant; t = $p<0.10$; * $p< 0.05$; ** $p<0.01$; *** $p<0.001$,

^{abc} Means in a row without a common superscript differ significantly at $p<0.05$

Table 5 Pearson correlation coefficients between the histology parameters, transepithelial transport at the proximal small intestine, interleukin 8 (IL8) mRNA expression level and dry matter intake of piglets fed either liquid or pelleted diets at restricted intake levels during days 0 to 6 post weaning.

	P_{app}GlySar [1]	Villous Height	Crypt Depth	Villous / crypt Ratio	10log (IL8 mRNA)	Daily dry matter intake	
						Day 1-2	Day 3-6
P_{app}Mannitol [1]	0.64 *** [2]	ns	ns	ns	ns	ns	ns
P_{app}GlySar		ns	-0.44 *	-0.42 *	ns	ns	ns
Villous height			ns	nr[3]	-0.42 *	ns	ns
Crypt depth				nr	ns	ns	ns
Villous / Crypt					-0.46 **	ns	ns
10log (IL8 mRNA)						ns	ns

1 PappGlySar indicates transcellular transport, PappMannitol indicates paracellular transport

2 P-value of the model: t = P<0.10; * P< 0.05; ** P<0.01; *** P<0.001

3 not relevant

Discussion

To evaluate the adaptation of the gut in pigs at weaning in relation to dietary factors several approaches can be taken: level of intake, composition of the feed and physical form of the feed. There is some evidence that feeding a dry pelleted diet to newly weaned piglets can cause adverse effects on feed intake and causes increased morbidity. *Ad libitum* access to manufactured wet diets however may improve ADG by up to 70% compared to sow-reared littermates (Jack Odle and Robert Harrell, researchers at North Carolina State University, personal communication). Liquid non fermented feeds resulted in increased feed intake and average daily gain during 4 to 5 weeks post weaning when supplied prior to weaning (Toplis *et al.*, 1999; Blanchard *et al.*, 2004) or after weaning (Chesworth *et al.*, 2001; Hurst *et al.*, 2001). Villous height in the small intestine of piglets was increased by liquid feeding compared to dry feeding at d11 (Deprez *et al.*, 1987) or d28 post weaning (Hurst *et al.*, 2001) but results are often entangled with feed intake.

Information on gut functional features in weaned piglets when feeding diets in wet and dry pelleted forms energy-balanced is scarce.

Therefore, in the present study the effects of the dietary physical form (wet versus dry pellets) when offered at similar restricted intake levels to weaned piglets were evaluated. Irrespective of the treatment, it appeared that the anticipated feed consumption was not achieved, particularly on day 2 post-weaning. This phenomenon of feed refusal due to a weaning stress is well known in practice, and it does not seem to be affected by the physical form of diets.

We found that feeding the dry pellets elevated significantly the transcellular permeability (P_{app}GlySar), whereas the paracellular permeability (P_{app}Mannitol), histomorphological indices (villous height and crypt depth) and the pro-inflammatory cytokine IL-8 expression along the small intestinal epithelium of weaned piglets were similar in animals on both physical dietary forms. Our histo-morphological findings imply that at the restricted feeding, dry pellets cased no gut damage or abrasion of villi. This diet slightly stimulated (mechanically) the proliferation of crypt cells, as indicated by numerically deeper crypts at the proximal small intestine. Deprez *et al.* (1987) concluded that feeding a pelleted diet led to higher villi on day 8 and day 11 post weaning and deeper crypts on day 11 in the distal jejunum and ileum compared to the same diet in wet form. Unfortunately, the authors provided no information on the level of feed intake. Therefore, it may only be speculated that piglets fed the liquid diet had longer villi and deeper crypts due to consuming greater amounts

of specific dietary precursors for epithelial cell proliferation. In contrast to our findings, (Pluske *et al.*, 1996) reported that piglets consuming cows' milk developed no villous atrophy and crypt hyperplasia, whereas feeding a pelleted starter diet at equally restricted level (2.5 x MR for ME) led to shorter villi and elongated crypts. These authors found that the histological differences had no repercussions on growth and gut function, as assessed by brush-border enzyme activity and xylose contents. (Van Beers-Schreurs *et al.*, 1998) observed similar villous heights and crypt depths in the small intestine of weaned piglets when feeding a commercial diet (at 2.8 MJ DE/day), and sow' milk (at 2.5 MJ DE/day).

Several cytokines are produced by the epithelium of the intestinal mucosa (Stadnyk, 1994), fibroblasts, and immune cells. Cytokines can affect epithelial differentiation and proliferation through epithelial-mesenchymal and epithelial-immune cell interactions. Cytokines play a central role in immune cell response, but they also participate in the maintenance of gut-wall integrity. Changes in the cytokine network at weaning are could be expected, because abrupt changes in dietary and environmental factors lead to important morphological and functional adaptations in the gut. Pié *et al.* (2004) concluded that cytokine response in the gut of pigs at weaning could be divided into two periods: an early acute response (0 to 2 d postweaning) and a late response (2 to 8 d postweaning). Between day 0 and day 2, the levels of IL-1ß, IL-6, and TNF-α messenger RNA (mRNA) increased. Upregulation of IL-1ß mRNA occurred in most parts of the intestine, whereas IL-6 and TNF-α mRNA increased only at specific sites in the intestine. Between d 2 and d 8, the levels of IL-1$_{ß}$, IL-6, and TNF-α mRNA rapidly returned to preweaning values, except that the level of TNF-α mRNA remained high in the distal SI.

To date, the importance of cytokines in post-weaning development of the piglet small intestine as affected by the physical form of diets has not been well established. We found IL-1 mRNA expression level above the detection limit in few gut-wall samples only. This is in line with our observations in weaned pigs fed dry pellets at graded intake levels (Verdonk et al, unpublished). In another study, the expression level of genes encoding the mediators of inflammation in the jejunal tissue were assessed qualitatively in weaned piglets fed a milk-based diet (Verdonk et al., 2002). We found significantly higher IL-1$_{\beta}$ levels at the low milk intake level compared to the high milk intake level.

Interleukin-8 (IL-8) is a powerful chemotactic factor. Epithelial cells can secrete IL-8 in order to attract neutrophils. Neutrophils are the first line of defence against invading bacteria. Although, in present study we found no effect of dietary physical form on mRNA expression

level of IL-8, the absolute mRNA expression level of IL-8 was numerically higher at days 2 and 6 compared to the mRNA level of IL-8 at the day of weaning. This implies that there is a possible increased synthesis and release of IL-8. Thus, this study shows that mucosal cytokines are developmentally regulated and that dietary physical form at restricted intake levels does not seem to be involved in this regulation in parallel with maturation of the gut mucosa after weaning of piglets.

