

Prebiotics in piglet nutrition?

Fermentation kinetics along the GI tract

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Prebiotics in piglet nutrition?

Fermentation kinetics along the GI tract

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Abstract

The generalized theory behind the carbohydrate to protein fermentation in the GIT is that in presence of fermentable carbohydrate substrate, microbes prefer to ferment carbohydrate source to derive energy and use the nitrogen available for their own growth. With this background information, it was hypothesized that inclusion of fermentable carbohydrates in the piglet diet will reduce the protein fermentation, which will be confirmed by reduced levels of ammonia and branched chain fatty acids in end product profile of the fermentation. The aim of this thesis was to study the effects of inclusion of fermentable carbohydrates in weaning piglets' diet, on GIT fermentation and any changes in microbial community composition and activity. Weaning process in an intensified pig production system brings many sudden changes in the environmental and physical factors in piglets' life. These sudden changes, especially in diet cause serious imbalance in the microbial community. Quicker stabilization and diversification of microbial community post weaning, is crucial in attending the gut health and reducing the risk of pathogenic infections by 'Colonization resistance'. As part of this overall aim, the *in vitro* cumulative gas production technique was used to study the fermentation of selected fermentable substrates. While these substrates namely lactulose, inulin, wheat starch and sugar beet pulp (SBP) were included in test diet and their effect on GIT fermentation was studied *in vivo*. The combination of microbial community analysis based on fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) with nutritional analysis of fermentation end product profiles, was used *in vivo* and *in vitro* studies. In *in vivo* trials, emphasis was given on using combination of slow fermenting carbohydrate sources such as, SBP and wheat starch with fast fermenting lactulose and inulin. The hypothesis behind this approach was to induce carbohydrate fermentation along the GIT, by providing carbohydrate substrate for the microbiota in different parts of GIT. Especially by taking in to account the difference in the transit time of feed in the different parts of GIT, it was expected that fast fermenting lactulose and inulin would be fermented in small intestine while wheat starch somewhere in the beginning of the large intestine while, SBP will reach the distal part of colon. It was found that fermentation along the GIT was improved or in other words skewed more towards the carbohydrate fermentation *in vivo*. It was observed *in vivo* that inclusion of fermentable carbohydrates in the diet reduces the protein fermentation in the GIT and ammonia concentration in end product profile. This decrease was observed along the GIT and in time in faecal fermentation end product profiles post weaning. Microbial community analysis using fingerprinting techniques revealed that inclusion of fermentable carbohydrates stabilized and diversified microbial community in the ileum as well as in the colon by day 10 post weaning. This way, the prebiotic effects of fermentable carbohydrates was evidenced.

Keywords: fermentation, gas production, piglets

To my parents and grand parents,

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General Introduction

“As set out in the White Paper on Food Safety, the Commission will pursue the prohibition or phasing-out of antibiotics used as growth promoters in the EU as part of its broad strategy to control and contain antibiotic resistance.

In the meantime, it is necessary to carry out studies on the most critical sectors (in particular for piglets and broiler production) to minimise possible economic losses or the increase in use of antibiotics for treatment under veterinary prescription. The studies should estimate the gap between the current situation and the husbandry standards which will be required following the abolition of antimicrobial growth promoters.”

Above strategy statement proposed in an official “communication from the EU commission on a community strategy against antimicrobial resistance” (http://europa.eu.int/comm/health/ph/others/antimicrob_resist/am_02_en.pdf), suggests the need for an alternative to in feed antibiotics, after the impending ban by the EU in near future. A potential alternative should be able to control the possible increase in the disease risk and also maintain the economic sustainability of pig production.

Potential Alternatives to in feed antibiotics

Several alternatives are suggested in previous studies. Some of them are summarised below;

i) Probiotics (live cultures of microbes)

The several mechanisms which are proposed in protecting piglets from intestinal pathogens can be referred as “colonization resistance”¹.

- ▷ Adherence to intestinal epithelium thereby preventing attachment of pathogens
- ▷ Competition with pathogens for nutrients
- ▷ Stimulation of mucosal immune response
- ▷ Production of antimicrobial compounds such as bacteriocins

There are several recent studies which have shown positive effects of probiotics in pig nutrition²⁻⁵.

ii) Enzymes

Newly weaned piglets may produce inadequate amounts of certain enzymes, which might cause the gastrointestinal disturbances predisposing piglets to post-weaning diarrhoea⁶. Addition of enzymes in weaning diet may facilitate the process of digestion and help reducing losses in production performance during post weaning. Some data indicate that addition of carbohydrate-degrading enzymes to a barley-based diet improved feed conversion and reduced incidence of diarrhoea in pre weaned and newly weaned pigs⁷. Bedford and Schulze⁸ have reviewed the currently available enzymes used as feed supplements and prospects for further developments of enzymes as alternatives to in feed antibiotics.

iii) Immune modulators

These are the immunologically active compounds which may have effect on the immune system of the animal. They include mainly antibodies in the form of colostrum from the sow vaccinated for a particular disease⁹ or in the form of freeze dried eggs containing antibodies¹⁰. Although the recent study by Chernysheva *et al.*¹¹ showed no efficacy of addition of chicken egg yolk antibodies on clinical response to *E. coli* K88⁺ challenge in weaned pigs. Growth promoting effects of immunomodulators are not pronounced, but they might help in reducing the incidence of the disease. However in some studies spray dried porcine plasma proteins have shown the growth promoting effects in newly weaned piglet^{12,13}.

iv) Organic acids

Post weaning period often result into a lag period with limited digestive and absorptive capacity due to insufficient production of HCL¹⁴ and pancreatic enzymes¹⁵. In post weaning period lowering of gastric pH by acidification of diet with weak organic acids has been reported to reduce the problems^{16,17}. Use of various organic acids in pig diets for performance

enhancement has been reviewed in depth by Partanen and Mroz¹⁸. Although, acidifiers look like a lucrative alternative, palatability of the diet may be a matter of concern¹⁹.

v) Herbal additives

Number of natural substances and herbal extracts which were used in alternative medicinal therapies are gaining interest and are looked on as a candidate for potential alternatives. Studies using herbal mixture containing great nettle, garlic and wheat grass showed the improved growth and feed efficiency in growing pigs²⁰.

vi) Fermentable carbohydrates

Inclusion of fermentable carbohydrates is considered to be a comparatively easy way to influence the composition and activity of the microbiota²¹. Being potential sources of energy for bacteria, fermentable versus digestible carbohydrates, can be effective in terms of influencing the gut microbiota beneficially²¹⁻²³. The addition of selected fermentable carbohydrates has been shown to increase bacterial diversity and lead to more rapid stabilization of the microbial community²⁴ in newly weaned piglets. In case of depletion in carbohydrates as energy source, fermentation in hind gut becomes more and more proteolytic²⁵. Furthermore, excess protein fermentation in large intestine leads to increased ammonia concentration in colon and predisposes early weaned piglets to diarrhoea²⁶. Inclusion of fermentable carbohydrate substrates in diet, has been shown to be an effective strategy to control large intestinal proteolysis^{25,27,28}.

To study several alternatives for in feed antibiotics and define a criterion for the gut health of weaning piglets, a project funded by EU 5th framework program was undertaken called “Healthy Piglet” (<http://www.rennes.inra.fr/healthypigut/>). We study a part of aspects of this large project, by concentrating on the possible use of fermentable carbohydrates in weaning pig nutrition. The out line of this thesis is based on some of the issues addressed in former review published by Williams *et al.*²³

The general scope of research in the present thesis is to investigate possibilities of inclusion of fermentable carbohydrates as prebiotics in weaning diet of the piglet, studied by in-vivo and in- vitro experiments. While the more specific objectives are:

- ▷ To determine whether inclusion of fermentable carbohydrates of varying fermentabilities in the weaning diet, could influence concentrations of fermentation end-products along the GIT. (Chapter 1&2)
- ▷ To determine whether enforced fasting at the beginning of weaning have an effect on fermentation end-products in later period. If there is any effect of fasting whether it is influenced by inclusion of fermentable carbohydrates. (Chapter 1&2)
- ▷ To investigate whether there are any gradual changes in the fermentation end product profiles in faeces with time after weaning. (Chapter 2)

- ▷ To investigate whether fermentable carbohydrates can be included as potential prebiotics in weaning diet for piglets. (Chapter 3)
- ▷ To evaluate difference in inocula from ileum and faeces, for their in-vitro microbial activity when incubated with fermentable carbohydrate sources known different fermentation kinetics. (Chapter 4)
- ▷ To determine whether the prior dietary exposure to fermentable carbohydrates by their addition to the host diet, influence *in vitro* fermentation of the added carbohydrates by the faecal microbiota of the host. (Chapter 5)

Chapter 1

Dietary carbohydrates with different rates of fermentation do affect fermentation end product profile in different sites of gastrointestinal tract of weaning piglet

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Abstract

An *in vivo* experiment was conducted to examine changes in fermentation in the gastrointestinal tract (GIT) of weaning piglets by the addition of fermentable carbohydrates in the diet. The experiment was repeated in three replicates of 36 piglets. Specially raised piglets (neither antibiotics nor creep feeding) were weaned abruptly at four weeks of age. Each replicate was conducted over a period of ten days. The piglets were offered one of two dietary treatments: control diet (CON), and fermentable carbohydrate enriched diet (CHO); and were subjected to one of the two fasting treatments (fasting for two days in the beginning of the experimental period and non-fasting). Piglets were slaughtered on the 1st, 4th and 10th day of each period. Digesta samples were collected from different parts of the intestinal tract. (first half of small intestine, second half of small intestine, caecum, and colon). The dry matter, volatile fatty acid (VFA) profile, and ammonia concentrations were analyzed. Feed intake, growth and feed conversion ratio were also recorded. There was no difference in production performances between the treatment groups. Concentrations of VFA and ammonia were significantly different between diets, sites of fermentation within GIT ($P < 0.001$), and the slaughtering day ($P < 0.05$). Fasting had no effect on fermentation end-products. This study concludes that the addition of fermentable carbohydrates of varying fermentabilities stimulated the production of fermentation end-products in four different areas of the GIT studied, in weaning piglets.

