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Effect of polydextrose on intestinal microbes and immune functions in pigs

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Dietary fibre has been proposed to decrease risk for colon cancer by altering the composition of intestinal microbes or their activity. In the present study, the changes in intestinal microbiota and its activity, and immunological characteristics, such as cyclo-oxygenase (COX)-2 gene expression in mucosa, in pigs fed with a high-energy-density diet, with and without supplementation of a soluble fibre (polydextrose; PDX) (30 g/d) were assessed in different intestinal compartments. PDX was gradually fermented throughout the intestine, and was still present in the distal colon. Irrespective of the diet throughout the intestine, of the four microbial groups determined by fluorescent *in situ* hybridisation, lactobacilli were found to be dominating, followed by clostridia and *Bacteroides*. Bifidobacteria represented a minority of the total intestinal microbiota. The numbers of bacteria increased approximately ten-fold from the distal small intestine to the distal colon. Concomitantly, also concentrations of SCFA and biogenic amines increased in the large intestine. In contrast, concentrations of luminal IgA decreased distally but the expression of mucosal COX-2 had a tendency to increase in the mucosa towards the distal colon. Addition of PDX to the diet significantly changed the fermentation endproducts, especially in the distal colon, whereas effects on bacterial composition were rather minor. There was a reduction in concentrations of SCFA and tryptamine, and an increase in concentrations of spermidine in the colon upon PDX supplementation. Furthermore, PDX tended to decrease the expression of mucosal COX-2, therefore possibly reducing the risk of developing colon cancer-promoting conditions in the distal intestine.

Intestinal bacteria: Immune responses: Polydextrose

The role of the gastrointestinal (GI) microbiota in health and disease has been widely studied in human subjects and animals. The GI microbiota is a highly diverse ecosystem established at birth, and its complexity is dependent on several factors, including the host's genotype and physiology, the availability of nutrients from the diet and competitive mechanisms amongst the bacteria themselves (Savage, 1977). The composition of the GI microbiota and its activities directly influence the host's health and may bring about beneficial effects or, on the contrary, pathogenic outcomes for the host (Gibson & Roberfroid, 1995; Salminen *et al.* 1998).

The main beneficial effects of an optimal microflora include resistance to colonisation by pathogens, for example, through adhesion mechanisms on the GI mucosa, competition for nutrients, the production of SCFA as endproducts of the fermentation of carbohydrates and proteins in the gut, and the production of antimicrobial compounds (for example, bacteriocins). In addition, the GI microbiota is important for mucosal integrity (Delzenne & Williams, 2002) and the development and regulation of intestinal immune responses, including oral tolerance towards food-borne antigen structures (Guarner & Malagelada, 2003; Mazmanian *et al.* 2005). The impact of the microbiota on chronic mucosal inflammation is rather unknown, but protective, anti-cancerous effects by probiotics and prebiotics via modulation of mucosal cyclo-oxygenase (COX) expression *in vitro* have been suggested (Mäkivuokko *et al.* 2005; Nurmi *et al.* 2005). Harmful activities of the GI microbiota include the production of potentially toxic and carcinogenic compounds deriving from proteolytic metabolic processes (for example, ammonia, phenols), the translocation of opportunistic pathogens across the mucosal barrier to the mesenteric lymph nodes and other extra-intestinal sites (Cummings & Macfarlane, 1991; Gibson & Roberfroid, 1995) and GI infections caused by ingested pathogens leading to diarrhoea. Therefore, the modulation of the GI microbiota through the diet may be advantageous for the host.

As in man, the large intestine of pigs is the anatomical region most heavily and diversely populated by bacteria, with over 400 different bacterial species and in concentrations ranging from 10^{10} to 10^{12} microbial cells per g intestinal contents (Ewing & Cole, 1994; Berg, 1996). Furthermore, nutritional studies have shown that the swine is generally

Abbreviations: COX, cyclo-oxygenase; FISH, fluorescent *in situ* hybridisation; G + C, guanine + cytosine; GI, gastrointestinal; PDX, polydextrose.
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considered as an excellent animal model of man's GI tract (Miller & Ullrey, 1987; Moughan *et al.* 1994). Several dietary components, such as fibre and prebiotics, can influence the intestinal functions and the digestive processes (digesta movement, digesta volume and digesta transit time) and therefore beneficially impact on the host's health (Roberfroid, 1993; Freire *et al.* 2003). Furthermore, an increase in dietary fibre intake appears to decrease the risk of colon cancer relative to the amount of consumed fibre (Bingham, 2006). The mechanisms are largely unknown. However, the role of COX, especially COX-2 in several different cancer types, such as colon, gastric, skin and lung cancer, has been documented (Muller-Decker *et al.* 1999; Prescott & Fitzpatrick, 2000; Arbabi *et al.* 2001). Especially, the risk of colon cancer can be decreased or development even reversed by the use of COX-2-specific inhibitors (Krause & DuBois, 2001).

Polydextrose (PDX) is a randomly polymerised glucose oligosaccharide with an average degree of polymerization of 12, which is used as a multi-purpose commercially available food ingredient. It is commonly used as a replacement for sugar, starch and fat in commercial food products, and has been previously shown to exhibit prebiotic properties and beneficial effects on mucosal activity (Jie *et al.* 2000; Peuranen *et al.* 2004; Probert *et al.* 2004; Mäkivuokko *et al.* 2005). In the present study, the fermentation of PDX was assessed in the small intestine, caecum and different parts of the colon in a porcine model. In addition, the microbial and immunological environment in the GI tract of pigs was further analysed by studying the composition and activity (SCFA and biomarkers of proteolytic activity) of the microbiota and immune functions, such as IgA secretion and mucosal COX gene expression.

Materials and methods

Animals and trial groups

The ethical approval for the trial was obtained from the Animal Care and Use Regional Ethical Committee of MTT (Agrifood Research Finland, Jokioinen, Finland). A total of twenty healthy pigs (eight females and twelve castrated males) were used in the trial. Of these, seven were Finnish Landrace, two Finnish Yorkshire, one cross of these breeds and ten were crosses of Landrace × Yorkshire sows and Duroc × Landrace boars. Their initial weight was 28 (SD 1·5) kg and age 67 (SD 9·5) d. The pigs were randomly allocated to two treatment groups which were balanced for sex, breed and litter origin. The pigs were housed individually in pens of 1·0 × 1·5 m with a slatted dunging area of 1·0 × 1·0 m. The acclimatisation period lasted 7 d and the experimental period 21 d. The purpose of the acclimatisation period was to familiarise the pigs with their experimental diets. Housing and environment of the pigs was kept similar during both periods.