The intestinal tract acts as a filter and barrier. Intestinal permeability is a highly regulated dynamic process determined by interactions among several components including the mucus layer and epithelial factors. Transcellular and paracellular pathways are two routes for transepithelial permeation. In this study, the paracellular transport of a small molecule (P_{app}Mannitol) across the gut in piglets was dependent on the days post-weaning, i.e., significantly greater at days 2 and 6 post-weaning (three and two fold, respectively) compared to the day of weaning. Similar results (a two fold increase of paracellular permeability at days 2 and 4 post-weaning compared to the day of weaning) were found with piglets fed low amounts of milk (Verdonk *et al.*, 2001). Results of another study with weaned pigs in our lab (Verdonk et al, unpublished) suggested an increased paracellular transport of small molecules (P_{app}Mannitol) at d4 and a decreased level at d7 post-weaning in pigs fed at increasing (1 x NE_m to 2 x NE_m) and high intake levels (2.5 x NE_m). Boudry *et al.* (2004) showed that paracellular permeability as assessed by transmucosal resistance decreased at d2 post-weaning in the proximal jejunum.

Conclusions

Weaning-stress in piglets resulted in low feed intake, irrespective of the dietary physical form. The dietary physical form did not significantly affect the villous height, crypt depth and villous height to crypt depth ratio at the proximal and mid small intestine of piglets.

The dry pellets elevated significantly the transcellular permeability (P_{app} GlySar), whereas the paracellular permeability (P_{app}Mannitol) along the small intestinal epithelium of weaned piglets was similar for both wet and dry pelleted feeding.

The paracellular transport (P_{app}Mannitol) across the gut in piglets was age-dependent, i.e., significantly greater at days 2 and 6 post-weaning (three and two fold, respectively) compared to the day of weaning.

Mucosal cytokines (IL-1 and IL-8) are probably developmentally regulated and the dietary physical form at restricted intake levels does not seem to be involved in this. The effect of day post-weaning on synthesis of IL-8 is significant.

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Chapter 7

General discussion

General

Weaning at 4 weeks of age is a stressful event for pigs and involves complex social, environmental and dietary changes that interfere with gut development and adaptation. Stress has been reported to affect gut mucosal structure and barrier function ((Perdue, 1999; Fioramonti, 2003). Stress may be an initiating factor for weaning-associated alterations in the stomach and gut resulting in changed gastric motility and emptying, mucus secretion, permeability and water absorption. Signalling pathways involve the central nervous system, the HPA axis (Fioramonti, 2003) as well as cross talk between gut microbes and gut epithelial cells (Gaskins, 2003). In metabolic stress conditions epithelial cells can perceive commensal bacteria as a treat (Nazli *et al.*, 2004).

The weaning stress is associated with an immediate but transient drop in feed intake, which affects the intestinal architecture and the intestinal micro flora and results in a growth check. Data on the impact of weaning in combination with feed intake level on gut inflammatory response and barrier function in pigs however are scarce. The period of immediate anorexia after weaning is followed by a regenerative phase during which feed intake resumes. The regenerative phase is characterized by down regulating of many intestinal disorders and these are most probably stimulated by the increased feed intake.

The aim of the experiments described in this thesis was to study the effect of feed intake and dietary physical form in the de- and regenerative phase on the inflammatory response and barrier function and their relationship with morphological indices.

Figure 1 shows the conceptual framework of the post-weaning processes in pigs. The weaning stress results in low feed intake and (local) inflammatory responses in the small intestine. This results in a shift in microbial composition and/or a different reaction of epithelial cells to (commensal) luminal microbes resulting in up-regulating of an inflammatory response and increased permeability of the gut wall. This chapter summarizes and discusses the important findings in relation to post weaning nutritional strategy.

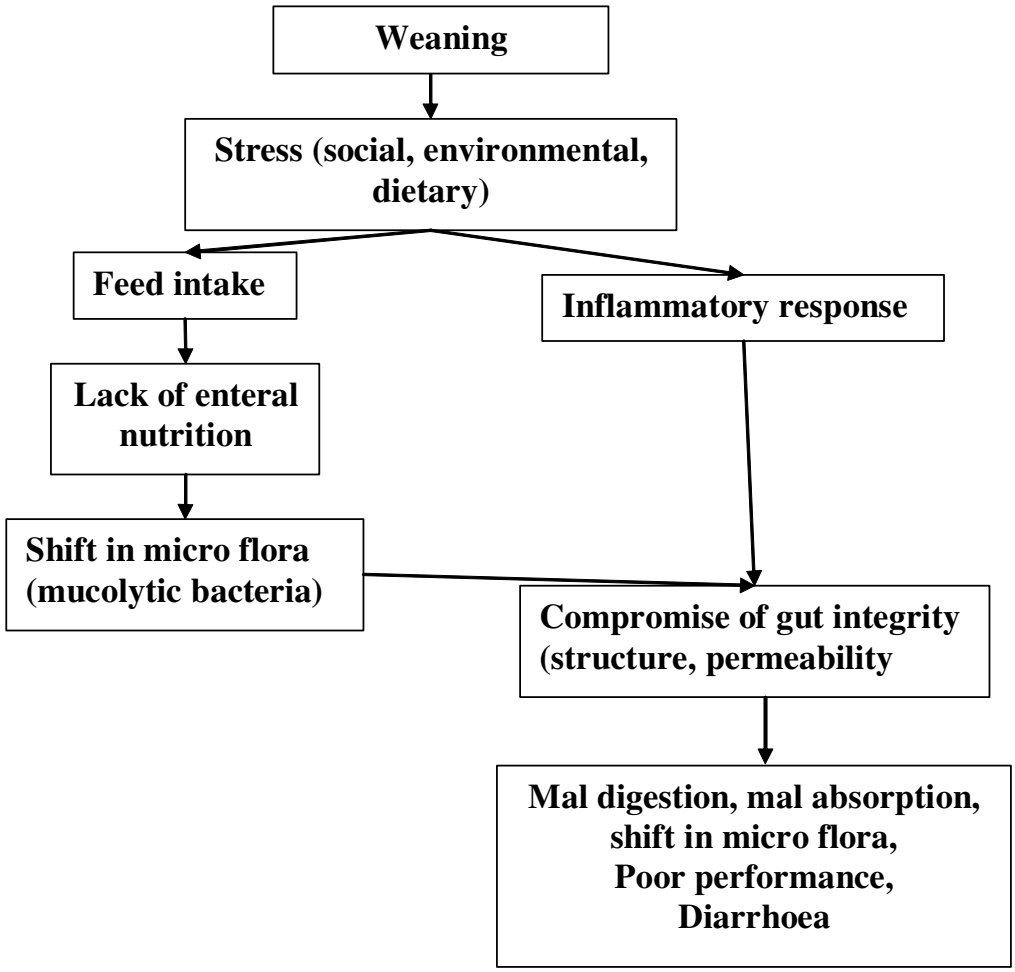

Figure 1 The weaning process and its consequences (adapted from Makkink (1993) and Spreeuwenberg 2002).

The literature shows an enormous variation in time after weaning at which responses were measured. This can be the reason for the large variation in responses between experiments with different designs. But even between experiments following a similar design the parameters related to gut wall integrity after weaning react in a very different magnitude to the change after weaning. This may be caused by differences in animals or small differences in treatments. Therefore we present a detailed design of each experiment in Table 5. In all experiments data on feed intake, body weight gain and gut morphology were collected. Samples of stomach and gut content, blood and intestinal tissue were taken to measure parameters on inflammatory response, barrier function and digestive capacity in a period of maximum 14 days post-weaning.