Introduction

Microbial fermentation in the gastrointestinal tract of monogastric animals is gaining interest in recent years especially, to find an alternative for in feed antibiotics in “Post in-feed antibiotic era.”

It is known that the microbial community is largely dependent upon the animal diet as the main source of substrate for its metabolism. Consequently, changes in the dietary composition or nutrient density can have dramatic effects on the intestinal microbial community²⁹, assuming that microbial requirements are taken into account. Manipulation of the diet by the use of fermentable carbohydrates is considered a comparatively easy way to influence the composition and activity of the microflora²¹. As potential sources of energy for bacteria, fermentable versus digestible carbohydrates can be effective in terms of influencing the gut microbiota beneficially²¹⁻²³. For example, the addition of selected fermentable carbohydrates has been shown to increase bacterial diversity and lead to more rapid stabilisation of the microbial community²⁴ in newly weaned piglets.

At weaning, exposure to a solid diet combined with other stresses, can lead to dramatic changes in the GIT microbiota, which leaves piglets potentially susceptible to the activity of potentially pathogenic bacteria and thus to the well-described “post-weaning syndrome”³⁰. Given the impending EU ban on the use of antimicrobial growth promoters in animal feeds, there is now an urgent imperative to examine alternative ways to improve pig health by stimulating the autochthonous gastrointestinal microflora³¹.

The post-weaning period in pigs is sometimes associated with a temporary anorexia. At first, some piglets avoid eating at all, and may then suddenly eat a large amount of feed between 24 and 72 hours after removal from the sow. This may lead to severe upset of digestive function and to diarrhoea³⁰.

In pigs, substantial fermentation has been observed also in the small intestine in addition to large intestine^{32,33}. By composing a diet which incorporates ingredients with different rates of fermentation, it should be possible to design a diet which will stimulate some degree of fermentation all along the GIT.

The aim of the present study was firstly to determine whether addition of fermentable carbohydrates of varying fermentabilities in the weaning diet, could indeed influence concentrations of fermentation end-products along the GIT. Secondly, to determine whether enforced fasting at the beginning of weaning had an effect on fermentation end-products, and whether there was a diet/fasting interaction.

Materials and Methods

Experimental Design

An *in vivo* experiment was designed as a split plot design. The experiment was conducted in three identical replicates. Each replicate was conducted over a period of ten days. For each replicate, four litters of nine piglets were used (in total 36 piglets per period- 108 piglet in

but no interference in dietary or stress treatment. This arrangement was to prevent cross-contamination between litters, but the continued contact with littermates was supposed to reduce stress.

Dietary Treatments

The control diet (CON) was semi-purified, and was designed in such a way as to be as low as possible in fermentable carbohydrates. The test diet with added fermentable carbohydrates (CHO) was based on this same diet, but had added carbohydrates in the form of unmolassed SBP, WST, lactulose and inulin. These ingredients had been chosen following testing for their fermentability by cumulative gas production (Williams *et al.*, submitted). Both diets contained neither antibiotics nor added copper. The diets were composed in such a way that total energy and protein contents were comparable. The composition of the diets is shown in Table 1.

Fasting Treatments

The animals with enforced fasting (fasting) were not offered any feed for 48 hours from the moment of arrival at the experimental facility. The non-fasted animals (non fasting) on the other hand, had free access to their diet from the moment of arrival at the facility. All piglets had free access to water at all times.

Slaughter of Piglets and Sampling

Piglets were slaughtered on D1, D4, and D10 post-weaning (see Figure1). The piglets were slaughtered as follows: Ketamin was used as a pre-anaesthetic, and 30 min later, the piglet was euthanatised by intra-cardiac injection of T61 (Hoechst Roussel Vet). Following an incision into the abdomen, the GIT was tied off at regular intervals using plastic strips to avoid mixing of digesta. The entire tract (from pyloric sphincter of stomach to anus) was then lifted from the abdominal cavity and taken to the laboratory. At the laboratory, the GIT was divided into four parts: first half of small intestine (SI1), second half of small intestine (SI2), caecum (CE) and colon (CO) and the digesta emptied into clean glass beakers. After mixing, the pH (pH meter – Hanna Instruments) was recorded. Samples were collected for DM, VFA and ammonia analysis. Additional samples were collected from SI1 and SI2, for lactic acid analysis.

Analyses

Dry matter was determined by drying to a constant weight at 103 °C³⁴ and ash by combustion at 550 °C³⁵. Volatile fatty acid (VFA) concentrations in the fermentation liquids were analysed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101, as carrier gas N₂ saturated with methanoic acid, at 190 °C and using *iso*-caproic acid as an internal standard.

Ammonia was determined according to the method described by Searle³⁶. In short, supernatant was deproteinized using 10% trichloro-acetic acid. Ammonia and phenol were oxidized by sodium hypochlorite in the presence of sodium nitroprusside to form a blue complex. The intensity was measured colorimetrically at a wavelength of 623 nm. Intensity of the blue colour is proportional to the concentration of ammonia present in the sample.

The lactic acid concentration of the digesta was analyzed according to the method described by Voragen *et al.*³⁷ using a Jasco HPLC unit fitted with Supelcogel HPLC column (C-610 H, 30cm x 7.8mm ID).

Table 1. Composition of the Diets (g/kg)

Ingredients	Diets	
	CON	CHO
Corn starch	504.8	368.1
Sugarbeet pulp	-	50.0
Inulin	-	7.5
Lactulose (~50%DM)	-	20.0
Wheat starch	-	50.0
Fishmeal	200.0	200.0
Soya isolate	50.0	45.0
Dextrose	150.0	150.0
Soya oil	15.0	30.0
Cellulose (Arbocel)	50.0	50.0
Premix	10.0	10.0
Chalk	2.5	1.5
Monocalcium phosphate	1.5	1.5
KHCO ₃	12.0	12.0
L-lysine HCl	0.6	0.7
DL-methionine	2.0	2.0
L-threonine	1.0	1.1
L-tryptophan	0.6	0.6
Calculated Analysis		
Dry Matter	916	911
Ash	43	46
Crude Protein	179	180
NE contents (MJ/Kg)	11	11

Statistics

Differences between the dietary and fasting treatments and the interactions between them were tested for significance by ANOVA using the repeated measurement procedure with the following model;

$$Y_{ijklm} = \mu + D_i + F_j + S_k + (D*F)_{ij} + (D*S)_{ik} + \epsilon 1_{ijkl} + G_m + (D*G)_{im} + (S*G)_{km} + \epsilon 2_{ijklm}$$

Where Y is the parameter to be tested, μ is the overall mean, D_i effect of the diet i; F_j effect of the fasting stress j; S_k effect of slaughter day k; $(D*F)_{ij}$, $(D*S)_{ik}$, $\epsilon 1_{ijkl}$ is the error term 1, which represents the random effect of animal within diet i, fasting stress j, slaughter day k level; G_m effect of site of GIT m; $(D*G)_{im}$, $(S*G)_{km}$ denotes the respective interactions and $\epsilon 2_{ijklm}$ is the error term 2, which represents the overall error including the GIT sites within the animal.

The non-significant interactions were removed from the model. The effect of replicate and litter was tested separately, and was not significant for any of the parameters. It was therefore removed from the statistical model. Differences between treatment lsmeans were evaluated using Tukey test of multiple comparisons. Differences were considered significant, when $p < 0.05$.

The observations on D1 were not included in statistical analysis as those piglets had not received any of the experimental treatment. The means of the observations on D1 are presented in separately in Table 3.

All statistical analyses were performed using the PROC GLM procedure of the statistical program SAS³⁸.

Results

Animal Performance

The mean values for total intake, growth and feed conversion ratio (FCR) by D10 are shown in Table 2. There was neither effect of diet, fasting nor any interaction between diet and stress for any of the parameters.

Table 2. Total intake, Growth and FCR of the piglets slaughtered on D10

Diet	Stress	Total intake (g)	Growth(g)	FCR
CON	Fasting	2099	1348	1.663
	Nonfasting	2177	1301	1.870
CHO	Fasting	2091	1446	1.494
	Nonfasting	2251	1520	1.606
Main Effects				
Diet		0.785	0.228	0.173
Stress		0.331	0.915	0.309
Diet*Stress		0.736	0.642	0.760
MSE ¹		120.7	129.6	0.15

¹MSE = mean standard error;

Fermentation End-Products

The lsmeans calculated for the main factors and the probabilities of significance, for the different variables and their interactions, are shown in Tables 4 and 5.

Slaughter day had a significant effect for all the parameters (except pH) shown in Table 4. Total VFA, acetic, propionic, butyric and ammonia concentrations all increased with time, while DM, pH, and lactic acid concentration decreased.

Differences between the different sites of the GIT were highly significant for the means of

parameters shown in Table 4. VFA and ammonia concentrations were higher in the large intestine, especially the caecum. Lactic acid was significantly higher for SI2 compared with SI1. The DM was much higher for the colon contents compared with other GIT sites. SI1 had a significantly lower pH, compared with the other sites. However, after SI1 the pH was lowest in the caecum.

Table 3. Mean concentrations of fermentation end products in the GIT of the piglets slaughtered on D1

GIT	Fermentation end products					
	TotVFA ¹	Acet ¹	Prop ¹	But ¹	Lact ¹	Ammo ¹
SI1	4.04	3.75	0.17	0.11	42.21	5.96
SI2	16.80	15.94	0.27	0.21	36.48	8.82
CE	98.70	63.20	21.19	7.38	-	63.58
CO	54.75	33.83	12.92	3.91	-	51.51

¹TotVFA= Total volatile fatty acid concentration (mmol/L digesta water); Acet = Acetic acid; Prop= Propionic acid; But= Butyric Acid; Lact= Lactic acid (mmol/ L digesta water); Ammo= Ammonia (mmol/ L digesta water);

Diet had a significant effect on most of the parameters shown in Table 4, except for butyric acid. It was observed that for the CHO diet, VFA and lactic acid concentrations were higher, while DM, pH, and ammonia concentration were lower, compared with CON.

Fasting seems to have had no effect on the pH, DM, VFA or lactic acid concentrations (see Table 4). However, there was a lower ammonia concentration for the non- fasting compared with fasting animals.