Diets

The experimental treatments were (1) control (basal high-energy-density pig diet; no added PDX) and (2) basal diet supplemented with 30 g PDX/pig per d (Litesse® *Ultra*™; Danisco Sweeteners, Redhill, Surrey, UK). In the basal diet, 19·9 % of gross energy came from protein, 29·7 % from fat and 50·4 % from carbohydrates (Table 1). The nutrient composition of the diet ingredients was adopted from Finnish food composition tables (Rastas *et al.* 1993).

The basal diet was supplemented with minerals, vitamins and amino acids to fulfil the Finnish nutrient requirements of growing pigs (Tuori *et al.* 1995). Protein in the diet was mainly of animal origin and wheat flour and potato flakes were used as the main carbohydrate source to restrict the intake of dietary fibre. The fibre intake of pigs was 1·8 g/MJ, which approximates to 60 % of human Finnish fibre intake recommendations (3·0 g/MJ). The total dietary fibre content was 43·4 g/kg food; 44–62 g dietary fibre/d (2·2 g/MJ) per pig. Lower fibre concentrations would have increased the risk of stomach ulceration (very low viscosity allows the free flow of acidic secretions to flush the walls of the stomach), and would therefore have been detrimental for the health of the pigs. Health status of pigs was assessed twice daily by clinical monitoring, and no signs of illness were detected during the 4-week trial. The average daily weight gain of the pigs was 834 (SD 72·7) g.

During the acclimatisation and experimental periods, the pigs were fed twice daily (07.00 and 15.30 hours) according to an age-based, restricted scale (Tuori *et al.* 1995). Their daily energy intake was 19·6–27·3 MJ. The feed intake of the pigs averaged 1378 g/d (32 g/kg live weight) and no

Table 1. Composition of the high-energy-density basal diet

Components (g/kg)	
Wheat flour*	500
Potato flakes	100
Soya protein flour†	40
Skimmed milk powder	50
Whey protein powder	60
Fish meal	60
Sugar	20
Butter	65
Rapeseed oil	50
L-Lysine HCl	6
DL-Methionine	4
L-Threonine	2
Limestone	2
Monocalcium phosphate	23
Mineral–vitamin premix‡	18
Calculated composition (g/kg)	
Gross energy, calculated (MJ/kg)	17·0
Crude protein, analysed	219
Crude fat, analysed	133
Dietary fibre, analysed	43·4
Ca, calculated	10·4
P, calculated	8·3

* Standard baking flour milled from wheat endosperm (Raisio Ltd, Raisio, Finland).
† Ground non-GMO soya protein product HP 310 for feed application purposes (Hamlet Protein A/S, Horsens, Denmark).
‡ Vitamin–mineral mixture provided (per kg/diet): P, 1·1 g; Ca, 3·1 g; Mg, 0·7 g; Fe, 143 mg; Cu, 31 mg; Se, 0·4 mg; Zn, 125 mg; Mn, 32 mg; I, 0·3 mg; NaCl, 4·5 g; vitamin A, 2·1 mg; vitamin D, 17·9 μg; vitamin E, 69 mg; vitamin K, 2·6 mg; vitamin B_1, 2·7 mg; vitamin B_2, 6·6 mg; vitamin B_6, 3·8 mg; vitamin B_{12}, 0·03 mg; biotin, 0·28 mg; pantothenic acid, 19·4 mg; niacin, 27·4 mg; folic acid, 4·6 mg.

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refusals were left. The aim was to maintain normal growth of the animals, however, with slightly restricted feed intake (80–85 % *ad libitum* feeding), ensuring 100 % consumption of PDX. In group 2, PDX was mixed in the basal control diet during the experimental period: 15 g/d in the morning and 15 g/d in the afternoon per pig.

Feed analyses

The N content of the diet was determined by a Dumas method (method 968·06) using a Leco FP 428 nitrogen analyzer (Leco Corp., St Joseph, MI, USA). The crude protein content was calculated by multiplying the percentage of N by 6·25. Diethyl ether extract was analysed after hydrolysis with 3 M-HCl (method 920·39) (Anonymous, 1971). The total dietary fibre was analysed according to AOAC 45·4·07/NMKL 129.

Sample collection

The pigs were killed with a stun gun and exsanguinations were performed immediately by cutting the jugular veins, alternating the pigs from control and PDX groups. Their live weight at slaughter was 51·1 (SD 2·67) kg. During the bloodletting, a blood sample was collected into 50 ml plastic tubes and blood cells and plasma were immediately separated by a short centrifugation (12 000 ***g***; 1 min). The small intestine, caecum and colon of each pig were dissected, and the last 4 m covering the distal part of the small intestine was isolated. At that point the intestines were tied with cable binders to avoid loss of digesta. After binding, approximately 2 cm long intestinal tissue pieces were excised from the point, which was 4 m from the ileo-caecal junction, and mucus was gently scraped from the tissue with a scalpel and submerged in RNAlater (see below in the section Determination of mucosal cyclo-oxygenase gene expression) (Qiagen, Hilden, Germany), kept first during the sampling at room temperature, and then stored at -20°C. In addition, a mucosal tissue sample was obtained from the tip of the caecum (after emptying the contents) and three mucosal tissue samples were obtained from the colon at proximal, middle and distal regions. All digesta from the proximal small intestine (approximately 20 m), including the last 4 m, were collected. The contents of the caecum and the large intestine, which was divided into three equally long segments, were similarly collected. Digesta from the distal small intestine, caecum and the three caecal segments were divided into subsamples for the determination of DM content, and IgA and chemical and microbial analysis. These subsamples were kept on ice during the sampling and subsequently stored at -20°C.

Determination of polydextrose concentrations

The concentration of native PDX was measured using an HPLC method designed to identify the presence of native PDX in foods as described previously (Craig *et al.* 2000).