Feed intake

Feed intake is important, because lack of intake is the main determinant for changed gut morphology after weaning ((Makkink, 1993; Pluske *et al.*, 1997; Spreeuwenberg, 2002). Feed intake also affects barrier function and inflammatory response (Gannesunker *et al.*, 1999; McCracken *et al.*, 1999).The individual feeding behaviour of piglets immediately after weaning shows considerable variation (Bruininx, 2002) and is affected by gender and body weight at weaning.

Intake of dry creep feed during lactation is generally small and variable and unlikely to significantly influence weaning weight, particularly in piglets weaned at 3 weeks of age or younger (Pluske *et al.*, 1995). Offering creep feed as a gruel/slurry (1:2 meal to water) may enhance the intake of dry matter before weaning (Toplis *et al.*, 1999; Blanchard *et al.*, 2000). Offering suckling pigs liquid milk diets significantly increased the intake of nutrients compared to dry creep feed and increased the pre-weaning growth rate (reviewed by King and Pluske (2003)). Supply of liquid milk replacer to piglets both prior to weaning and during the first week post weaning resulted in 10% heavier pigs at 120 days of age compared to no supplementation before weaning and weaning onto dry starter feed (Dunshea *et al.* 1997 cited by King and Pluske (2003)). Bruininx (2002) showed that supplementation of a highly digestible and complex creep feed resulted in a higher creep feed intake than a feed with a composition as in practice, which is less digestible. In the experiments described in this thesis piglets were not supplemented during the suckling period to prevent additional variability between piglets because of variability in pre weaning creep feed intake. We compared individually housed pigs with high voluntary feed intake (VFI) with pigs which were fed restricted at either a predetermined percentage of VFI of pigs fed *ad libitum* or a maximum amount of feed. In the experiments using dry feed, piglets with high VFI were selected based on daily VFI figures at d3 post weaning from a pool of pigs of pigs fed *ad libitum*. The number of piglets in the pool was 2 or 3 times the required number of pigs with high VFI. Pigs fed with high VFI were compared with pigs fed at restricted (0.25 to 1 x requirement for maintenance) / increasing feeding level during the during the immediate post weaning period.

In general with this approach the average intake of pigs fed *ad libitum* or high intake was 2 to 7 times higher compared to the average intake of the pigs fed restricted or at the low level.

The factual contrast depends on experiment and day post weaning. However, in some experiments variation in feed intake between animals within experiments at both the high and low level was still high (Table 6). This might be related to the weaning procedure as well as genetic factors determining individual stress responsiveness of the pigs. The amount of feed offered was not always consumed by pigs fed both a high or low predetermined amount. This indicates that these amounts can still be considered as *ad libitum*. The energy intake of pigs fed restricted reached maintenance level at day 4 post weaning in most experiments. So this shows the importance of comparisons within experiments.Starvation and under-feeding result in lack of nutrients in the gut lumen. This lack of luminal nutrition leads to a compromise in gut integrity (McCracken *et al.*, 1995; McCracken *et al.*, 1999; Spreeuwenberg *et al.*, 2001)).

Morphology

The data in Table 1 and 2 suggest a non linear relationship between time and villous height (VH) showing a transient decline. The largest reduction in VH was seen at d1-d4 at the proximal and at d4-d7 at the mid small intestine. High intake of liquid milk resulted in the smallest and shortest dip in VH. The VH was maintained by a high milk intake. Also at d14 VH reached pre-weaning values at a high intake level irrespective of the dietary composition. In general a high intake level resulted in higher VH compared to low intake levels.

Table 1 Villous height (%) at the proximal small intestine relative to the value at the day of weaning for pigs fed at a low (L), increasing (I) or high (H) feeding level. The initial value at weaning is taken as 100.

Ch	2		3				4			5		6	
	milk		smp	smp	fm	fm	dry crumble			dry pellet		slurry	dry
day	H	L	H	L	H	L	H	I	L	H	I	I	I
1	90	85											
2	93	64										68	76
3							62	39	39				
4	94	75	65	51	49	47				67	74		
5													
6												68	83
7			63	51	61	52	65	59	57	76	63		
14			91	65	89	63							

Table 2 Villous height (%) at the mid intestine relative to the value at the day of weaning for pigs fed at a low (L), increasing (I) or high (H) feeding level. The initial value at weaning is taken as 100.

Ch	2		3				4			5		6	
	milk		smp	smp	fm	fm	dry crumble			dry pellet		slurry	dry
day	H	L	H	L	H	L	H	I	L	H	I	I	I
1	114	115											
2	110	80										80	78
3							70	59	59				
4	92	83	70	57	57	51				81	70		
5													
6												64	83
7			68	65	63	62	55	50	44	89	72		
14			128	86	99	90							

Crypts were shallower during d1-4 compared to the value at weaning especially for the pigs at a low intake level. At high intake level crypt depth (CD) was increased compared to weaning values up to 50% and 100% at d7 and d14 respectively. Data on crypt depth are shown in Table 3 and 4.

A diet based on skimmed milk protein (SMP, highly digestible protein) resulted in higher VH at d4 compared to a diet containing feather meal protein (FM, low protein digestibility). Diets were formulated on estimated requirements for growing pigs of 60 kg and made equal for ileal digestible lysine, methionin and cystin, threonin and tryptophan. Maybe that the SMP diet resulted in higher content of nutrients (indispensable amino acids, functional components, growth factors) in the digesta supporting the gut mucosa compared to the FM diet. The higher content of indigestible protein in the FM diet may have resulted in increased proteolytic fermentation in large and possibly small intestine, negatively affecting gut integrity. This effect of dietary protein source is in contrast with earlier findings of Makkink (1993) and McCracken *et al*. (1999), who did not find an effect of protein source on gut morphology, but, they compared protein sources like SMP, soy bean meal and fish meal which have similar ileal protein digestibility.

Table 3 Crypt depth (%) at the proximal small intestine relative to the value at the day of weaning for pigs fed at a low (L), increasing (I) or high (H) feeding level. The initial value at weaning is taken as 100.

Ch	2		3				4			5		6	
	milk		smp	smp	fm	fm	dry crumble			dry pellet		slurry	dry
day	H	L	H	L	H	L	H	I	L	H	I	I	I
1	94	88											
2	106	103										77	89
3							119	106	106				
4	125	116	138	89	110	103				144	113		
5													
6												123	122
7			151	117	151	116	154	133	126	155	135		
14			194	117	161	145							

Table 4 Crypt depth (%) at the mid small intestine relative to the value at the day of weaning for pigs fed at a low (L), increasing (I) or high (H) feeding level. The initial value at weaning is taken as 100.