Interaction between site of the GIT and slaughter day had significant effect on production of most of the fermentation end-products (see Table 4). There was a significant interaction between diet and GIT site, for propionic acid, DM, pH and ammonia concentrations.

Table 5 shows that site in the GIT and diet had a significant influence on proportional production of VFA. For the small intestinal digesta (SI1 and SI2), the acetic acid proportion was significantly higher compared with that of the large intestine, while the propionic and butyric acid proportions were greater for the large intestine compared with the small intestinal digesta. Diet had a significant effect on the acetic acid proportion, while the diet and GIT interaction had an effect on the acetic and propionic acid proportions.

Discussion

During the experimental periods, none of the piglets showed any signs of diarrhoea or any other illness. This was not unexpected, given that this was not an infection challenge study, and was conducted in an experimental facility where husbandry may be more fastidious than may be possible on a commercial farm.

It had been expected that fasting would have a significant effect on the total intake and/or growth parameters, but this was not the case. This might be due to the fact that some of the piglets without enforced fasting also consumed very little in the first 48 hours. It was also interesting to observe that the piglets with enforced fasting immediately caught up with the

non-fasting group from D3 (Chapter 2). This might account for the absence of any effect of fasting seen for most of the parameters. However, the results therefore disagree with the theory of temporary anorexia after weaning, followed by a large feed intake leading to post-weaning diarrhoea³⁰. It would be interesting to consider the possibility that anorexia in response to stress may be an individual characteristic. If this were true, a future experiment should offer all animals feed, and then separate them into groups according to whether or not they actually consume any, rather than enforcing fasting, as was done here.

In a study by Jensen & Jorgensen³², where seven-month old piglets were fed with high and low fibre diets there was a trend of a lower DM content with the high fibre diet, and increasing DM contents from the beginning of small intestine towards the end of large intestine. The results of the study reported here, are in agreement with this, though of course, for much younger animals. The significantly higher DM contents in the colon were not surprising, given the physiological fact of water re-absorption in the colon. However, in this study, there was also a significant interaction between GIT and diet for the DM content (Table 4). It was observed that the CHO piglets had lower DM contents in colon than the CON piglets (data not shown). This was in agreement with previous authors, who also reported lower DM contents for animals fed high fibre diets^{39,40}.

The pH in the different GIT sites, showed a trend of lower pH at the beginning of small intestine with an increase towards the end of the small intestine, a slight drop in the caecum, and then a rise in colon, which is in agreement with the study already mentioned³². In the present study, the significant effect of interaction between diet and GIT, showed the lower pH values for CHO diet compared to CON diet along the complete GIT for both the small and large intestine. On the other hand, the study of Jensen and Jorgensen³², showed pH lowering effect of high fibre diet in large intestine only. This is most likely due to the difference in the diet composition between the two studies. In the Danish study, the high fibre diet contained supplemented pea fibre and pectin along with barley. Being slow fermenting carbohydrate sources, the effect on pH, due to higher VFA production, was seen in large intestine only. While in the present study, CHO diet contents fermentable carbohydrates with variable fermentability, from rapidly fermenting lactulose⁴¹ to the very slow fermenting SBP containing pectins and other complex substrates⁴², which was supposed to stimulate the carbohydrate fermentation along the whole GIT.

Both diet and GIT had a significant effect on fermentation end-products such as total VFA, acetic, propionic, lactic acid and ammonia, although the interaction was only significant for ammonia and propionic acid concentrations. It would seem that the higher contents of fermentable carbohydrates, indeed resulted in higher VFA concentrations in the piglets fed the CHO diet compared with the CON diet. The large intestine, probably due to its larger and more diverse microbial population and longer transit time of its digesta⁴³ had higher VFA concentrations compared with the small intestine. Table 4 clearly shows higher VFA production for the caecal digesta compared with the colon. However, as reviewed by Williams *et al.*,²³ it is also important to note that there is a higher rate of absorption of VFA in colon, due to more specialized colon epithelium for VFA absorption compared with other

parts of the GIT. This may also have influenced the lower VFA concentrations in the colon compared with the caecum. Furthermore, according to Macfarlane and Macfarlane⁴⁴, higher production of short chain fatty acids (SCFA) in the proximal large intestine is due to greater carbohydrate availability compared with the distal large intestine.

Table 4. DM, pH, and end product profile of digesta of the weaning piglets according to the main effects of diet, stress, slaughter day and GIT area

Levels	TotVFA ¹	Acet ¹	Prop ¹	But ¹	Lact ¹	NH3 ¹	DM ¹	pH
Diet								
CON	56.79	37.97	10.66	4.94	36.87	24.46	152.35	6.22
CHO	63.43	43.31	12.53	5.28	47.37	16.44	140.66	6.13
MSE ³	1.23	0.86	0.25	0.19	1.30	0.549	2.415	0.021
P value	0.019	0.010	0.001	0.381	0.001	<0.001	0.064	0.083
Stress								
Fasting	61.99	41.87	11.95	5.15	44.27	21.53	151.97	6.16
Nonfasting	58.23	39.87	11.25	5.07	39.98	19.38	141.05	6.18
MSE ³	1.24	0.86	0.25	0.19	1.30	0.549	2.415	0.021
P value	0.179	0.228	0.177	0.838	0.163	0.038	0.084	0.683
Slday ²								
4	55.09	38.27	10.06	4.66	46.63	18.39	163.82	6.22
10	65.13	43.01	13.13	5.56	37.61	22.54	129.19	6.14
MSE ³	1.24	0.86	0.25	0.19	1.30	0.549	2.415	0.021
P value	0.001	0.022	<0.001	0.022	0.004	0.001	<0.001	0.068
GIT								
SI 1	5.02	3.66	0.20	0.41	32.26	4.92	113.87	5.57
SI 2	9.79	8.73	0.19	0.46	51.99	4.91	112.02	6.55
CE	118.33	70.47	24.42	9.98	-	35.59	137.23	6.18
CO	107.28	70.46	21.59	9.58	-	36.37	222.92	6.41
MSE ³	1.75	1.22	0.36	0.27	1.30	0.77	3.41	0.030
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Interaction	Probabilities							
Diet*Stress	0.263	0.422	0.242	0.674	0.241	0.103	0.204	0.953
Diet*Slday	0.776	0.713	0.678	0.536	0.178	0.202	0.602	0.288
Diet *GIT	0.431	0.471	0.001	0.541	0.696	<0.001	0.001	<0.001
GIT*Slday	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	0.087	0.271

¹TotVFA= Total volatile fatty acid concentration (mmol/L digesta water); Acet = Acetic acid; Prop= Propionic acid; But= Butyric Acid; Lact= Lactic acid (mmol/ L digesta water); NH3= Ammonia (mmol/ L digesta water); DM= Dry matter (g/Kg);

²Slday = Slaughtering day;

³MSE= mean standard error;

In this study, there was a higher lactic acid concentration detected both in SI1 and SI2 for CHO piglets compared with CON piglets, which tends to confirm the more active fermentation occurring with the CHO diet (assuming that diet itself had no effect on absorption across the mucosa). In this experiment, lactic acid was only measured in the small intestine (SI1 and SI2). The SI2 had significantly higher lactic acid concentration compared to SI1. This GIT effect is might be due to the rapid flow of digesta in the beginning of small intestine compared with end of small intestine⁴³. That could also lead to flow of lactic acid from SI1 to SI2 part of the GIT, as well as increased production. No information is available quantifying the flow versus absorption of lactic acid in different areas of the small intestine.

In the present study where especially; the piglets had no creep feed, but only sow's milk during the suckling period, it is important to note the decrease in lactic acid concentration by D10. Whilst the piglet is suckling the small intestinal microbiota is dominated by *lactobacilli* and *streptococci*³⁰ and the major fermentation product is lactic acid. With time,

there is development of the small intestinal microbiota both in number and diversity. This might change the end-product profile from lactic acid as a major product to more diverse VFA profile with time (Table 4).

Table 5. Proportional VFA production in weaning piglets, as expressed by % of the total VFA, according to the main effects of diet, stress, slaughter day and GIT area

Levels	AP ¹	PP ¹	BP ¹	BCP ¹
Diet				
CON	73.72	12.37	7.99	2.72
CHO	77.03	11.83	7.28	2.10
MSE ³	0.805	0.23	0.52	0.40
P value	0.009	0.185	0.429	0.261
Stress				
Fasting	75.92	12.02	6.86	2.74
Nonfasting	74.84	12.19	8.40	2.07
MSE ³	0.81	0.23	0.52	0.40
P value	0.375	0.676	0.086	0.223
Slday ²				
4	76.48	11.70	7.00	2.70
10	74.28	12.50	8.26	2.12
MSE ³	0.81	0.23	0.52	0.40
P value	0.078	0.049	0.159	0.292
GIT				
SI 1	79.82	4.44	7.82	4.55
SI 2	88.70	2.89	5.68	1.20
CE	67.13	20.96	8.24	1.46
CO	65.87	20.12	8.79	2.43
MSE ³	1.14	0.33	0.74	0.57
P value	<0.001	<0.001	0.019	0.001
Interaction	Probability			
Diet*Stress	0.917	0.951	0.674	0.506
Diet*Slday	0.802	0.833	0.205	0.378
Diet *GIT	0.027	0.001	0.262	0.997
GIT*Slday	0.907	0.275	0.218	0.698

¹ AP= Acetic acid proportion (%); PP= Propionic acid proportion (%); BP= Butyric acid proportion (%); BCP= Branched chain fatty acid proportion (%);

²Slday = Slaughtering day;

³ MSE= mean standard error;

Interestingly, the proportions of VFA also differed significantly according to site of the fermentation in the GIT (Table 5). The production of different VFA is very well regulated by the availability of substrate to the bacteria, and also to cross-feeding between species. An example of this, would be the metabolism of intermediate products such as lactic acid into VFA such as propionic acid^{44,45}. The substrate energy availability plays major role in determining the proportions of end-products of fermentation⁴⁴. The proportionally higher acetic acid production by the small intestinal microbiota may also be an effect of faster transit of the substrate, resulting essentially in reduced availability of the substrate. The reverse would be true for the large intestine, where propionic and butyric acid proportions are higher than that in the small intestine, as substrate energy availability may be increased due to longer transit time in large intestine.