Determination of dry matter contents and short-chain fatty acid concentrations

The DM content of digesta was determined by weighing it before and after drying at 105°C for 24 h. The SCFA in digesta or plasma were analysed as follows: 1 ml of an internal standard (20 mM-pivalic acid) and 5 ml water were added to 1 g of the sample. After thorough mixing, the sample was centrifuged at 5000 ***g*** for 5 min. Following centrifugation 0·25 ml of saturated oxalic acid solution was added to 0·5 ml of the supernatant fraction and the mixture was incubated at 4°C for 60 min, and then centrifuged at 16 000 ***g*** for 5 min. The supernatant fraction or blood plasma samples were analysed by GC essentially as described previously (Holben *et al.* 2002). The concentrations of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, 2-methylbutyric acid and lactic acid were determined. Biogenic amines were determined from caecal digesta samples according to Saarinen (2002). The results are expressed as mmol SCFA/ml plasma, μmol SCFA/g digesta (wet weight), nmol biogenic amines/g digesta (wet weight) and μmol NH_3/g digesta (wet weight).

Determination of intestinal immunoglobulin A concentrations

IgA was determined from individual digesta samples treated with an equal volume of 1·0 % bovine serum albumin solution in 50 mM-tri(hydroxymethyl)-aminomethane (pH 7·5), 0·15 M-NaCl for 60 min at room temperature. The samples were then briefly centrifuged at 50 000 ***g*** and the supernatant fractions were used for IgA measurement. IgA was determined with ELISA utilising specific antibodies and standard samples obtained from Bethyl Laboratories, Inc. (Montgomery, TX, USA) according to the manufacturer's instructions. The results were expressed as μg IgA/g digesta (wet weight).

Determination of mucosal cyclo-oxygenase gene expression

For the quantitative determination of COX-1 and -2 expression levels, tissue specimens were rinsed briefly in sterile 0·9 % (w/v) NaCl solution and approximately 35 mg of the epithelial cells were scraped off using a sterile scalpel. Total RNA was stabilised immediately with the RNAlater reagent (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Total RNA was extracted from the samples using the RNeasy Mini Kit (Qiagen) and the contaminating genomic DNA was digested during RNA extraction using the same manufacturer's RNase-free DNase (Qiagen) according to instructions provided by the manufacturer. Then 2 μg RNA was reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. Absolute quantitative TaqMan PCR assays were set up for primers (Applied Biosystems) detecting specifically porcine COX-1 and COX-2, the sequences of which are shown in Table 2. All assays were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the instrument's default settings for thermal cycling and fluorescence measurements. Standard curves showing the inverse log-linear relationship between initial template copy number and the PCR cycle at which fluorescence intensity crosses a background threshold value (the threshold cycle) were prepared using spectrophotometrically quantified synthetic oligonucleotides representing the antisense cDNA sequence of each target transcript (data not shown). Results are expressed as absolute transcript copy numbers/100 ng

Table 2. The sequences of oligonucleotide primers, probes and standards used in the present study

Oligonucleotide	Sequence (5′ → 3′)
COX-1 forward primer	CAAGATGGGTCCTGGCTTCA
COX-1 reverse primer	CCATAAATGTGGCCGAGGTCTA
COX-1 probe	FAM-CAAGGCGTTGGGCCACGGG-TAMRA
COX-1 standard	CCATAAATGTGGCCGAGGTCTACCCCGTGGCCCAACGCCTTGGTGAAGCCAGGACCCATCTTG
COX-2 forward primer	CATTGATGCCATGGAGCTGTA
COX-2 reverse primer	CTCCCCAAAGATGGCATCTG
COX-2 probe	FAM-CTGCCCTTCTGGTAGAAAAGCCTCGC-TAMRA
COX-2 standard	CTCCCCAAAGATGGCATCTGGGCGAGGCTTTTCTACCAGAAGGGCAGGATACAGCTCCATGGCATCAATG

COX, cyclo-oxygenase.

total cellular RNA in oligonucleotide equivalents. When the effect of the PDX-supplemented diet on COX-gene expression was investigated, a relative percentage change for each part of the intestine was calculated by using the following formula:

$$\begin{aligned}&\%\,\text{Change in COX} - \text{gene expression}\\&= ((\text{absolute copy number in PDX piglet}\\&- \text{mean absolute copy number in control group})/\\&\text{mean absolute copy number in control group}) \times 100.\end{aligned}$$

Determination of microbial numbers by flow cytometry

In order to determine the total number of microbes in caecal digesta samples, two methods were utilised: a fluorescent *in situ* hybridisation (FISH) method (explained later) and a flow cytometric method. The cells were separated from the collected digesta by differential centrifugation (Apajalahti *et al.* 1998). For counting, a sample of separated bacterial cells from each digesta sample was appropriately diluted and the cells were stained with a fluorescent, nucleic acid-binding dye (Syto 24; Molecular Probes, Eugene, OR, USA) (Apajalahti *et al.* 2002). Microbial numbers were determined by flow cytometry as previously described (Apajalahti *et al.* 2002). The results were reported as cells/g digesta (wet weight) and used for converting the relative proportions of bacteria in the percentage guanine + cytosine (G + C) profiling to numbers of bacteria.

Microbial percentage guanine + cytosine profiling

The %G + C profiling was performed as described previously (Apajalahti *et al.* 2001). In short, bacteria were separated from digesta samples by differential centrifugation after which the bacterial DNA was recovered from the cells by a combination of physical, chemical and enzymic lyses. DNA recovered from the total microbial community was then profiled based on the G + C content of the chromosomal DNA in the individual bacterial members of the community. Chromosomal DNA molecules (minimum of 300 μg DNA as pools of one to three samples) with different G + C contents were separated by CsCl density gradient centrifugation and the abundance of DNA monitored by pumping the solution through a UV flow cell. In order to analyse the effects of treatments on %G + C profiles, the profiles were divided into five increments, each covering 10 % (covering an area between 26–75 %G + C). The proportion of microbes with %G + C belonging to a certain range of %G + C was calculated integrating the fractions from the %G + C profile.