Ch	2		3				4			5		6	
	milk		smp	smp	fm	fm	dry crumble			dry pellet		slurry	dry
day	H	L	H	L	H	L	H	I	L	H	I	I	I
1	94	89											
2	90	95										77	93
3							112	102	102				
4	118	106	120	96	104	91				129	116		
5													
6												108	108
7			138	110	127	101	145	155	150	150	119		
14			161	93	151	112							

Weaning is associated with villous atrophy caused by an increased rate of cell loss and a decreased rate of cell renewal (Pluske *et al.*, 1997). An increased rate of cell loss can be due to apoptosis, which is an active process of gene directed cellular self-destruction or programmed cell death. Two main cellular signaling pathways are involved in the apoptotic program

i) an intrinsic pathway, primarily induced by endogenous stressors such as oxidative stress and

ii) an extrinsic pathway activated by binding of special ligands such as tumor necrosis factor α to receptors on the cell surface (Fleck & Carey, 2005).

The small intestine is a sensitive organ for the induction of apoptosis following patho-physiological and physiological stressful conditions including lack of luminal nutrition, ischemia/reperfusion, burn trauma, treatment with anti cancer agents, zinc deficiency, aging (Fukuyama *et al.*, 2001). Apoptotic cell clearance is normally efficient and non-inflammatory and may often be mediated by neighboring cells (Henson *et al.*, 2001). Starvation clearly induces a hypoproliferative state in the whole gastro intestinal tract but the response to refeeding is very rapid in most parts of the gut (Ortega *et al.*, 1996; Dou *et al.*, 2001; Marion *et al.*, 2002) . The structural and functional changes that occur during the intestinal adaptation are dramatic. The mechanisms that initiate, maintain and ultimately terminate the process of hyperplasia and hypertrophy are unknown. During adaptation the expression of about 1700 genes (10% were significantly changed mainly related to 14 physiological pathways of which one was the proteasome pathway (Otterburn *et al.*, 2005).

Activated or stressed intestinal epithelial cells can produce a large number of chemokines and cytokines but the repertoire of the cells nonetheless is limited. The response mainly serves to express and secrete multiple cytokines with proinflammatory and chemotactic functions (Eckmann *et al.*, 1993; Jung *et al.*, 1995). In our studies we found expression of IL-1 at the mid small intestine at d1 to d4 post weaning especially at the low intake level, whereas IL-1 values were above the detection level in few samples of the proximal small intestine at d2 to d7 in 2 other experiments. TNFα expression in samples of the mid small intestine was not found. IL-8 expression levels were detectable in samples and were affected by day post-weaning but not by feed intake level. Pié *et al.* (2004) showed that gene expression of the proinflammatory cytokines IL-1 and TNFα was increased during the first 2 d post-weaning but not during d5-d8. They showed IL-8 expression only in the colon during d5-d8.

Intestinal epithelial cells stimulated by enteric microbes also produce a range of products and inflammatory mediators other than cytokines like prostaglandin E_2 and NO.

In our experiment we did not find any evidence for increased gene expression of COX2 and iNOS, enzymes involved in the arachidonic acid pathway and the NO pathway respectively.

Permeability

Intestinal permeability comprises a passage of molecules between epithelial cells (paracellular permeability) and through epithelial cells (transcellular permeability). Increased transepithelial permeation of macromolecules is found to occur in neonates and might me important for acquiring passive and active immunity.

Transcellular passage of macromolecules is greatly diminished by cytoplasmatic or lysosomal digestive activities within the enterocytes. Passage of molecules by the paracellular pathway (the extracellular route) via the so called tight junctions depends on passive diffusion. The permeability and size selectivity of the paracellular route varies along both, the longitudinal and the crypt-villous, axis of the gastrointestinal tract. Stress, bacterial microflora, viruses and epithelial cell damage have been reported to affect gut integrity and gut barrier function. Exposure of humans and rodents to acute stress can alter epithelial functions, stimulating secretion of ions and water. Adherence of enteropathogenic Escherichia coli (EPEC) to intestinal epithelial cell mono-layers disrupted tight junction and barrier function The cumulative flux of the paracellular marker mannitol was four-to-fivefold with wild-type EPEC compared to non-adherent isogenic type (Spitz *et al.*, 1995). Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. Environmental as well as genetic factors are important in determining stress responsiveness of the intestinal mucosa. Stress-induced mucosal barrier defect was a combination of increased paracellular leakage via the tight junctions as shown by increased conductance and trans cytosis of macromolecules, as shown by endosomal uptake and flux of horse radish peroxidase (HRP) (Söderholm *et al.*, 2002). The permeability of the porcine jejunal epithelium to HRP did not change after ETEC infection in weaned pigs (Egberts *et al.*, 1993). Rotavirus infection of Caco-2 cells caused disruption of the TJ and loss of TER in the absence of cell death, accompanied by increased transepithelial permeability to macromolecules of 478 and 4,000 Da. Infection was associated with increased production of lactate and reduced cellular ATP content (Dickman *et al.*, 2000). Altered jejunal permeability to macromolecules

during transmissible gastroenteritis virus was shown. An increased permeation of HRP occurred only during the early hours of infection when the virus disrupts the epithelium and not during diarrhoea when the epithelium is relatively undifferentiated but intact (Keljo *et al.*, 1985).On the other hand, mucosal colonization with Lactobacillus casei mitigated the barrier injury. The bacterial translocation was quantitatively lower and less frequent in test than in control rats (Llopis *et al.*, 2005). Most findings implicate pro inflammatory cytokines as agents which reduce barrier integrity. IL-1 and TNF_{α} increase the permeability whereas anti-inflammatory cytokines like trans-forming growth factor β and IL-10 decrease the permeability. The regulation of tight junction by cytokines occurs and many actors increase epithelial permeability acutely with no long term consequences. The size, shape and charge and other properties of probe molecules will determine the permeability. *In vivo*, increased permeability is important only if it results in patho-physiology (Perdue, 1999).

Permeability of intact horse radish peroxidase (HRP) and mannitol were both used as markers for the paracellular route. HRP is a molecule with high molecular weight and mannitol with low molecular weight.

Figure 2 shows the results of the permeability coefficients for mannitol with time after weaning. Data suggest a non linear response for mannitol permeability with transiently increased values except for the pigs fed the low milk intake.

At the proximal small intestine (group 5H, 5In, 6In_slurry and 6In_dry) the restricted intake level resulted in higher values for mannitol permeability than the increasing or high intake level. The permeability at the proximal small intestine of the large molecule HRP showed decreased values at d4 and d7 compared to the values at weaning irrespective of the intake level (see chapter 5).

At the mid small intestine (group 2H and 2L), the data suggest transiently increased values for mannitol permeability at the high intake level (black square). At the low intake level (black triangle) the mannitol permeability at d4 was increased even more and did not show any decrease at d4 (black triangle).