Conclusion

Before concluding, it must be emphasized that the semi-purified diets used in this experiment do not resemble the normal commercial diets used by farmers. As shown by Bauer,⁴⁶ ingredients commonly used in normal pig diets can vary quite considerably in their fermentability. In order to obtain two diets with a clear variation in the presence of fermentable (vs. digestible) carbohydrates, it was necessary to choose basal ingredients which were highly digestible.

This study certainly concludes that the forced fasting at the beginning of weaning period has no effect on end product profile in the post-weaning period. However, author would like to suggest that, in future studies piglets should divide into two groups of fasting and nonfasting piglets, based on their voluntary feed intake at the beginning of the weaning.

The present study also emphasise that the addition of fermentable carbohydrates with different fermentability leads to more carbohydrate fermentation compared to protein fermentation along the GIT. This might be important in designing diets with fermentable carbohydrates for their prebiotic effects, in “post in feed antibiotics era”.

Acknowledgement

The authors would like to thank Walter Gerrits, Jan Dijkstra for their assistance with the statistical analysis, and Meijke Booi, Jane-Martine Muijlaert, Dick Bongers and Huug Boer of the Animal Nutrition Group for their assistance with the laboratory analyses. Tamme Zandstra and personnel of the animal experimental facilities at “De Haar” are thanked for their co-operation during this study.

Chapter 2

Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in faeces of weaning piglets

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Abstract

An *in vivo* experiment was conducted to monitor changes in fermentation end products in the faeces of weaning piglets by the inclusion of selected fermentable carbohydrates in the diet. The experiment was repeated in three replicates of 16 piglets. Specially raised piglets (neither antibiotics nor creep feeding) were weaned abruptly at four weeks of age. Each replicate was conducted over a period of ten days. The piglets were offered one of two dietary treatments: control diet (CON), or fermentable carbohydrate enriched diet (CHO); and were subjected to one of the two fasting treatments (fasting for two days in the beginning of the experimental period and non-fasting). Fecal samples were collected per rectum everyday during the experimental period. Piglets were slaughtered at the end of the experimental period and digesta samples collected from different parts of gastrointestinal tract (GIT): first half of small intestine, second half of intestine, caecum and colon. The dry matter, volatile fatty acid (VFA) profile, and ammonia concentrations were analyzed from the fecal and digesta samples. Daily feed intake was also recorded. There was no difference in concentrations of VFA in faeces, between the treatment groups. Ammonia concentration was significantly lower in piglets fed with CHO diet compared with CON diet, ($P < 0.05$) in both faeces and digesta from different parts of GIT. Fasting had no effect on fermentation end-products in faeces. This study concludes that the inclusion of fermentable carbohydrates with different fermentabilities in weaning diets; significantly reduce the protein fermentation along the GIT and fecal concentration of ammonia.

Introduction

Weaning in piglets is associated with a great deal of stress, including exposure to a solid feed. This can lead to dramatic changes in the gastrointestinal tract (GIT) microflora, which leaves piglets susceptible to the activity of pathogens and to the “post-weaning syndrome”³⁰. There are possibilities to improve pig health by stimulating the comensal microflora.

The microflora in GIT is mainly dependent upon the animal’s diet as its main source of substrate for its metabolism. Any changes in dietary composition may have dramatic effects on the intestinal microflora³⁰. Depletion in carbohydrates as energy source, leads to more proteolytic fermentation in hind gut²⁵. Excess protein fermentation in large intestine results in increased ammonia concentration in colon and predisposes early weaned piglets to diarrhoea²⁶. Sometimes temporary anorexia post-weaning may be followed by sudden increase in feed intake large amounts of feed, which leads to severe upset of digestive function and to diarrhoea^{30,47}.

Inclusion of fermentable carbohydrate substrates in diet, is an effective strategy to control large intestinal proteolysis^{25,27,28}. In pigs, substantial fermentation has been observed also in the small intestine^{32,33,48}. By selecting ingredients with different rates of fermentation, it should be possible to design a diet which will stimulate fermentation all along the GIT. Therefore, in present study, four fermentable carbohydrates with different rates of fermentation were included in one of the dietary treatment. The carbohydrate substrates were chosen based on their *in vitro* fermentation characteristics (Figure 1), tested by *in vitro* gas production test (Williams *et al.* submitted).

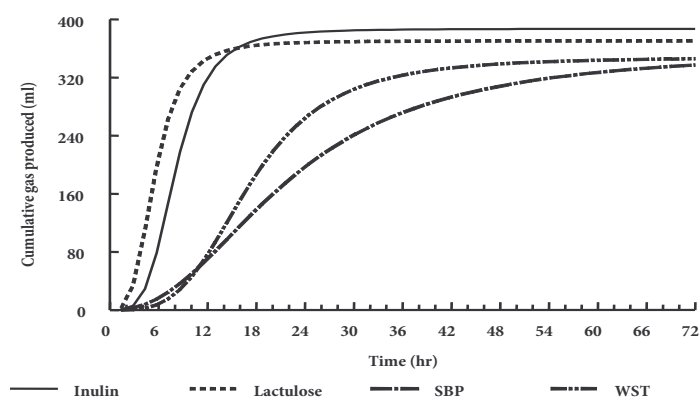


Figure 1. Gas production profiles after 72 hrs for fermentation of four carbohydrates, by fecal inoculum of suckling piglets.

The present Study aims to investigate i) whether the inclusion of fermentable carbohydrates with variable rates of fermentation in weaning diet influence fermentation along the GIT and reduce the protein fermentation ii) whether starvation due to enforced fasting at the beginning of weaning had an effect on concentration of fermentation end-products in later period, and iii) whether there are any gradual changes in the fermentation end product profiles with time after weaning.

Materials and Methods

Experimental Design

An *in vivo* Experiment was designed as 2 x 2 factorial design. The experimental treatments were the two dietary treatments with and without inclusion of fermentable carbohydrates and two treatments with or without enforced fasting in the beginning of experimental period. Experiment was conducted in three identical replicates. Each Replicate was conducted over a period of ten days. For each replicate, four piglets from four litters were used (in total 16 piglets per period- 48 piglets in total). At the beginning of each period, on Day 1 (D1), fecal samples were collected before piglets were subjected to any treatment. Then piglets from each litter were assigned to one of the four treatment combinations. Daily fecal samples were collected. At the end of each period piglets were slaughtered and samples were collected from different parts of GIT. All the procedures involving animals were conducted in accordance with the Dutch law on experimental animals and had been approved by the Wageningen University Animal Experimental Committee (Dier Experimenten Commissie).

Animals and Housing

The 48 crossbred piglets (Hypor x Pietrain, male-female mixed group) were weaned at four weeks of age, and transported to the experimental facility. The piglets had no access to creep feed prior weaning, nor any antibiotic treatment before and during the experimental period. During the experimental period of ten days, the piglets had free access to their diet (except the fasted piglets for the first 48 hours) and clean drinking water. Daily feed intake was measured per piglet during the experiment. Piglets from the same litter were kept in adjacent pens separated by a wire mesh, so that they could have visual contact with their littermates, but no interference in dietary or fasting treatment. This arrangement was to prevent cross-contamination between litters, but the continued contact with littermates was supposed to reduce stress.

Dietary Treatments

The control diet (CON) was semi-purified, and was designed to have very low levels of fermentable carbohydrates (see Table 1, Chapter 1). The test diet with added fermentable carbohydrates (CHO) was based on this same diet, but had added carbohydrates in the form of unmolassed sugar beet pulp (SBP), native wheat starch (WST), lactulose and inulin. These ingredients had been chosen following testing for their fermentation kinetics by cumulative

gas production (Figure 1). Lactulose and inulin were more rapidly fermentable compared to wheat starch and SBP. For both the diets, the main source of starch used was native corn starch, with ileal digestibility ~97%,⁴⁹ to have a better contrast in substrate reaching large intestine. The diets were composed in such a way that total energy and protein contents were comparable. Both diets contained no antibiotics no extra added copper. The composition of the diets is shown in Chapter 1 (Table 1).

Fasting Treatments

The animals with enforced fasting were not offered any feed for 48 hours from the moment of arrival at the experimental facility. The non-fasted animals (non fasting) on the other hand, had free access to their diet from the moment of arrival at the facility. All piglets had free access to water at all times.

Slaughtering and sampling

Daily fecal samples were collected in the morning with a gloved finger for dry matter (DM), volatile fatty acid (VFA) and ammonia concentration analyses. The piglets were slaughtered on day 10. First Ketamin was used as a pre-anaesthetic, and 30 min later, the piglet was euthanatized by intra-cardiac injection of T61 (Hoechst Roussel Vet). After the dissection into the abdomen, the entire GIT was separated from the abdominal cavity. The gastro-intestinal tract was ligated at regular intervals to avoid mixing of digesta. Then in the laboratory, the GIT was divided into four parts: first half of small intestine (SI-1), second half of small intestine (SI-2), caecum (CE) and colon (CO). From each site, digesta was properly mixed and samples were collected for DM, VFA and ammonia analysis.

Analyses

Dry matter was determined by drying to a constant weight at 103°C³⁴ and ash by combustion at 550°C³⁵. Volatile fatty acid (VFA) concentrations in the fermentation liquids were analysed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101, as carrier gas N₂ saturated with methanoic acid, at 190°C and using iso-caproic acid as an internal standard. Ammonia was determined according to the method described by Searle.³⁶

Calculations

The branched chain proportion (BCP) was calculated as an indicator of the protein fermentation:

$$\text{BCP (\%)} = [(\text{isoButyric} + \text{isoValeric}) / \text{Total VFA}] \times 100 \dots\dots\dots(1)$$

The acetic (AP), propionic (PP) and butyric acid proportions (BP) were calculated similarly.

Statistics

Concentrations of the fermentation end product in faeces and daily feed intake of the animal were measured repeatedly during the experiment. The statistical analysis was done for repeated measurements as explained by Gerrits *et al.*⁵⁰ In short, the separation of time from treatment effects is illustrated in Figure 2. A straight line was fitted through the end product concentrations or daily feed intake values against time starting with measurement from Day 2. The existence of a quadratic component in this relationship was tested, but since it failed to improve the statistical fit over the linear, the later one was preferred.