Microbial enumeration by fluorescent in situ hybridisation analysis

For microbial enumeration using FISH, the homogenised digesta slurries in sterile ice-cold PBS (0·1 M-phosphate, pH 7·0) were centrifuged at 1500 ***g*** for 3 min to remove particulate matter and then fixed overnight in 4 % (w/v) paraformaldehyde. The bacterial cells were subsequently washed and re-suspended twice in sterile PBS and finally stored in PBS–ethanol at −20°C until hybridisation with appropriate molecular probes targeting 16S rRNA, as described by Ames *et al.* (1999). The probes used were Bif164 (Langendijk *et al.* 1995), Bac303 (Manz *et al.* 1996), CHis150 (Franks *et al.* 1998), Lab158 (Harmsen *et al.* 1999), specific for bifidobacteria, bacteroides, clostridia (*Clostridium perfringens/histolyticum* subgroup) and *Lactobacillus/Enterococcus* spp., respectively. The nucleic acid stain DAPI (4′6-diamidino-2-phenylindole) was used for total bacterial counts. The DNA probes were tagged with the Cy3 fluorescence, enabling the examination of hybridised samples with fluorescence microscopy. Results are expressed as $\log_{10}$ cells/g digesta (wet weight).

Statistical analyses

To describe the intestinal environment in pigs, mean levels of measured characteristics (pH, NH_3, SCFA, biogenic amines, FISH, COX-1, COX-2 and IgA) were compared between different compartments of the intestine. These mean levels of measured characteristics were compared in each part of the intestine between the control group and the group receiving PDX using two-sample *t* tests. %G + C profiling was also compared between the two groups using a *t* test. Furthermore, the statistical significances of the effect of PDX on COX-1 and COX-2 expression between different intestinal segments, and plasma concentrations of SCFA were calculated with a *t* test. *P* values $<0{\cdot}05$ were considered as significant.

Results

Twenty pigs were fed with a high-energy-density diet. The diet for half of the pigs was supplemented with 30 g PDX/d.

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Measurement of the PDX content of digesta DM taken from different parts of the intestine from the PDX group showed that concentrations of PDX decreased at an even rate, starting from approximately 53 mg/g DM in the distal small intestine and ending up at 35 mg/g DM in the distal colon, indicating that PDX was fermented more or less evenly throughout the intestine and was still present in the most distal part of the colon (Fig. 1).

Microbial metabolites

Several characteristics describing the microbial metabolites were measured from the different compartments of the intestine. The pH declined from the average of 6·5 measured in the distal small intestine to an average of 6·1–6·2 in the caecum. The pH increased gradually from the caecum to the most distal part of the colon (on average 6·5) even though the number of microbes remained high. The concentration of ammonia, on the other hand, increased steadily from the average of 10 μmol/g in the distal small intestine to over 40 μmol/g towards the distal colon. PDX supplementation had no effect on the pH or NH_3 concentrations (Fig. 2 (A)).

The differences of the total SCFA and biogenic amine concentrations for both treatments in different compartments of the intestine are depicted in Fig. 2 (B). The PDX addition to the diet had strongest influence on the colon fermentation pattern. Concentrations of the main SCFA (acetate, propionate, butyrate and valerate) were decreased in the colon in the group receiving PDX compared with the control group (Table 3). The changes became more evident towards the distal end of the colon where significant decreases could be observed with all SCFA including also all branched-chain fatty acids (iso-butyric acid, 2-methylbutyric acid and iso-valeric acid). Concomitant to lowered luminal SCFA, the plasma concentrations of the SCFA and lactic acid increased in the PDX-fed pigs. The mean plasma concentrations of acetic acid (11·5 (SE 1·70) mmol/l in control pigs and 12·4 (SE 1·32) mmol/l in PDX pigs), and especially lactic acid (7·5 (SE 1·32) and 10·8 (SE 2·12) mmol/l, respectively), appeared higher in pigs with the PDX-supplemented diet. Taken together, a tendency of a higher sum of the SCFA and lactic acid in the PDX group could be demonstrated ($P=0·0758$). Only trace amounts of butyrate and propionate could be detected (0·2 and 0·03 mmol/l, respectively), and no difference between the two diet groups could be detected. However, the plasma sum of branched SCFA (iso-butyric acid, 2-methylbutyric acid and iso-valeric acid) appeared lower in the PDX-supplemented group than in the control group (0·03 (SE 0·01) and 0·06 (SE 0·02) mmol/l, respectively; $P=0·11$; data not shown).

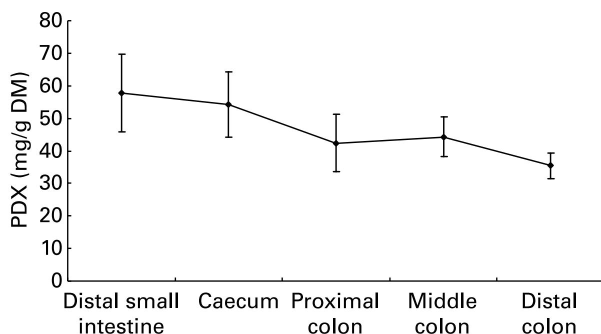

Fig. 1. Fermentation of polydextrose (PDX) in the pig intestine described as the luminal concentrations of PDX (mg/g DM) remaining in the five different compartments of the intestine (*n* 10) fed with the PDX-supplemented diet. Values are means, with their standard errors represented by vertical bars.