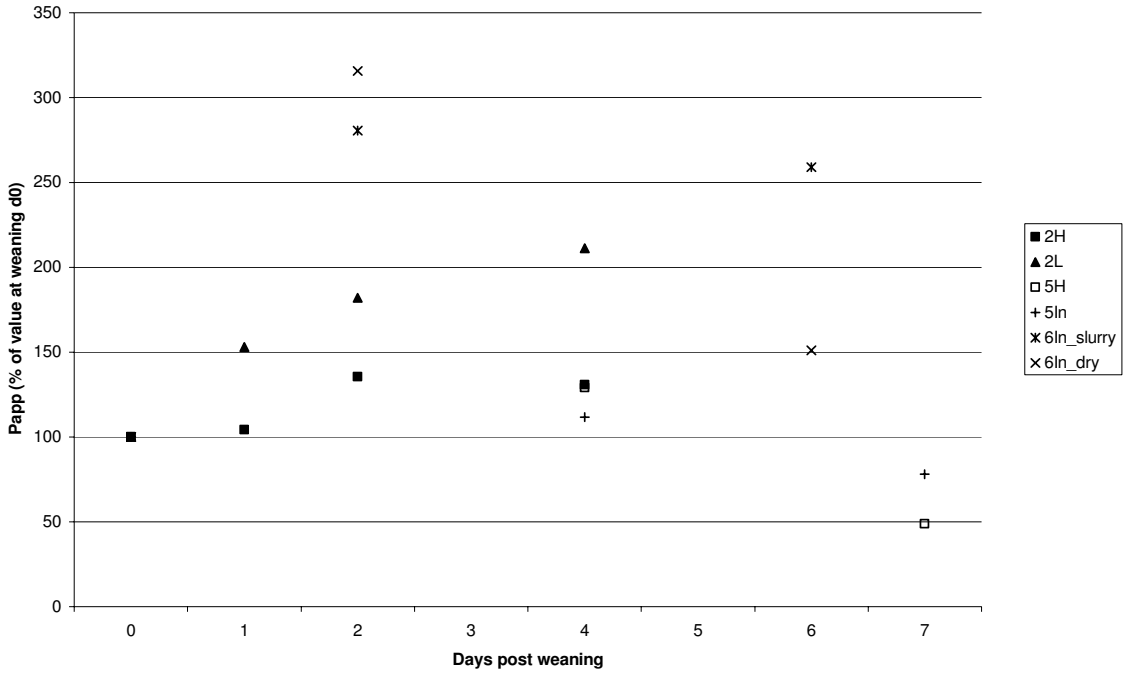

Figure 2 Permeability coefficients relative to the value at the day of weaning for mannitol in experiments from chapter 2 (middle small intestine) and chapter 5 and 6 (proximal small intestine) at low (L), increasing (I) or high (H) feeding level. The initial value at weaning is taken as 100.

The experiments described in this thesis suggest that on d1 after weaning gene expression of proinflammatory cytokines is changed concomitantly with a change in (paracellular) permeability of the mucosa followed by changes in gut morphology. Recovery of villous length occurs from day 4 onwards after an increase in CD has taken place. Changes in gut integrity are related to feed intake level. Individual feed intake was correlated in most experiments with body weight gain, morphology and in some experiments with inflammatory response parameters and barrier function. Therefore, individual feed intake is still a very important determinant for farmers. Farm management should be aimed at reducing stress at weaning and preparing the pig for the weaning process. The latter could be done by adapting the piglet pre-weaning already to the conditions post weaning like temporary weaning (removal from the sow or no possibility to drink milk) and mixing with piglets from other litters pre-weaning. Stimulation of intake of creep feed also will minimize post weaning problems. This could be realized formulating diets which i) support intake (size, physical form, smell, taste), ii) support a rapid shift of the micro flora to a diverse and stable micro flora and iii) supply specific nutrients which support mucus synthesis, maintain antioxidant status and exert anti inflammatory properties.

Future research using molecular techniques and (jejunal) pig cell lines may help to better understand the changes which occur in the gut tissue and micro flora in pigs post-weaning.

Table 5 Schematic overview over the experiments described in this thesis.

Chapter	2	3	4	5	6
Feeding frequency	4 x daily	4 x daily	4 x daily	3 x daily	3 x daily
Last feeding before dissection	2h	1h	1h	2h	2h
Weaning age	26	27	26	26	26
Weaning process	transport in truck for 10 km	transport in truck for 10 km	move from sow unit to experimental facilities	move from sow unit to experimental facilities (500m)	transport in truck for 60 km
Feeding form	liquid milk	dry pellets	crumbled pellets (diameter 4mm)	dry pellet (ø 2.5mm)	dry pellet (ø 2.5mm) soaked with water
Feeding level	high (voluntary DE intake NRC) low (1/3 of voluntary DE intake NRC)	*ad libitum* (piglets with high voluntary intake selected at d3) and restricted (1/3 of ad lib intake)	high (2.5 x NEm; piglets with high voluntary intake were selected at d3),increasing (d1: 0.25 to d7: 2.5 x NEm)low (d1: 0.25 NEm to d7 1.0 x NEm)	high feeding level (2.5 x NEm; piglets with high voluntary intake were selected at d3), increasing level (d1: 0.5 NEm to d7 2.0 x NEm)	increasing (d1: 0.5 NEm to d6 2.0 x NEm)
Feed composition	dairy protein and lactose, butter oil and vegetable oil	barley, corn and 23% skimmed milk powder or 10% feather meal, 12 lactose, 1% corn)	corn, barley, cornstarch, soy bean meal and wheat gluten	barley, corn, wheat gluten, soy flakes	barley, corn, soy flakes, fish meal, potato protein
Dissection days	0, 1, 2, 4	0, 4, 7, 14	0, 3, 7	0, 4, 7	0, 2, 6
Pigs sampled	48	84	40	48	30
Body weight	d0, at dissection	d0, at dissection	d0, at dissection	d0, at dissection	d0, at dissection
Faeces score	daily	daily	Daily	daily	daily
Feed intake	daily	daily	Daily	daily	0-2d, 3-6d
Morphology[1]	VH, CD	VH, CD; shape	VH, CD	VH, CD	VH, CD
Microflora + pH[1]	-	stomach, jejunum	-	-	-
BBB enzymeactivity[1]	-	-	aminopeptidase, sucrase-isomaltase	-	-
Mucus[1]	crypt goblet cells (number and type)	-	muc type 1, 2 and 3 in ileal digesta	-	-
Inflammatory response[1]	expression level of IL1, TNF, iNOS, COX2 Crypt CD4-CD8 T lymphocytes;	blood leukocytes	expression level of IL1, IL8	-	expression level of IL1, IL8
Permeability[1]	[^{3}H]Mannitol, [^{14}C]GlySar	-	-	[^{3}H]Mannitol, [^{14}C]GlySar, HRP, Na-Flu	[^{3}H]Mannitol, [^{14}C]GlySar

[1] At dissection

Table 6 Feed intake (FI) (KJ NE/piglet/day) for the experiments described in chapters of this thesis.