The slope of the line (α , see Figure 2) represents the effect of time (t) and the intercept (at $t = \bar{t}$), the average end product concentration or daily feed intake during the experiment. Both the slope and intercept were treated as dependent variables and treatment effects on slope and intercept were analysed by ANOVA (see Equation 2).

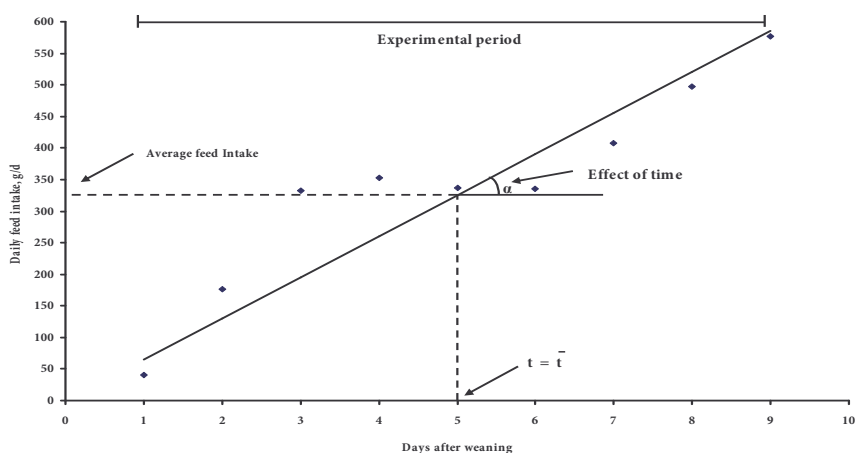


Figure 2. Example of separating time from treatment effects on daily feed intake. The individual measurements of daily feed intake are presented for one animal (\bullet). A straight line is fitted to this data. The slope of the line (α) represents the effect of time. The average daily feed intake is read at time, averaged between the start of the experiment and the time at the end of the experiment ($t = \bar{t}$).

The values from Day 1 faeces were not considered in analysis, as on Day 1 fecal samples were collected before the piglets were exposed to the treatments. The difference between the intercept for different treatment combinations and Day 1 values for the end product concentrations is considered as “post-weaning change”. This change was analysed for significant difference among different treatment combinations by ANOVA (see Equ. 2).

$$Y_{ijk} = \mu + D_i + F_j + (D \cdot F)_{ij} + \epsilon_{ijk} \dots\dots\dots (2)$$

Where Y is the parameter to be tested, μ is the overall mean, D_i effect of the diet I ($i=1,2$); F_j

effect of the fasting treatment j ($j=1,2$); $(D \times F)_{ij}$ interaction, ϵ_{ijk} is the error term. The effect of period and litter was tested separately and having no effect on any of the parameters tested, was removed from the model.

In case of the digesta samples on Day 10, the effect of diet, fasting and the site within GIT, where faeces on Day 10 were also considered as an additional site and interaction between diet and site within GIT, was analysed by ANOVA (see Equ. 3).

$$Y_{ijklm} = \mu + D_i + F_j + (D \times F)_{ij} + \epsilon_{1ijk} + G_l + (D \times G)_{il} + (F \times G)_{jl} + (D \times F \times G)_{ijl} + \epsilon_{2ijklm} \quad \dots (3)$$

Where Y is the parameter to be tested, μ is the overall mean, D_i effect of the diet i ; F_j effect of the fasting stress j ; $(D \times F)_{ij}$, ϵ_{1ijk} is the error term 1, which represents the random effect of animal within diet i and fasting stress j , G_l effect of site of GIT l ; $(D \times G)_{il}$, $(F \times G)_{jl}$, $(D \times F \times G)_{ijl}$ denotes the respective interactions and ϵ_{2ijklm} is the error term 2, which represents the overall error. Differences were considered significant, when $p < 0.05$.

All statistical analyses were performed using the PROC GLM procedure of the statistical program SAS³⁸.

Table 1. Changes in time in daily feed intake, faeces dry matter content, ammonia, total VFA concentration and proportions of different VFAs, in piglets during post weaning period

Variable	Slope	P< ^a
Daily feed intake, g/d	37.88	0.001
Dry matter, g/Kg/d	14.11	0.001
^b Ammonia, mmol/L/d	-2.21	0.076
^b Total VFA, mmol/L/d	2.06	0.119
Acetic Proportion, %/d	-0.96	0.001
Propionic Proportion, %/d	0.61	0.001
Butyric Proportion, %/d	0.62	0.001
Branched chain Proportion, %/d	-0.45	0.001

^aProbability for the test, if regression coefficient of variable, associated with time, averaged over the experiment = 0;

^bExpressed per unit of fecal water;

Treatment effects on these time related changes were analysed as explained in the text (Equation 2) and were found not significant, except for daily feed intake, which was affected by fasting ($P < 0.05$)

Results

Effect of time

The changes in daily feed intake and fermentation end product concentration in faeces with time are presented in Table 1. Most of the parameters including daily feed intake and fecal DM content changed significantly with time except ammonia and total VFA concentration. This effect of time was independent of the experimental treatments, with exception of the effect of time on daily feed intake was significantly affected by fasting.

Effect of experimental treatments

The effect of experimental treatments on daily feed intake, fermentation end product concentration in faeces and fecal DM content, are presented in Table 2. Fasting had no effect on any of the parameters. Nor there was any interaction with diet. Diet had a significant effect on the ammonia concentration and BCP, which were both lower for the CHO diet compared to CON diet. The diet had an effect on dry matter content of the faeces ($p = 0.05$), but no effect on the VFA concentrations.

Table 2. Effects of diet composition and fasting treatments on Average daily feed intake, fecal dry matter content and fermentation end product profile in faeces of weaning piglets.

Variable	Diet		Fasting		SEM ^b	P-value		
	Control	CHO	Fasted	Non fasted		Diet	Fasting	Diet* Fasting
No. of observations	24	24	24	24				
Feed intake, g.d ⁻¹	248	257	245	260	14	0.63	0.46	0.50
Fecal characteristics ^a								
Dry matter content, g.kg ⁻¹	330.7	299.8	311.7	318.8	10.9	0.05	0.65	0.21
Ammonia, mmol.L ⁻¹	77.8	63.7	71.0	70.5	4.6	0.03	0.93	0.92
Total VFA, mmol.L ⁻¹	117.9	120.3	116.5	121.7	3.9	0.66	0.36	0.89
VFA molar proportions, %								
Acetic	64.7	66.7	65.2	66.1	1.2	0.25	0.61	0.91
Propionic	18.4	17.7	18.3	17.7	0.7	0.52	0.53	0.25
Butyric	8.1	8.2	8.1	8.3	0.5	0.93	0.75	0.12
Branched-chain	6.0	5.1	5.6	5.5	0.3	0.04	0.74	0.93

^aExpressed per unit fecal water

^bSEM= mean standard error;

Effect of dietary treatments over the “post-weaning change” on fecal characteristics

The “post-weaning change” i.e. the difference in fecal concentrations of different fermentation

end products between Day 1 and the average of the first 10 days on the treatments and dietary effect on this “post-weaning change” are shown in Figure 3. For both dietary groups, there was a post-weaning increase in the total VFA concentrations. However, the branched chain fatty acids concentrations (BCFA) particularly iso-butyric and iso-valeric acid, ammonia concentration, AP and BCP, were decreased post-weaning. The diets had significant effect on the “post-weaning change” in terms of BCFA concentrations, BCP and ammonia concentration. The CHO diet was observed to be more effective in lowering of BCP, BCFA and ammonia concentrations, but failed to increase total VFA concentration significantly.

Effect on digesta characteristics

The CHO diet significantly reduced the ammonia concentration, and BCP in different sites within the GIT (Figure 4). Although the total VFA concentrations were higher for the CHO group (Awati *et al.* submitted) the difference between dietary groups did not reach the significance. GIT had significant effect ($p < 0.001$) on all parameters, including DM content, VFA and ammonia concentrations. However, fasting had no effect on the fermentation end products in the different sites within GIT, nor any interaction with diet.

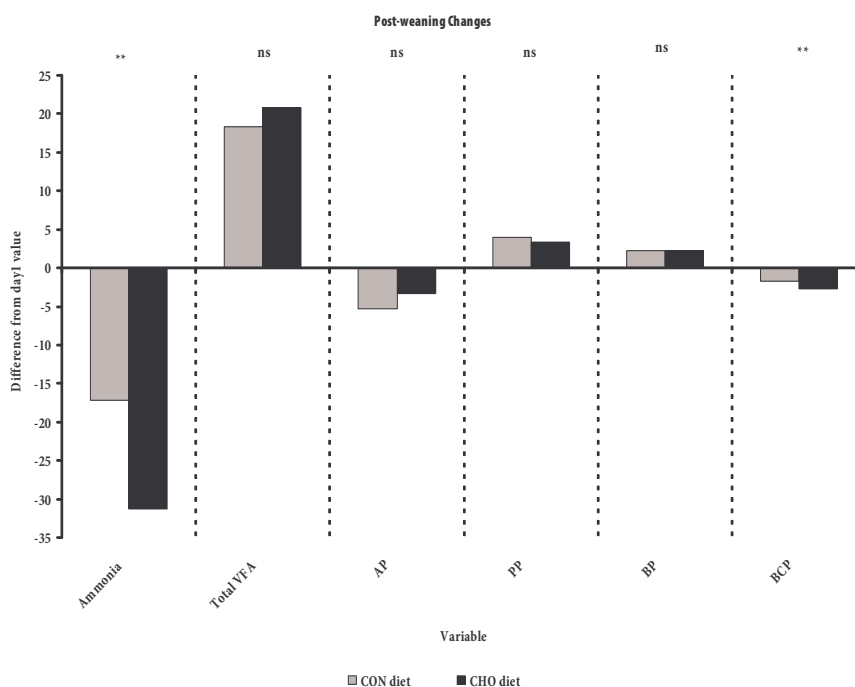


Figure 3. Post-weaning changes in the fermentation end product profile of faeces, affected by dietary treatments in weaning piglets. Ammonia mmol*L-1 fecal water, Total VFA mmol*L-1 fecal water, Acetic proportion (AP %), Propionic proportion (PP %), Butyric proportion (BP %), Branched chain proportion (BCP %). (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Discussion