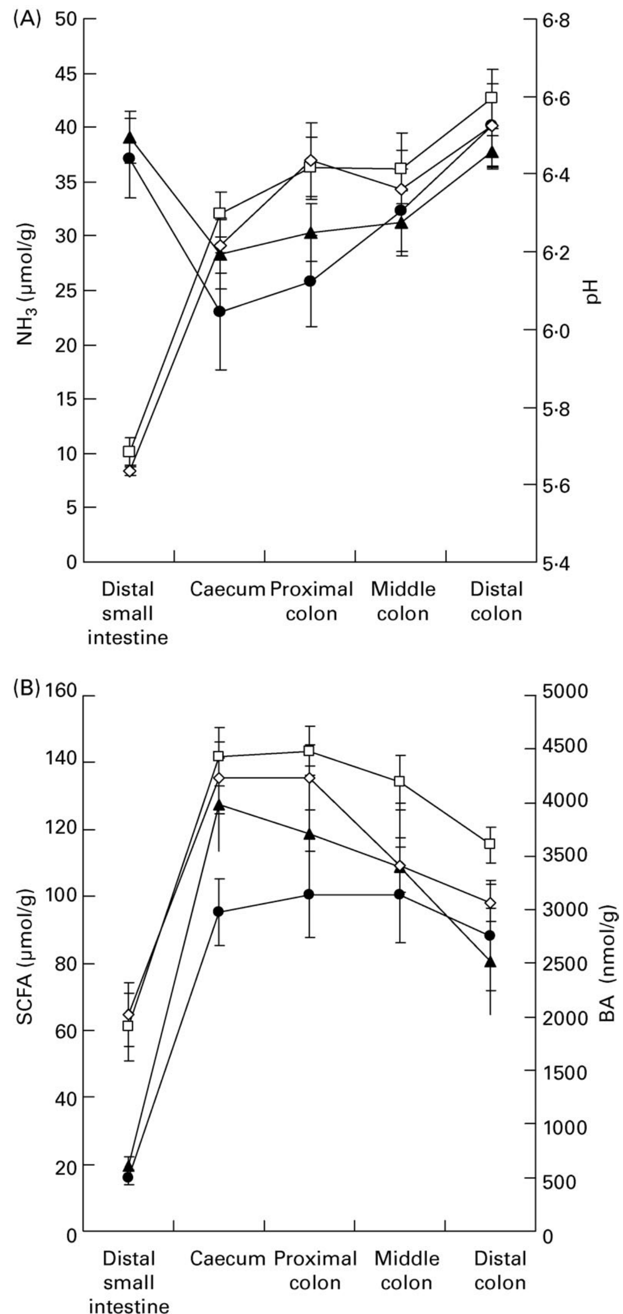

Fig. 2. The microbial and immunological intestinal environment in five different compartments; distal small intestine, caecum, proximal colon, middle colon and distal colon in pigs fed a control diet (*n* 10) and a polydextrose (PDX)-supplemented diet (*n* 10). (A) Luminal pH of control (▲) and PDX-fed (•) animals, and NH_3 concentrations (μmol/g digesta) of control (□) and PDX-fed (◇) animals. (B) Luminal concentrations of SCFA (μmol/g digesta) of control (□) and PDX-fed (◇) animals, and biogenic amines (BA; nmol/g digesta) of control (▲) and PDX-fed (•) animals. Values are means, with their standard errors represented by vertical bars.

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Table 3. The effect of polydextrose (PDX) on individual short-chain fatty acids and luminal concentrations of spermidine, spermine and tryptamine in the proximal, middle and distal colon

(Mean values)

	SCFA (μmol/g digesta)							Biogenic amines (nmol/g digesta)		
	Acetate	Propionate	Butyrate	Valerate	Isobutyrate	2-Methyl-butyrate	Isovalerate	Spermidine	Spermine	Tryptamine
Proximal colon										
Control	75·35	36·47	18·51	7·18	2·50	1·33	2·11	357·7	71·93	64·46
PDX	72·87	31·22	16·19	6·08	1·92	0·98	1·62	385·4	71·68	39
*P**	NS	0·101	NS	NS	NS	NS	NS	NS	NS	0·044
Middle colon										
Control	68·02	34·21	17·32	7·41	2·93	1·67	2·46	358·2	73·11	90·94
PDX	58·78	25·04	13·51	5·75	2·37	1·42	2·05	488·8	81·86	57·42
*P**	NS	0·013	0·069	0·112	0·096	NS	NS	0·012	NS	0·0043
Distal colon										
Control	58·72	26·27	14·55	6·13	3·51	2·25	3·01	369·7	69·42	84·17
PDX	52·97	21·53	11·20	5·16	2·42	1·54	2·10	507·6	85·50	51·86
*P**	0·12	0·053	0·042	0·19	0·021	0·034	0·024	0·001	0·0788	0·0018

**P* value for the difference between the PDX and control groups.

The concentrations of the most dominant biogenic amines, i.e. spermidine, putrescine, cadaverine, and methylamine, were low in the distal small intestine in control pigs (on average 83, 138, 188 nmol/g, and below detection level, respectively), increased approximately 3- to 9-fold in the beginning of the caecum, and then remained more or less constant throughout the large intestine or even decreased towards the end of the colon (see Table 3; data partially not shown). The concentration of tryptamine was also below detection level in the distal small intestine in control pigs, and increased starting from the caecum. The concentration increased slowly towards the distal colon (on average 84 nmol/g), but did not reach concentrations as high as the four major biogenic amines (data not shown). Concentrations of biogenic amines were influenced by the PDX-supplemented diet. More specifically, concentrations of spermidine (and spermine) were increased in the distal colon in the group receiving PDX (Table 3). In contrast, the concentrations of tryptamine were decreased in the entire colon. As with SCFA concentrations, the PDX-induced changes on biogenic amine concentrations became more evident towards the distal end of the colon.

Microbial community

The composition of intestinal microbiota was characterised by G + C% profiling. A clear shift in the G + C% profiles from the proximal to the distal intestine was demonstrated irrespective to the diet (Fig. 3). However, the composition of the microbial community remained relatively unchanged by the PDX intervention as measured by %G + C profiling, though individual variation was high between animals (data not shown). After the profiles were divided into five increments,

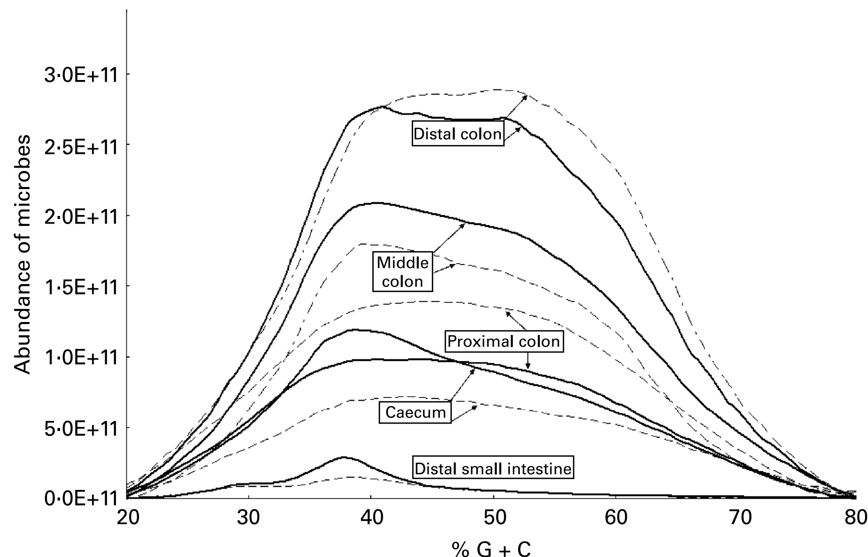

Fig. 3. Composition of the microbiota in different gastrointestinal compartments depicted as percentage guanine + cytosine (G + C) profiles. The chromosomal DNA was extracted from the samples and subjected to %G + C profiling, which separates the DNA strands according to their G + C content, thus enabling a culture-independent comparison of sample DNA. The profiles have been corrected with the corresponding microbial numbers. Each line represents the pooled average of the DNA of ten individual animals.(–-), Controls; (——), polydextrose supplemented.