Exp	1				2				3						4				5	
Chap	2				3				4						5				6	
FI	L		H		R		A		L		I		H		I		H		R	
day	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
1	434	174	2971	1616	80	100	370	530	64	107	11	11	139	278	29	200	466	551	200	238
2	1320	591	4135	1008	270	170	1720	780	567	225	235	300	1626	599	105	95	1473	931	485	447
3	1388	591	4378	1320	360	140	2670	730	1027	107	931	396	2226	1038	1074	608	2081	732	931	684
4	1233	678	4378	1181	770	260	2740	830	1391	407	1455	171	2161	1327	1701	874	2594	979	1425	732
5					880	80	2690	1010	1509	193	2022	503	2643	1177	1805	1131	3164	1340	1729	646
6					810	120	2810	1050	1487	268	2301	835	2996	899	2195	1226	3031	1587	1748	770
7					960	100	3130	1190	1455	139	2589	1017	2600	696	2005	1492	2993	1435		
8					1010	110	3910	1080												
9					1260	140	4310	1270												
10					1450	60	4820	1040												
11					1600	80	5440	1330												
12					1800	210	5960	1730												
13					1980	100	5630	2300												
14					1880	170	6790	2360												

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Summary

In the Netherlands, piglets are weaned abruptly at an age of 24-28 days. The weaning process is generally regarded as a stressful event and involves complex social, environmental and dietary changes. Some of these can impair gut development and adaptation. Normally, the weaning stress is associated with an immediate but transient drop in feed intake and if that can be avoided also gut morphology is maintained much better. So, feed intake is an important determinant of performance and gut morphology in weaned pigs. The drop in feed intake and the change in composition affect the intestinal micro flora and the small intestinal architecture. The period of immediate anorexia after weaning is followed by a regenerative phase during which feed intake resumes. The regenerative phase is characterized by down regulating of many intestinal disorders most probably stimulated by the increased feed intake.
The research described in this thesis was conducted within the framework of a research programme investigating the effect of dietary strategy immediately after weaning on gut integrity. In this thesis the effect of feed intake and dietary physical form in the de- and regenerative phase on the inflammatory response and barrier function and their relationship with morphological indices was assessed. In the experiments several markers for different types of transepithelial transport (*in vitro*) and for inflammatory response were studied. In the experiments we compared animals with high voluntary feed intake (VFI) with pigs fed restricted.
High intake of cows or sows milk can maintain the gut morphology post weaning. In **Chapter 2** the effect of intake level of milk on gut integrity parameters like barrier function, inflammatory response and histo-morphological characteristics was studied in pigs during a 4 day period post weaning. It appeared that sufficiently high intake of liquid milk replacer (above maintenance requirement) maintained gut integrity (i.e. morphology and permeability) compared to the level at weaning whereas low feed intake resulted in villous atrophy as well as increased gut wall permeability and inflammatory response. Dry matter intake was related positively with villous height and negatively with paracellular transport.

Milk protein is highly digestible. However, in weaner diets also protein sources with lower digestibility are sometimes being used. In **Chapter 3**, the effect of protein digestibility and intake level (restricted, high) of dry feed on micro flora (stomach and jejunum) and small intestinal morphology during the first 14 day period post-weaning is described.
Results showed that the feed intake level affected the morphology of the small intestine as well as the pH and micro flora (especially in the stomach). The dietary protein source affected

the number of leukocytes in the blood and the BWG till d7 and d14 post weaning. The dietary protein source did neither affect the pH and micro flora nor the morphology in the small intestine except for the villous height at the mid small intestine.

We also tested whether or not maintenance of gut morphology and body weight gain is related to sufficiently high feed intake. **Chapter 4** describes the effect of feed intake level (restricted, high) during the degenerative phase and feed intake level (restricted, increasing and high) during the regenerative phase on inflammatory response, morphology and digestive capacity of weaned pigs fed a crumbled feed during a 7 day period. Results showed that the feed intake level affected the villous architecture and weight of the small intestine. At d3 villous height and crypt depth were affected by feeding strategy but at d7 post weaning, differences in villous height between the 3 feed intake strategies (low, increasing, high) were not significant. The specific activities of amino peptidase and sucrase-isomaltase in the brush border and the mRNA gene expression level of interleukin (IL) 1β and IL-8 in the epithelium were not affected by feed intake level. It was concluded that a feed intake level lower than maintenance level negatively affects gut integrity at d 3 post weaning. High intake of dry feed could not prevent the changes in the gut wall induced by weaning but resulted in less compromised gut architecture. We hypothesized that high feed intake attenuates the changes in gut integrity and stimulates the intestinal repair in newly weaned piglets compared to low feed intake level. Feed intake levels between 1 x (low) and 2.5 x (high) maintenance requirement for energy resulted in similar gut integrity at d7 post weaning. Furthermore, weaning induced changes in villous architecture start proximal and spread distal.

Chapter 5 describes the effect of feed intake level of a dry pelleted feed during the degenerative and regenerative phase on morphology, inflammatory response and barrier function using 4 marker molecules. The modulating effects of feed intake level on the onset and recovery of changes in the small intestine of pigs post weaning were studied. Results showed that weaning induced acute and long lasting changes in gut morphology and permeability. Intake level of dry pelleted feed affects villous architecture but intake level of dry feed did not affect mucosal permeability to small (mannitol) or large (horse radish per-oxydase) molecules. A restricted feed intake level, below maintenance level, negatively affected gut morphology at d 4 post-weaning. This effect could be alleviated but not completely prevented by a high voluntary feed intake of dry pelleted feed directly after

weaning.

Feeding *ad libitum* milk or liquid diets maintains or supports the gut morphology in weaned pigs. **Chapter 6** describes the effect of physical form of a feed (dry versus slurry) when fed at a low level during the degenerative phase and an increasing level in the regenerative phase on gut barrier function, inflammatory response and morphology is described. In general, the results showed that the days post-weaning exerted more impact on the measurements than the physical form of the diet. The trans-cellular transport was elevated ($P < 0.05$) in piglets fed the pelleted diet. The histo-immunological changes in the intestine such as villous heights, crypt depths and mRNA expression levels of interleukin 8 (IL-8) were not affected by the dietary physical form.

In the **General discussion** the importance of feed intake level and factors affecting feed intake in weaned piglets were evaluated. Feed intake is important, because lack of intake is a very important determinant for gut morphology, barrier function and inflammatory response after weaning. In the experiments described in this thesis piglets were not supplemented during the suckling period. Individually housed pigs with high voluntary feed intake (VFI) with pigs which were fed restricted at either a predetermined percentage of VFI of pigs fed *ad libitum* or a maximum amount of feed were compared. In general with this approach the average intake of pigs fed *ad libitum* or at high intake level was 2 to 7 times higher compared to the average intake of the pigs fed restricted or at the low level. However, in some experiments variation in feed intake between animals within experiments at both the high and low level was still high showing the importance of comparisons within experiments.

The data described in this thesis suggest a non linear relationship between time and villous height (VH) showing a transient decline. The largest reduction in VH was seen at d1-d4 at the proximal and d4-d7 at the middle small intestine. The VH was maintained by the high milk intake and also at d14 VH reached pre-weaning values at the high intake level irrespective of the dietary composition. In general the high intake level resulted in higher VH and deeper deeper crypts compared to low intake levels.

In this thesis gene expression of several inflammatory was studied. In one experiment an effect of feed intake was found. In other experiments, values for inflammatory mediators were smaller than the detection level or not significantly affected by feed intake level.

Data on in vitro gut permeability suggest a non-linear response for small molecules (mannitol) permeability with transiently increased values. High intake feed (milk) resulted in lower permeability values compared to a low intake level.

The permeability for large molecules (horse radish peroxydase) showed decreased values after weaning irrespective of the intake level.