Fasting had a significant effect on daily change of feed intake with time, which was expected as one of the two groups of piglets was fasted in the beginning of the experimental period. However, fasting had no effect on any of the average fermentation end product profiles in faeces. This might be the result of the lower feed intake on Day 1 and 2, by the piglets from non-fasted group as well. In non-fasted group, independent of diet, the average cumulative feed intake on first two days post-weaning was 52 g, which was comparable with <100 g which was reported by McCracken *et al.*⁵¹, while very low compared to 110 g observed on first day by Pluske *et al.*⁵², from the piglets offered dry starter diet. In present study both fasted and non-fasted groups had comparable feed intake from Day 3 (Figure 5) onwards. There was no difference in two groups for average daily feed intake (see Table 2). The average daily feed intake for both groups was comparable with that of 286 g observed by Pluske *et al.*⁵² During the experimental periods, none of the piglets showed any signs of diarrhoea

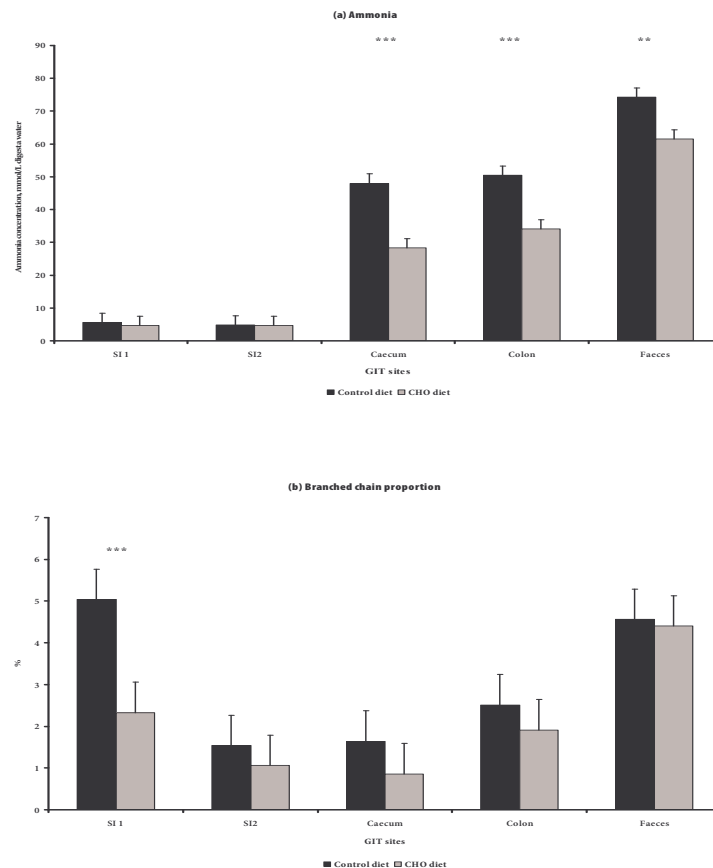


Figure 4. Effect of diet on the (a) ammonia concentration and (b) branched chain proportions, in different sites of GIT on day 10

or any other illness. These observations lead to the conclusion, either; i) The results of the present study, deny the theory that temporary anorexia after weaning, is followed by a large feed intake leading to higher protein fermentation⁶ or post-weaning diarrhoea³⁰. Or ii) the sudden rise of approximately 200 g in feed intake on Day 3 observed in fasted group, was not big enough to cause overeating and subsequent gastric disturbances.

Considering the fact that post-weaning anorexia is a precautionary measure on animal behalf to adjust to new environment and the time required for adjustment and severity of the anorexia can be an individual characteristic of an animal, authors would like to suggest for the future experiments studying effects of post-weaning anorexia, offering all animals feed from beginning, and then separate them into groups of “good eaters”, “moderate eaters” and “non eaters” according to amount of feed consumed by particular piglet, might be helpful. It was done earlier by Bruininx *et al.*⁵³ for creep feed intake studies.

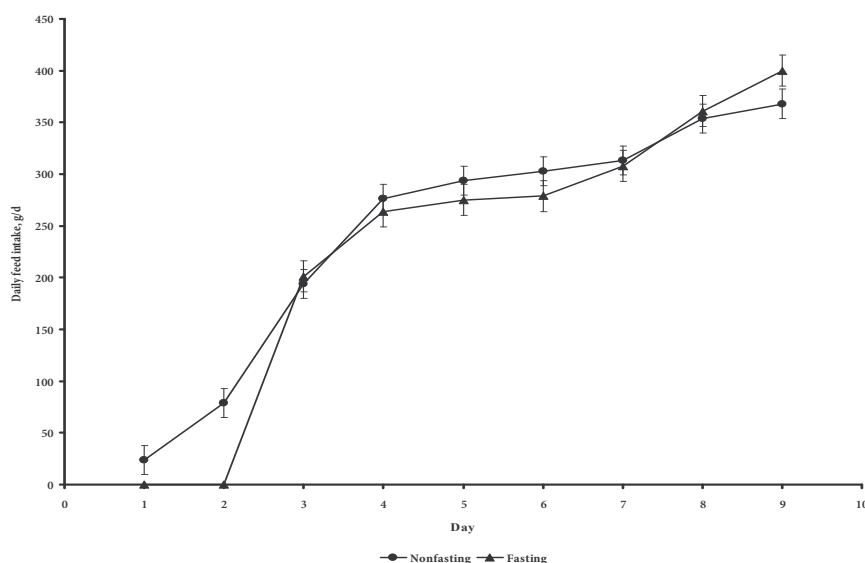


Figure 5. Daily feed intake of weaning piglets without (non fasting) or with enforced fasting (fasting) for two days in the beginning of the weaning period.

Time after weaning plays a major role in fermentation end product profiles in the faeces (Table 1). AP, PP and BP were significantly time dependent, although, the effects of time on ammonia and total VFA concentration were not significant. It is interesting to notice that, while the PP and BP were increased, the AP decreased significantly with time after weaning. This might be due to post-weaning development of microbiota, both in terms of numbers as well as in diversity. Furthermore, the daily feed intake was increased with time (Table1), so availability of the substrate for the large intestinal microflora was increased which resulted in higher PP and BP while, AP had gone down significantly⁴⁴. The DM content of the faeces was increased in time (Table 1). Absorption of short chain fatty acids stimulates sodium

absorption from the intestinal lumen, this adds to the efficient re-absorption of the water in the large intestine⁵⁴. Furthermore colon increases in size, in post weaning period⁵⁵, which adds to the absorptive capacity of colon, resulting in increasing DM content of faeces.

Although in comparison with CON diet, the CHO diet had shown significant lower ammonia concentrations and BCP, which are mainly products of protein fermentation,⁵⁶⁻⁵⁸ there were no differences in dietary treatments for VFA concentrations in the faeces (Table 2). It was expected that corn starch with ~97% ileal digestibility⁴⁹ will reach large intestine in negligible amount. But the depression in amylase activity during the first week after weaning⁵⁹ might have led to some extra amount of corn starch escaped the enzymatic digestion in small intestine, and became available to microbial fermentation in large intestine. This may therefore have masked the contrast in the two dietary treatments in the present study.

“Post-weaning change” in fermentation characteristics showed a decrease in ammonia and BCP. This decrease was significantly facilitated by CHO diet (see Figure 3). In the present study, both the diets contained the native corn starch as the main starch source, along with fermentable carbohydrates in CHO diet (see Table1, Chapter1). This clearly demonstrates a shift from protein fermentation towards carbohydrate fermentation.

In different parts of GIT, significant reduction in ammonia (caecum, colon and faeces) and BCP (SI1) on Day 10, was also observed with the CHO diet compared to CON diet, (Figure 4). Similar results had been confirmed in an earlier *in vivo* study in our laboratory (Awati *et al.* submitted) for animals fed the same diets. This supports the hypothesis that inclusion of fermentable carbohydrates with different fermentation rates in weaner diet, helps improving carbohydrate fermentation along the GIT, with reduction in the protein fermentation.

Conclusion

This study emphasizes that, inclusion of fermentable carbohydrates with different fermentation rates, significantly reduce the protein fermentation along the GIT and fecal concentrations of ammonia. This study also reveals that post-weaning temporary anorexia, being individual characteristic of piglet not necessarily, the reason for post weaning diarrhoea. But certainly, the protein fermentation occurring due to digestive and absorptive dysfunction, as a consequence of temporary anorexia, and the risk of eventual diarrhoea in weaning piglets, can be reduced by inclusion of fermentable carbohydrate substrates in diet.

Acknowledgement

The authors would like to thank Meijke Booij, Jane-Martine Muijlaert, Dick Bongers and Huug Boer of the Animal Nutrition Group for their assistance with the laboratory analyses. Tamme Zandstra and personnel of the animal experimental facilities at “De Haar” are thanked for their co-operation during this study.

Chapter 3

Specific Response of a Novel and Abundant *Lactobacillus amylovorus* – Like Phylotype to Dietary Prebiotics in the Guts of Weaning Piglets

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Abstract

Using 16S rRNA gene-based approaches, we analyzed the response of ileal and colonic bacterial communities of weaning piglets to dietary addition of four fermentable carbohydrates (inulin, lactulose, wheat starch and sugar beet pulp). A diet, enriched in, and a control one, lacking these fermentable carbohydrates, were fed to piglets for 4 days (n=48), and 10 days (n=48), and the lumen associated microbiota was compared using denaturing gradient gel electrophoresis (DGGE) analysis of amplified 16S rRNA genes. Bacterial diversity in the ileal and colonic samples was measured by assessing the number of DGGE bands and Shannon index of diversity. A higher number of DGGE bands in the colon (24.2 ± 5.5) compared to the ileum (9.7 ± 4.2) was observed in all samples. In addition, significantly higher diversity as measured by DGGE fingerprint analysis was detected in the colonic microbial community of weaning piglets fed the fermentable carbohydrate-enriched diet for 10 days compared to the control. Selected samples from the ileal and colonic lumen were also investigated using fluorescent in situ hybridization (FISH), and cloning and sequencing of the 16S rRNA gene. This revealed a prevalence of *Lactobacillus reuteri* in the ileum and *Lactobacillus amylovorus*-like populations in the ileum and the colon of the piglets fed with fermentable carbohydrates. Newly developed oligonucleotide probes targeting these phylotypes allowed their rapid detection and quantification in the ileum and colon by FISH. The results indicate that addition of fermentable carbohydrates supports the growth of specific lactobacilli in the ileum and colon of weaning piglets.