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each corresponding to an area of 10 %G + C (range 26–75 %G + C), and multiplied with the corresponding total microbial numbers from fluorescence-activated cell-sorting analysis, a statistical comparison of the microbial abundances was possible. The abundance of microbes was increased by PDX treatment in the distal small intestine in the area of %G + C 26–35 ($P=0{\cdot}04$), 36–45 ($P=0{\cdot}0004$) and 66–75 ($P=0{\cdot}0025$), and in the caecum in the area of %G + C 26–35 ($P=0{\cdot}05$) and 36–45 ($P=0{\cdot}01$). In the proximal colon the abundance of microbes was decreased in four sequential increments (%G + C 36–75; $P=0{\cdot}02$, $P=0{\cdot}007$, $P=0{\cdot}004$ and $P=0{\cdot}03$, respectively). In the middle colon the abundance of microbes was increased in all five increments ($P=0{\cdot}05$, $P=0{\cdot}07$, $P=0{\cdot}05$, $P=0{\cdot}05$ and $P=0{\cdot}0002$, respectively) and in the distal colon in two increments: 36–45 ($P=0{\cdot}05$) and 56–65 ($P=0{\cdot}09$).

More detailed information on the intestinal distribution of bacterial population levels was obtained using FISH and oligonucleotide probes targeting important groups of the intestinal microbiota including *Bifidobacterium* species, *Bacteroides*, lactobacilli/enterococci and *Clostridium perfringens/histolyticum* subgroup. The FISH measurements of total microbial numbers ranged from 1×10^9 to 1×10^{10} cells/g (Table 4). Proportions of selected bacteria, bifidobacteria, clostridia, lactobacilli and *Bacteroides* are given in Table 4. Of these four groups, lactobacilli appeared to be the most dominant, followed by clostridia and *Bacteroides*, the least abundant being bifidobacteria. No differences between the control and the PDX group were detected by the FISH measurements. The numbers of clostridia had a tendency to increase in the distal colon of PDX-fed animals; however, the difference was not significant when compared with the control group ($P=0{\cdot}08$).

Mucosal biomarkers

In addition to the changes in the luminal metabolites and microbes, biomarkers of immunological status in the intestine were monitored by measuring luminal IgA concentrations and mucosal COX-1 and COX-2 gene expression. Independent of the treatment in pigs the concentrations of IgA were highest in the distal small intestine (on average 380 μg/g in the control pigs), decreasing to approximately one-tenth in the large intestine. No significant differences were found between the PDX and the control group in IgA concentrations in any of the different compartments (Table 5).

In general, the COX-1 and COX-2 expression remained fairly constant throughout the intestine, except in the distal small intestine, in which both COX-1 and COX-2 expression was significantly lower when compared with other parts of the intestine (Table 5). No significant differences between control or PDX diet were observed, when the effects of the two diets on the respective parts of the intestine were compared with each other. However, when the effect of PDX on COX gene expression was calculated as a percentage difference compared with the control, it was shown that PDX had generally a lowering effect on COX-2 expression, as its expression was 50·6, 22, 33 and 64·7 % lower in the distal small intestine, caecum, middle colon, and distal colon, respectively, compared with the respective controls. Although these differences only reached statistical significance for the distal small intestine ($P<0{\cdot}001$), trends were observed for the caecum and distal colon ($P=0{\cdot}074$ and $P=0{\cdot}065$, respectively). The only significant difference in the COX-1 expression pattern was observed in the caecum and proximal colon, in which the PDX induced a lower expression of COX-1 compared with the caecum ($P<0{\cdot}05$).

Discussion

In the present study the pigs were fed with two diets, the control high-energy-density diet, and the other high-energy-density diet supplemented with PDX. When the concentration of PDX was measured from the DM of digesta collected from various parts of the intestine, we observed, confirming the earlier results obtained with a human colon simulator, that PDX was fermented more or less evenly throughout the large intestine (Mäkivuokko *et al.* 2006).

In accordance with previous analysis of the swine intestinal microflora, the numbers of total bacteria were found to be higher in the large intestine than in other parts (Jensen & Jorgensen, 1994). Also the *Lactobacillus/Enterococcus* population was found to be dominant in all of the animals, confirming previous results on the characterisation of porcine intestinal microbiota showing that this bacterial population is present at higher numbers in pigs compared with man (Barnes, 1986; Sghir *et al.* 2000; Mountzouris *et al.* 2006).

The %G + C profiling technique is a culture-independent method capable of depicting the total bacterial community within the GI tract in a single analysis. This method is capable of revealing large-scale shifts in the microbial community as a response to dietary changes (Apajalahti *et al.* 1998, 2002) without the need for conventional plating techniques. Differences in specific bacterial composition were observed for different regions of the gut irrespective of diet. The %G + C-profiling technique does not allow differentiation between different bacterial genera, but important information regarding the populations of major bacterial genera along the large intestine of pigs was generated by FISH, highlighting the possibility of monitoring the microbial ecology of the porcine gut. However, as a limitation to the present study, not all bacterial groups present in the porcine gut were enumerated with this selection of oligonucleotide probes. The existing FISH probes have been developed principally for human studies and may not give sufficient coverage for bacterial species relevant to porcine physiology. For instance, the porcine *Lactobacillus* microbiota differs from that found in man in both population size and species composition (Sghir *et al.* 2000). The highest concentration of the *Lactobacillus* population is present in the stomach and small intestine of pigs and it decreases towards the end of the pigs' GI tract. Lactobacilli represent the major bacterial group in the swine GI microbiota, constituting 100 % of the microflora in the stomach and between 90 and 100 % of the microbiota in the duodenum of weaning piglets. In adult pigs lactobacilli constitute 30 % of the microbiota in the stomach, while they are found at lower concentrations in the distal part of the GI tract (Sghir *et al.* 1998). On the contrary, lactobacilli are not found in the stomach of man and constitute less than 1 % of the faecal microbiota (Mueller *et al.* 2006).