In summary, this thesis shows that on d1 after weaning gene expression of proinflammatory cytokines is changed concomitantly with a change in (paracellular) permeability of the mucosa followed by changes in gut morphology. Recovery of villous length occurred from day 4 onwards after an increase in crypt depth has taken place. Changes in gut integrity are related to feed intake level. Individual feed intake was also correlated in most experiments with body weight gain, morphology and in some experiments with inflammatory response parameters and barrier function. Farm management should be aimed at reducing stress at weaning and preparing the pig for the weaning process. Stimulation of intake of creep feed also will minimize post weaning problems. Variation in parameters related to gut integrity was high emphasizing the necessity to minimize variation in feed intake and to standardize experimental protocols regarding sampling procedures (i.e, sampling day post weaning, site of the intestine).

Future research using molecular techniques and (jejunal) pig cell lines may help to better understand the changes which occur in the gut tissue and micro flora post-weaning.

Samenvatting

In de Nederlandse varkenshouderij worden biggen gespeend op een leeftijd van ongeveer 25 dagen. Het spenen vindt abrupt plaats en gaat gepaard met veranderingen in o.a. voer, huisvesting en sociale omgeving. Het spenen wordt beschouwd als een stressvolle periode in het leven van een varken. Het spenen gaat vaak gepaard met een acute maar tijdelijke daling in voeropname.

Voeropname bepaalt in belangrijke mate de prestatie (o.a. de groei) en de morfologie van de dunne darm. Een verlaagde voeropname in combinatie met een verandering in voersamenstelling beïnvloedt de microflora in de darm maar ook de structuur van de darm. Na een korte periode met verslechtering van de darm integriteit en verlaagde voeropname (degeneratieve fase) herstelt de voeropname en de darmintegriteit zich (regeneratieve fase). Het onderzoek dat beschreven is in dit proefschrift is uitgevoerd in het kader van een groter onderzoeksprogramma dat als doel had het effect van voerstrategie op de integriteit van de darm tijdens de speenperiode van biggen te onderzoeken.

In dit proefschrift is onderzocht wat het effect is van voeropname niveau en fysieke vorm van het voer in de de- en regeneratieve fase op ontstekingsreactie en barrière functie in relatie tot de morfologie van de darm bij biggen net na het spenen.

In de experimenten zijn verschillende indicatoren en markers voor transport door het darmepitheel en voor ontstekingsreactie bestudeerd. In de experimenten zijn biggen met een hoge vrijwillige voeropname vergeleken met beperkt gevoerde biggen.

In **hoofdstuk 2** is het effect van opname niveau van melk op darmintegriteit zoals barrière functie, ontstekingsreactie en histo-morfologische parameters onderzocht in biggen gedurende een studie van 4 dagen na spenen. De resultaten toonden dat bij een hoog opname niveau van melk de darmintegriteit (morfologie en permeabiliteit) niet veranderd was ten opzichte van het moment van spenen, terwijl een laag opname niveau leidde tot kortere villi en een toegenomen darmpermeabiliteit en een verhoogde expressie van ontstekingsmediatoren. De voeropname (droge stof) was positief gerelateerd aan de hoogte van de villi en negatief aan het paracellulaire transport.

Melkeiwit is goed verteerbaar voor biggen. Echter, in de praktijk worden in voeders voor gespeende biggen ook grondstoffen verwerkt met een lagere eiwitverteerbaarheid. In **hoofdstuk 3** zijn de effecten beschreven van eiwitverteerbaarheid en opname niveau (beperkt, onbeperkt) van droog voer op microflora in de maag en dunne darm en morfologie van de dunne darm bij biggen gedurende 14 dagen na spenen. De resultaten lieten zien dat

voeropname niveau zowel de morfologie in de dunne darm als ook de pH en microflora (met name in de maag) beïnvloedt. De eiwitbron in het voer (melkpoeder, verenmeel) had een effect op het aantal leukocyten in het bloed and op de lichaamsgroei tot dag 7 en dag 14 na spenen. De eiwitbron had geen effect op de pH en de microflora in de maag en jejunum. De eiwitbron had alleen effect op de villushoogte in het midden gedeelte van de dunne darm maar niet op villushoogte (op andere plekken in de dunne darm) of op de crypt diepte. Dit geeft aan dat het meten op een enkel moment of slechts op een enkele plaats in de darm niet zinvol is.

In **hoofdstuk 4** is het effect bestudeerd van het voeropname niveau tijdens de degeneratieve fase (laag, hoog) en tijdens de regeneratieve fase (laag, oplopend, hoog) op de ontstekingsreactie, morfologie en de enzymactiviteit in de dunne darm van biggen die gevoerd werden met een droge kruimel gedurende 7 dagen na spenen. Het voeropname niveau beïnvloedde de morfologie en het gewicht van de dunne darm. Het voeropnameniveau had langere villi en diepere crypten op dag 3 na spenen maar op dag 7 waren de verschillen tussen de 3 voeropname niveaus (laag, oplopend, hoog) niet significant. Zowel de specifieke activiteiten van de brush border enzymen, amino peptidase en sucrase-isomaltase als ook het mRNA expressie niveau van de ontstekingsmediatoren interleukine (IL) 1β en IL-8 werden niet beïnvloed door het voer opnameniveau. Conclusie was dat een voeropnameniveau op of lager dan onderhoudsniveau een negatief effect heeft op de darmintegriteit op dag 3 na spenen. Een hoge opname van droogvoer na spenen kon een verslechtering van de darmintegriteit na spenen niet voorkomen maar resulteerde in minder verslechtering dan een laag voeropnameniveau. Voeropnameniveaus in de range van 1 x (laag) tot 2,5 x (hoog) onderhoudsbehoefte voor energie resulteerden in vergelijkbare darmintegriteit op dag 7 na spenen. De veranderingen in de morfologie van de darm begonnen in het eerste deel van de darm en verspreidde zich daarna naar het tweede deel van de dunne darm.

In **hoofdstuk 5** is het effect beschreven van voeropname niveau van droog voer (pellet) tijdens de de- en regeneratieve fase op morfologie en barrière functie van de dunne darm gebruikmakend van 4 verschillende marker moleculen. Het effect van voeropname niveau op het ontstaan van veranderingen en het herstel van de darmintegriteit zijn bestudeerd bij biggen gedurende 7 dagen na spenen.

De resultaten laten zijn dat er veranderingen in de morfologie en permeabiliteit van de dunne darm zijn van korte maar ook van iets langere aard. De opname van droge pellets had effect op de morfologie van de darm maar had geen invloed op de paracellulaire permeabiliteit van kleine (mannitol) en grote (horse radish peroxydase) moleculen. Een beperkt voerniveau, (beneden onderhoudsniveau voor energie) had een negatief effect op de morfologie van de darm op dag 4 na spenen. Een hoog voeropnameniveau na spenen kon de verslechtering in morfologie beperken maar niet helemaal voorkomen.