Introduction

The diets, microbiota and gastrointestinal tract (GIT) interactions of mammals are extremely complex, and are the result of millions of years of co-evolution between the higher vertebrates and their microbiota. As a consequence, any major changes in lifestyle and diet are likely to place stress on the stability of these interactions and affect the entire gastrointestinal (GI) tract ecophysiology. In contrast to the gradual weaning of human babies, piglets within a production environment are weaned at an early stage with solid feed and transported to production farms. This combination of stress factors can lead to diarrhoea, a reduced growth rate, and in some cases, even death⁶⁰. In order to enhance growth and suppress the activity of the gut microbiota, antimicrobial compounds have been fed to weaning pigs for more than four decades⁶¹. Nowadays, the emergence of antibiotic resistance in the human commensal bacteria has raised concerns about the impact of antimicrobial compounds for agricultural use⁶² and has accelerated the search for alternative nutritional strategies, such as the addition of probiotics and prebiotics. These approaches have become an increasingly important consideration in swine nutrition, because of accumulating evidence of their potential benefits in animals and humans and the possibility that they replace in-feed antibiotics⁶³⁻⁶⁶. The development of such dietary strategies requires a combination of *in vitro*, *in vivo*, and challenge studies, involving both expertise in animal nutrition, and an evaluation of the composition and activity of the indigenous microbiota throughout the GI tract.

In the past the microbial community in the GI tract of pigs has been studied intensively, but most attention was paid to easily cultivable commensal bacteria and a number of opportunistic pathogens^{67,68}. Many of the strictly anaerobic GI tract bacteria are still difficult to cultivate and therefore remain undetectable using conventional techniques^{69,70}. Recent phylogenetic analysis based on the *in vitro* amplification of 16S rRNA gene and other phylogenetic markers by polymerase chain reaction (PCR) techniques have revealed dramatically higher diversity than described previously by cultivation⁷¹⁻⁷³. While molecular approaches based on PCR can introduce different types of bias,^{74,75} a combination of PCR and fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) has led to new insights into GI microbial ecology, and the effects of different dietary strategies and host factors on the bacterial community composition⁷⁶.

It has been recognized that a stable indigenous microbiota in the intestine can prevent colonization by pathogens^{1,77}. This so-called colonization resistance may be of utmost importance for animals, especially at stressful times such as weaning. Promotion of colonization resistance through the addition of prebiotics has been suggested as a comparatively easy way to improve enteric health^{23,78,79}. Prebiotics have been used to induce the colonization of bacteria such as lactobacilli and bifidobacteria, considered to be beneficial for the host^{21,65,80}. Stimulation of the *Lactobacillus* population within the gastrointestinal tract of piglet is of specific importance, not only due to their potential effect on gut function and health^{68,81}, but also because of their possible antagonistic activities toward other bacteria^{82,83}. Lactobacilli establish early in the piglet intestine, and although succession occurs throughout

the pig's lifetime, they remain a predominant part of the intestinal bacterial community^{68,84-86}. Numerous studies have suggested that some prebiotics may specifically stimulate intestinal lactobacilli. The application of lactobacilli as probiotics or therapeutic supplements has also been studied^{87,88}. However, little is known of the response of the bacterial community to such dietary interventions.

This work describes changes in the predominant ileal and colonic bacterial populations in weaning piglets that were fed a diet containing four added fermentable carbohydrates, namely inulin, lactulose, wheat starch and sugar beet pulp. The data indicate that the incorporation of these four ingredients in the diet results in outgrowth of lactobacilli in the small intestine and higher diversity in the colon. Two particular phylotypes related to *L. amylovorus* and *L. reuteri* were the most prevalent throughout the gut of piglets fed with the prebiotics as demonstrated by DGGE of 16S rRNA gene amplicons in combination with sequence analysis. Newly developed DNA oligonucleotide probes targeting these key species allowed their rapid detection and quantification in the ileum and colon of piglets by fluorescent in situ hybridization (FISH).

Materials and Methods

Animals, diets and sampling

All the procedures involving animals were conducted in accordance with the Dutch law on experimental animals and had been approved by the Animal Experimental Committee of Wageningen University.

Three identical, but independent feeding experiments including a total of 108 piglets (crossbred Hypor×Pietrain) were started immediately at the time of weaning (25–28 days old). Each experiment used 36 piglets. At the start of the experiment (day 1), four piglets were sacrificed. The remaining 32 piglets were offered one of two diets (16 piglets per diet): the HF diet containing four added fermentable carbohydrates namely, lactulose, inulin, sugar beet pulp and wheat starch, and the LF diet with a low concentration of fermentable carbohydrates (see Table 1). The diets were composed in such a way that total energy and protein content were comparable. On day 4 and 10 of each experiment, 8 piglets were sacrificed per treatment.

The samples were divided into aliquots, one of which was used for genomic DNA extraction followed by 16S rRNA gene-targeted PCR-DGGE analysis, cloning and sequencing. In parallel, aliquots from the same samples were fixed for FISH and for determination of the lactic acid concentration.

DNA isolation

DNA isolation from lumen samples (0.2 g) was done by using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA). Agarose gel (1.2% [w/v]) electrophoresis in the presence of ethidium bromide was used to check visually for DNA quality and yield.

PCR amplification

All primers used in this study are listed in Table 2. Primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 were used to amplify the V6 to V8 regions of 16S rRNA gene. PCR was performed using the Taq DNA polymerase kit from Life Technologies (Gaithersburg, MD). PCR mixtures (50 µl) contained 0.5 µl of Taq polymerase (1.25 U), 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 3.0 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 5 pmol of the primers, 1 µl of DNA diluted to ~1 ng/µl and UV-sterilized water. The samples were amplified in a thermocycler T1 (Whatman Biometra, Göttingen, Germany) and the cycling consisted of 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 68°C for 40s, and 68°C for 7 min (final extension). Aliquots (5µl) were analyzed by electrophoresis on 1.2% (w/v) agarose gel containing ethidium bromide to check for product size and quantity.

Table 1. Composition of the diets (g/kg),

Ingredients	Diets	
	LF ¹	HF ¹
Corn starch	504.8	368.1
Sugarbeet pulp	-	50.0
Inulin	-	7.5
Lactulose (~50%DM)	-	20.0
Wheat starch	-	50.0
Fishmeal	200.0	200.0
Soya isolate	50.0	45.0
Dextrose	150.0	150.0
Soya oil	15.0	30.0
Cellulose (Arbocel)	50.0	50.0
Premix	10.0	10.0
Krijt	2.5	1.5
Monocalcium phosphate	1.5	1.5
KHCO ₃	12.0	12.0
L-lysine HCl	0.6	0.7
DL-methionine	2.0	2.0
L-threonine	1.0	1.1
L-tryptophan	0.6	0.6
Calculated Analysis		
Dry Matter	916	911
Ash	43	46
Crude Protein	179	180

¹LF - a diet with a low concentration of fermentable carbohydrates, HF- a diet containing lactulose, inulin, sugar beet pulp and wheat starch.

To investigate the *Lactobacillus*-specific GI-tract bacterial community by DGGE, a specific nested PCR approach was chosen. For the initial amplification, S-D-Bact-0011-a-A-17 and S-G-Lab-0677-a-A-17 primers were employed⁸⁹ using the following cycling conditions: predenaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 66°C for 20 s, at 68°C for 40 sec, and a final extension at 68°C for 7 min. The PCR products were then used as templates in nested PCRs with S-G-Lab-0159-a-S-20 and S*-Univ-0515-a-A-24-GC. The cycling program was identical with the one used for the amplification of the V6 to V8 regions of the 16S rRNA gene.

DGGE analysis

The amplicons obtained from the lumen-extracted DNA were separated by DGGE according to the specifications of Muyzer *et al.*⁹⁰ using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was performed in an 8% polyacrylamide gel (37.5:1 acrylamide–bisacrylamide; dimensions 200 by 200 by 1 mm) using a 38–48% denaturing gradient⁹¹ for separation of PCR products obtained with primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17, while gradients of 30–60% were employed for the separation of the S-G-Lab-0159-a-S-20 and S-*Univ-0515-a-A-24-GC generated amplicons. The gels were electrophoresed for 16 h at 85 V in 0.5×TAE buffer⁹² at a constant temperature of 60°C and subsequently stained with AgNO₃⁹³.