In vitro studies suggest that pig intestinal microbes ferment diverse types of oligosaccharides differently. Both the type

Table 4. The bacterial composition of digesta: numbers of *Bifidobacterium*, *Bacteroides*, lactobacilli/enterococci, *Clostridium perfringens/histolyticum*, and total number of cells measured by fluorescent *in situ* hybridisation

(Mean values with their standard errors)

		Bifidobacterium		*Bacteroides* spp.		Lactobacilli		Clostridia		Total bacteria	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Distal small intestine	Control	$6·86 \times 10^{6}$	$2·46 \times 10^{6}$	$1·68 \times 10^{7}$	$8·00 \times 10^{6}$	$1·61 \times 10^{8}$	$8·06 \times 10^{7}$	$1·85 \times 10^{7}$	$5·94 \times 10^{6}$	$3·05 \times 10^{9}$	$5·49 \times 10^{8}$
	PDX	$3·70 \times 10^{6}$	$8·34 \times 10^{6}$	$2·25 \times 10^{7}$	$4·78 \times 10^{6}$	$1·73 \times 10^{8}$	$6·28 \times 10^{7}$	$5·53 \times 10^{7}$	$4·85 \times 10^{7}$	$3·50 \times 10^{9}$	$7·40 \times 10^{8}$
Caecum	Control	$7·33 \times 10^{6}$	$2·91 \times 10^{6}$	$5·42 \times 10^{7}$	$2·97 \times 10^{7}$	$6·21 \times 10^{8}$	$1·69 \times 10^{8}$	$5·56 \times 10^{7}$	$3·20 \times 10^{7}$	$2·01 \times 10^{10}$	$5·06 \times 10^{9}$
	PDX	$5·07 \times 10^{6}$	$9·56 \times 10^{5}$	$7·54 \times 10^{7}$	$5·29 \times 10^{7}$	$4·10 \times 10^{8}$	$5·13 \times 10^{7}$	$5·36 \times 10^{7}$	$2·40 \times 10^{7}$	$1·39 \times 10^{10}$	$9·34 \times 10^{8}$
Proximal colon	Control	$1·08 \times 10^{7}$	$3·47 \times 10^{6}$	$1·13 \times 10^{8}$	$7·54 \times 10^{7}$	$3·75 \times 10^{8}$	$1·20 \times 10^{8}$	$1·30 \times 10^{7}$	$7·63 \times 10^{6}$	$6·42 \times 10^{9}$	$8·29 \times 10^{8}$
	PDX	$7·14 \times 10^{6}$	$2·79 \times 10^{6}$	$3·71 \times 10^{7}$	$1·09 \times 10^{7}$	$3·10 \times 10^{8}$	$4·76 \times 10^{7}$	$2·58 \times 10^{7}$	$1·04 \times 10^{7}$	$5·51 \times 10^{9}$	$6·64 \times 10^{8}$
Middle colon	Control	$2·29 \times 10^{7}$	$8·22 \times 10^{6}$	$1·17 \times 10^{8}$	$4·04 \times 10^{7}$	$2·28 \times 10^{8}$	$6·37 \times 10^{7}$	$1·09 \times 10^{8}$	$2·64 \times 10^{7}$	$4·55 \times 10^{9}$	$5·64 \times 10^{8}$
	PDX	$1·98 \times 10^{7}$	$1·06 \times 10^{7}$	$1·63 \times 10^{8}$	$6·93 \times 10^{7}$	$2·56 \times 10^{8}$	$4·50 \times 10^{7}$	$5·03 \times 10^{7}$	$1·27 \times 10^{7}$	$6·37 \times 10^{9}$	$1·04 \times 10^{9}$
Distal colon	Control	$3·00 \times 10^{7}$	$8·48 \times 10^{6}$	$1·65 \times 10^{8}$	$4·54 \times 10^{7}$	$5·79 \times 10^{8}$	$1·16 \times 10^{8}$	$4·08 \times 10^{7}$	$8·37 \times 10^{6}$	$9·83 \times 10^{9}$	$1·25 \times 10^{9}$
	PDX	$2·34 \times 10^{7}$	$6·49 \times 10^{6}$	$1·37 \times 10^{8}$	$3·75 \times 10^{7}$	$6·87 \times 10^{8}$	$1·53 \times 10^{8}$	$2·61 \times 10^{8}$	$1·63 \times 10^{8}$	$9·17 \times 10^{9}$	$1·13 \times 10^{9}$

PDX, polydextrose.

Table 5. Luminal concentrations of immunoglobulin A, expression of cyclo-oxygenase (COX)-1 and COX-2 in tissue samples obtained from the intestinal compartments within control and polydextrose (PDX) groups, and effect of PDX on the COX expression (calculated as percentage difference compared with control)

(Mean values with their standard errors)

	COX-1 (mRNA copies/100 ng total RNA)				COX -2 (mRNA copies/100 ng total RNA)				Difference in COX gene expression (%)				IgA (μg/g digesta)			
	Control		PDX		Control		PDX		COX-1		COX-2		Control		PDX	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Distal small intestine	8952	789	8093	927	13 425	3779	6635	1245	− 9·6§	10·4	− 50·6‡	9·3	379·50	59·03	355·93	62·33
Caecum	25 212	2788	30 968	3831	43 978	9591	34 227	7935	22·8‖	15·2	− 22·2	18·0	38·25	13·54	48·59	19·41
*P**	$<0·0005$		$<0·0005$		$<0·05$		$<0·01$						$<0·0005$		$<0·001$	
Proximal colon	37 965	6947	32 112	2656	46 621	11 063	68 971	23 386	− 15·4†	7·0	47·9	50·2	41·33	12·26	34·42	13·73
*P**	$<0·005$		$<0·0001$		$<0·05$		$<0·05$						$<0·0005$		$<0·001$	
Middle colon	27 919	2820	30 638	3205	59 994	16 354	40 029	13 121	9·7	11·5	− 33·3	21·9	36·66	19·14	13·89	4·88
*P**	$<0·0001$		$<0·0001$		$<0·05$		$<0·05$						$<0·0005$		$<0·0005$	
Distal colon	32 021	5795	27 690	6384	125 782	46 470	44 425	13 659	− 13·5	19·9	− 64·7	10·9	55·12	32·84	19·48	5·49
*P**	$<0·05$		0·053		$<0·05$		$<0·05$						$<0·0005$		$<0·0005$	

* *P* value for the comparison with the distal small intestine (*t* test).
† Mean value was significantly different from that of the caecum ($P<0·05$) (*t* test).
‡ Mean value was significantly different from that of COX-1 ($P<0·01$) (*t* test).
§ Mean value was significantly different from that of the COX-2 ($P<0·01$).
‖ Mean value was significantly different from that of the proximal colon ($P<0·05$).