Ad libitum opname van melk of vloeibare voeders laat de darm morfologie van gespeende biggen intact of heeft een positief effect op morfologie in vergelijking met lage opname al dan niet in combinatie met droog voer. In **hoofdstuk 6** zijn de effecten beschreven van de fysieke vorm (droog versus slurry) bij een laag voerniveau tijdens de degeneratieve fase en een oplopend voerniveau tijdens de regeneratieve fase op de permeabiliteit, ontstekingsreactie en morfologie van de darm van biggen gedurende 6 dagen na spenen.
In het algemeen lieten de resultaten zien dat het spenen op zich een grotere impact had op de gemeten darmparameters dan de fysieke vorm van het voer. De transcellulaire permeabiliteit was hoger voor biggen die gevoerd werden met het droge voer in vergelijking met de biggen gevoerd met slurry. De veranderingen in histo-immunologische parameters in de darm zoals villus hoogte, crypte diepte en mRNA expressie niveau van IL-8 waren niet beïnvloed door de fysieke vorm van het voer.

In de **General discussion** is het belang van voeropname aangegeven. Tevens zijn factoren die van invloed kunnen zijn op de voeropname bij biggen besproken. De voeropname is belangrijk en bepaald belangrijke mate de morfologie, de barrière functie en de ontstekingsreactie in de darm van biggen na het spenen. In dit proefschrift zijn individueel gehuisveste biggen met een hoge voeropname vergeleken met beperkt gevoerde biggen (met lage voeropname) op een vooraf vastgestelde hoeveelheid of percentage van de voeropname van de ad libitum gevoerde biggen. Het gemiddelde voeropname niveau van de biggen die op een hoog niveau of ad libitum gevoerd werden was 2 tot 7 keer hoger in vergelijking met de voeropname van biggen die op een laag niveau of beperkt gevoerd werden.
Echter, in sommige experimenten was de variatie in voeropname tussen biggen binnen hetzelfde experiment voor zowel het hoge als het lage voerniveau groot. Dit geeft aan dat het lastig is om resultaten van darmintegriteit tussen experimenten te vergelijken vooral als de

monsters genomen zijn op verschillende tijdstippen na spenen in verschillende delen van de darm. De data die in dit proefschrift beschreven zijn suggereren een non-lineair verband tussen de tijd na spenen en de villus hoogte met een tijdelijke dip. De grootste reductie van villus hoogte vond plaats tussen dag 1 en dag 4 na spenen in het voorste deel van de dunne darm en tussen dag 4 en dag 7 na spenen in het midden dan de dunne darm. Een hoge opname van melk zorgde voor een onveranderde morfologie maar ook op dag 14 na spenen werden villus hoogten gevonden vergelijkbaar met waarden op het moment van spenen onafhankelijk van de voersamenstelling. In het algemeen resulteerde een hoge voeropname in langere villi en diepere crypten vergeleken met lage voeropname.

In een aantal experimenten beschreven in dit proefschrift is ook het mRNA expressie niveau van ontstekingsmediatoren bestudeerd. In één experiment (hoofdstuk 2) werd een significant effect van voeropname op expressie niveau van ontstekingsmediatoren gevonden. In de andere experimenten waren de waarden lager dan de detectie limiet of er was geen effect van voeropname niveau of fysieke vorm van het voer. De gegevens van paracellulaire darm permeabiliteit suggereren een non-lineair verband voor kleine moleculen (mannitol) met een tijdelijke toename in permeabiliteit na spenen. Een hoge opname van melk leidde tot lagere waarden dan een lage opname van melk. De paracellulaire permeabiliteit voor grote moleculen (horse radish peroxidase) was verlaagd na het spenen vergeleken met waarden op het moment van spenen onafhankelijk van het voer opnameniveau.

Samenvattend, dit proefschrift laat zien dat op dag 1 na spenen het expressieniveau van ontstekingsmediatoren en de (paracellulaire) permeabiliteit van de darmwand is veranderd gevolgd door veranderingen in de morfologie. Het herstel van de villus hoogte vond plaats vanaf dag 4 na spenen nadat een toename in crypte diepte zichtbaar is geworden. De veranderingen in de darmintegriteit waren gecorreleerd met voeropname. De individuele voeropname was gecorreleerd met toename in lichaamsgewicht, morfologie van de darm en in sommige experimenten met ontstekingsmediatoren en darmpermeabiliteit.

Management op varkensbedrijven gericht op het verminderen van stress rond spenen of het wennen van de biggen aan stresssituatie voor het spenen zou de voeropname na spenen kunnen stimuleren en zo de speenproblemen kunnen minimaliseren. Toekomstig onderzoek met moleculaire technieken en varkens (darm) cellijnen kan wellicht een bijdrage leveren aan een beter inzicht in de veranderingen die plaatsvinden in de darmflora en in het darmweefsel bij biggen na het spenen dan het meten van morfologie.

Appendices

Publications

Curriculum Vitae

Publications

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Curriculum Vitae

Han (Johannes Maria Allegonda Johannes) Verdonk is geboren op 9 juli 1964 in het agrarische dorpje Liempde in Noord Brabant. Na het behalen van het diploma Atheneum B aan het Jacob Roelandslyceum te Boxtel startte hij in september 1982 met de studie Zoötechniek (Veeteelt) aan de toenmalige Landbouwhoge school te Wageningen. In september 1988 rondde hij zijn studie af met de afstudeervakken Tropische Veehouderij, Veevoeding en Pluimveeteelt.

Van 1990 tot 1996 werkte hij als nutritionist in technisch-commerciële functies bij Nutrifeed en Borculo Whey Products, allebei producenten op gebied van jongdiervoeding. Hij was verantwoordelijk voor onderzoek en product ontwikkeling en technische ondersteuning van klanten. Eind 1996 trad hij in dienst bij het ILOB, de afdeling Diervoeding van het toenmalige TNO Voeding als wetenschappelijk onderzoeker. In 1998 werd het project gestart waarbinnen het onderzoek is uitgevoerd dat beschreven is in dit proefschrift.
Na achtereenvolgens te hebben gewerkt voor ILOB, ID TNO Diervoeding en de Animal Sciences Group van WUR is Han in juni 2005 in dienst getreden bij CCL bv (het onderzoeksbedrijf van Cehave-Landbouwbelang) als Nutritionist Varkens en Pluimvee.

Han (Johannes Maria Allegonda Johannes) Verdonk was born on July 9th 1964 in the agricultural village Liempde (province of Noord Brabant). After finishing secondary school (Jacob Roelandslyceum in Boxtel) in september 1982 he started to study Animal Science at Wageningen University. He completed his MSc in September 1988 with subjects Tropical Animal Husbandry, Pig Nutrition and Poultry Nutrition.

From 1990 till 1996 Han was employed as nutritionist at Nutrifeed en Borculo Whey Products, which are both active in young animal feed. Han was responsible for research & product development and technical sales support. In 1996 he joined ILOB, the Department of Animal Physiology and Nutrition of the former TNO Nutrition and Food Research Institute as scientific researcher. The experiments described in this thesis were conducted within the framework of a project which was started in 1998.
After ILOB, ID TNO Animal Nutrition and the Animal Sciences Group of Wageningen University and Researchcentre Han joined CCL bv (the Research Institute of Cehave-Landbouwbelang) as Nutritionist Pigs and Poultry in June 2005.

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