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and amount of SCFA produced vary, as well as the amount of gas produced (Smiricky-Tjardes *et al.* 2003). Fermentation of PDX was observed to have significant effects on the microbial metabolite profiles but having rather small effects on the composition of the microbial community. In human clinical trials, and in the *in vitro* models, PDX has been shown to reduce putrefaction metabolites (branched-chain fatty acids) and increase SCFA production (Jie *et al.* 2000; Probert *et al.* 2004; Mäkivuokko *et al.* 2005; Mäkeläinen, In the press). However, more variable results have been obtained regarding the changes in composition of the microbial community (Jie *et al.* 2000; Probert *et al.* 2004; Mäkivuokko *et al.* 2005). Lack as well as presence of a bifidogenic effect has been shown both *in vitro* and *in vivo*.

Various factors could be considered as a reason for the lack of a prebiotic effect when certain oligosaccharides have been applied to swine diets. First, experimental animals are in good health and optimally reared using uniform balanced and highly nutritious diets in controlled and hygienic environments (Mikkelsen *et al.* 2003). Second, other non-digestible oligosaccharides or NSP in the diet could induce a dilution effect. Third, the intestine of pigs may harbour a high number of lactobacilli and bifidobacteria (Barnes, 1986; Gabert *et al.* 1995; Mathew *et al.* 1998; Franklin *et al.* 2002) that can reduce their response to prebiotic supplementation measured in the colon or in faeces. In human subjects, the bifidogenic effect of prebiotic oligosaccharides is inversely related to the volunteer's initial counts (Van Loo *et al.* 1999). As with other single-stomached animals, pigs acquire a more stable GI microbiota only after weaning (Ewing & Cole, 1994; Adami & Cavazzoni, 1999). Perhaps the most appropriate times for demonstrating prebiotic effects in swine are stressful periods such as weaning. Stress is known to influence the GI microbiota and affect total lactobacilli populations (Mathew *et al.* 1998; Tannock, 2001; Franklin *et al.* 2002). Adult animals gradually adapted to the experimental diets were not under stressful conditions and might have had a more stable microbiota, showing less or no prebiotic effect.

In the present study, decreased concentrations of SCFA by the PDX treatment were measured. *In vitro* colon simulation experiments utilising complex microbiota (Probert *et al.* 2004; Mäkivuokko *et al.* 2005), all major SCFA (acetic acid, propionic acid and butyric acid) were increased upon PDX fermentation. Therefore, the decrease in the pig lumen may indicate increased uptake by epithelial cells rather than reduced production by microbes (Vogt & Woleyer, 2003). Results obtained from the blood samples support this hypothesis – higher plasma concentrations of acetic acid and lactic acid in the PDX-supplemented group indicate improved absorption rate from the GI tract. Availability of butyrate is of great importance, since it has been shown to function as a primary energy source for the colon, and have anticancer effects (for a review, see Miller, 2004). Therefore, supplementation of the diet by sustainable fermented fibre could reduce the risk of developing colon cancer in that respect, too (Perrin *et al.* 2001). In human subjects PDX has been shown to be carried through the whole GI tract, to promote butyrate production, and therefore increasing the availability of butyrate throughout the colon (Jie *et al.* 2000; Mäkivuokko *et al.* 2005).

Increased spermidine, and a tendency of spermine, concentrations in the PDX group in the distal large intestine indicated improved epithelial cell growth (Heby, 1981). Furthermore, the principal reduction in production of branched-chain fatty acids as an indication of reduced putrefaction observed upon PDX supplementation in the distal large intestine is noteworthy, since the risk of developing colon cancer in the distal colon is especially high, and some of the putrefactive metabolites, such as indoles and phenols, have been shown to have carcinogenic effects (Smith & Macfarlane, 1997). By supplementing a low-fibre high-energy-density diet with such non-digestible carbohydrates that can persist until the distal colon and modify the microbial metabolism there towards a reduction in putrefaction may impact on the risk of developing colon cancer. Importantly, it was shown in the present study that PDX is fermented gradually throughout the colon and reaches also the most distal parts of the colon.

Prostaglandins derived from COX-2 are important in the healing of mucosal injury, in protecting against bacterial invasion, and in down regulating the mucosal immune system. However, increased COX-2 expression has been observed in colorectal carcinogenesis (Wendum *et al.* 2004; Wallace & Devchand, 2005). In the present paper we report for the first time that in healthy piglets fed with a high-energy-density diet the expression of COX-2 increased towards the distal end of the colon, although individual variation was quite high. Here, supplementation of the diet with PDX had a tendency to decrease the COX-2. As it is likely that in man expression of COX-2 varies from one individual to another, these data obtained from pigs may reflect the human situation in a reasonable way. A low-fibre diet may create an environment in the distal colon in sensitive individuals that favours cancer development. Increasing the weight of these preliminary findings, in a chemically induced colon cancer model, PDX has been shown to reduce numbers of aberrant crypt foci, especially in the distal colon (Ishizuka *et al.* 2003) and in an *in vitro* model, combining a colon fermentation simulation model with a Caco-2 cell model (Mäkivuokko *et al.* 2005), a dose-dependent reduction towards the distal colon in COX-2 expression was noted by PDX.

In a rat model an immune-stimulatory effect in the intestine by PDX has been previously suggested (Peuranen *et al.* 2004). In the present study no increase in IgA concentrations was detected. If stimulation is mediated by specific microbes, the difference may be explained by different microbiota in the two animal species, rat and pig.

In conclusion, the stable nature of the microbial community structure in pigs appears not to allow detection of minor effects induced by diet. Metabolic changes, on the other hand, are more readily detected and effects induced by PDX were evident and, furthermore, appeared similar to those retrieved from previous *in vitro* models and clinical studies. A gradual fermentation of PDX was found to take place throughout the large intestine, explaining detection of beneficial effects even in the most distal colon.

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