Table 2 Oligonucleotide primers and probes used in this study

Primer/Probe	Sequence (5'-3')	References
Primer		
^a S-D-Bact-0011-a-A-17	AGA GTT TGA T(C/T)(A/C) TGG CTC AG	73
^a S-G-Lab-0159-a-S-2	GGA AAC AG(A/G) TGC TAA TAC CG	89
^a S-*Univ-0515-a-A-24-GC	CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G ATC GTA TTA CCG CGG CTG CTG GCA	89
^a S-G-Lab-0677-a-A-17	CAC CGC TAC ACA TGG AG	89
^a S-D-Bact-0968-a-S-GC	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	24
^a S-D-Bact-1401-a-A-17	CGG TGT GTA CAA GAC CC	24
^a S-Univ-1100-a-A-15	GGG TTG CGC TCG TTG	24
^a S-D-Bact-1492-a-A-19	GGT TAC CTT GTT ACG ACT T	73
T7	TAA TAC GAC TCA CTA TAG G	Promega
Sp6	GAT TTA GGT GAC ACT ATA G	Promega
Probes		
^a S-D-Bact-0338-a-A-17	GCT GCC TCC CGT AGG AGT	94
^a S-G-Lab-0158-a-A-20	GGT ATT AGC A(C/T)C TGT TTC CA	94
^a L*-OTU171-0088-a-A-18	CGC TTT CCC AAC GTC ATT	This study
^a L*-OTU173-0085-a-A-18	CCA TCG TCA ATC AGG TGC	This study

^aNomenclature according to Alm *et al.*⁹⁵

Analysis of the DGGE gels

Analysis of all DGGE samples was done as described previously.²⁴ Briefly, all gels were scanned at 400 dpi and analysed using the software of Molecular Analyst/PC (version 1.12, Bio-Rad, Hercules, CA). First, a number of bands were assessed per lane using the bands searching algorithm within the program. A manual check was done and the DGGE fragments constituting less than 1% of the total area of all bands were omitted from further analysis. Second, as a parameter for the structural diversity of the microbial community, the Shannon

index of general diversity, $H'^{24,96-98}$ was calculated using the following function: $H' = -\sum P_i \log P_i$, where P_i is the importance probability of the bands in a lane. H' was calculated on the basis of the bands on the gel tracks that were applied for the generation of the dendrograms by using the intensities of the bands as judged by peak height in the densitometric curves. P_i was calculated as follows: $P_i = n_i/n''$, where n_i is the height of a peak and n'' is the sum of all peak heights in the densitometric curve. The similarity between the DGGE profiles was determined by calculating a band similarity (Dice) coefficient SD [$SD = 2nAB/(nA+nB)$], where nA is the number of DGGE bands in lane 1, nB represents the number of DGGE bands in lane 2, and nAB is the number of common DGGE bands]^{24,82,99}

Table 3. Bacterial strains, their sources, media used for their cultivation and FISH results

Strain	Source ^a	Medium ^b	Probe results ^c	
			I	II
<i>Lactobacillus amylovorus</i>	DSMZ 20531	MRS	+	-
<i>Lactobacillus crispatus</i>	DSMZ 20584	MRS	-	-
<i>Lactobacillus reuteri</i>	DSMZ 20015	MRS	-	+
<i>Lactobacillus reuteri</i>	VTT E92142	MRS	-	+
<i>Lactobacillus acidophilus</i>	VTT E96276	MRS	-	-
<i>Lactobacillus acidophilus</i>	ATCC4356	MRS	-	-
<i>Lactobacillus brevis</i>	VTT E91458	MRS	-	-
<i>Lactobacillus buchneri</i>	VTT E93445	MRS	-	-
<i>Lactobacillus plantarum</i>	VTT E79098	MRS	-	-
<i>Lactobacillus rhamnosus</i> LGG	ATCC 53103	MRS	-	-
<i>Enterococcus faecalis</i>	DSMZ 20478	WW	-	-

^aDSMZ, German Collection for Microorganisms and Cell Culture, Braunschweig, Germany; VTT, VTT culture collection, FIN-02044, Finland; ATCC, American Type Culture Collection, Manassas, Va.

^bMRS, Lactobacillus MRS broth (Difco, Sparks, Md.); WW, Wilkins West broth.

^c(I), L-S-OTU171-a-A-0088; (II), L-S-OTU173-a-A-0085; +, hybridization signal; -, no hybridization signal.

Statistical analysis

For statistical analysis, the number of DGGE bands, the Shannon index of general diversity, and the band similarity coefficient (SD) were calculated. Differences between these parameters for the two diets were tested for significance using Tukey's Studentised range test of multiple comparisons¹⁰⁰ according to the following equation: $Y = \mu + D_i + \epsilon_{ij}$, where Y is the result, μ the mean, D the effect of the diet, and ϵ_{ij} the error term. All statistical analyses were performed using the SAS GLM procedure³⁸.

Generation and screening of 16S rRNA gene clone libraries

PCR was performed with a Taq DNA polymerase kit from Life Technologies using primers S-D-Bact-0011-a-S-17 and S-D-Bact-1492- a-A-19. Amplification was carried out as described previously.²⁴ The PCR product was purified with the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. Purified PCR product was cloned into pGEM-T (Promega, Madison, WI) using competent *E. coli* JM109 as a host. The colonies of ampicillin-resistant transformants were transferred with a sterile toothpick to 15 μ l TE buffer and boiled for 15 min at 95 °C. PCR was

immediately performed with vector specific primers T7 and SP6 to check the size of the inserts using the cell lysate as a template. Plasmids containing an insert of ~ 1.6 kb were used to amplify V6 to V8 regions of the 16S rRNA gene. The amplicons were compared with the bands of DGGE profiles that comprised more than 1% of the total area of all bands. Clones representing an insert corresponding to a dominant band were grown in Luria Broth liquid medium (5 ml) with ampicillin (100 µg ml⁻¹). Plasmid DNA was isolated using the Wizard Plus purification system (Promega), and used for sequence analysis of the cloned 16S rRNA gene by using a Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, UK) according to the manufacturer's specifications and using either the T7 and SP6 primers or S-Univ-1100-a-A-15 labelled with IRD-800. Sequences were automatically analysed on a LiCOR (Lincoln, Neb.) DNA Sequencer 4000L and corrected manually. Sequences were also compared to those available in public databases by using BLAST analysis.¹⁰¹ The partial and complete 16S rRNA gene sequences were checked for chimeric constructs by the Ribosomal Database Project CHECK_CHIMERA program.¹⁰² None of the sequences were found to be PCR-generated chimeras.

Cloning and sequencing of DGGE bands after *Lactobacillus* specific PCR amplification

Representative bands were excised from DGGE gels using a QIAEXII Gel extraction kit (Westburg) according to the instructions in the manual. After reamplification using the original S-G-Lab-0159-a-S-20 and S*-Univ-0515-a-A-24-GC primer set, cloning and sequencing analysis were carried out as previously described.

Design and validation of oligonucleotide probes for FISH analysis

Nearly complete 16S rRNA sequences of *L. amylovorus*-like and *L. reuteri*-like isolates (this study) and closely related *L. amylovorus*-like (OTU171) and *L. reuteri*-like (OTU173) isolates from pig intestine⁷³ were aligned, and probes targeting these sequences were designed using the ARB software package (<http://www.arb-home.de>). Probes were designed taking into consideration the types and positions of nucleotide mismatches with sequences of related species with a G_C content of ~50% and a length of ~18 nucleotides. Sequence comparisons using the ARB, Check Probe, and BLAST programs confirmed that the targeted regions were conserved among the 16S rRNA sequences of OTU171 (*L. amylovorus*-like) isolated from pig intestine and *Lactobacillus kitasatoi* isolated from chickens¹⁰³ (Figure 1, band B) and to *L. reuteri*-like OTU173 (Figure 1, band A). Probe OTU171-0088-a-A-18 was found to also match the partial sequence of *Lactobacillus galinarum* (National Center for Biotechnology Information accession no. X97898). However, a comparison among the *L. galinarum*, OTU171, and *L. kitasatoi* sequences showed 99 to 100% homology among them based on 600 bp (*E. coli* positions 20 to 620).

The reference strains *L. amylovorus* DSMZ 20531 and *L. reuteri* DSMZ 20015 were used as positive controls (Table 3). Ten reference *Lactobacillus* strains and *Enterococcus faecalis* DSMZ 20478, frequently found in the GI tract, were used as negative controls to evaluate the

specificities of the newly designed probes. The temperature of hybridization was 50°C, and if needed, formaldehyde was added to increase the specificity.¹⁰⁴

The reference strains used in this study were obtained from the sources indicated in Table 3. The strains were cultivated as recommended by the culture collections in the respective catalogues. Exponentially grown cells were harvested at 5,000 x g for 10 min, washed with 0.2- μ m-pore-size-filtered phosphate-buffered saline (PBS; per liter, 8 g of NaCl, 0.2 g of KCl, 1.44 g of NaH₂PO₄, and 0.24 g of KH₂PO₄, pH 7.2), and diluted 1:3 with 4% paraformaldehyde in PBS. After fixation at 4°C for 16 h, the cells were stored in 50% ethanol-PBS at -80°C for subsequent FISH analysis.

Collection and preparation of ileal- and colonic-lumen samples for FISH

Ileal- and colonic-lumen samples from 108 experimental piglets were processed as described previously.¹⁰⁵ In short, 0.5 g of lumen samples was resuspended in 4.5 ml of PBS and vortexed with five or six glass beads (diameter, 3 mm) for at least 3 min to homogenize the sample. After centrifugation at 700 x g for 1 min, 1 ml of the supernatant was added to 3 ml of 4% paraformaldehyde in PBS and stored for 16 h at 4°C. After being washed twice with PBS, the fixed cells were stored in 50% ethanol-PBS at -80°C until further use.

Enumeration of bacteria by FISH

For microscopic analysis, fixed cells were spotted on gelatin-coated glass slides and dried for 20 min at 50°C. The optimal cell concentration for counting using the different probes was determined using dilution series of the lumen samples. After drying of the slides, the cells were dehydrated for 3 min in 50%, 70% and finally 96% ethanol/H₂O. Ten microliters of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.1% [wt/vol] sodium dodecyl sulfate) containing 10 ng/ μ l Cy3-labelled lactobacilli probes/ μ l or 5 ng of fluorescein-isothiocyanate-labeled S-D-Bact-0338-a-A-17/ μ l (Table 2) was added to each well, followed by incubation at 50°C for 16 h. After hybridization, the slides were washed in 50 ml hybridization buffer for 10 min. For total cell counts, DAPI (4', 6-diamino-2-phenylindole) at a final concentration of 100 ng/ml was added to the washing buffer. After the slides were rinsed in double distilled water, they were immediately air-dried and mounted in Vectashield (Vector Labs, Burlingame, CA). Digital images of the slide were analyzed and fluorescence positive cells were counted using Qwin image analysis software (Leica Microsystems, The Netherlands). For each analysis 25 microscopic fields were counted.

Lactic acid analysis

The lactic acid concentration in ileal lumen was analyzed by High Performance Liquid Chromatography (Jasco instruments) using column (Supelcogel, C-610H, 30cm*7.8mm ID) and precolumn (Supelcoguard, C-610H, 5cm*4.6mm ID) with 1% H₂SO₄ as mobile phase. The concentrations were determined by UV detection at 210 nm.

Table 4. Clones with the percentage of identity to known sequences in Genbank^a

No.	Closest relative in Genbank	GenBank accession n ^o	Number of clones		Avg. similarity
			HF	LF	
1	<i>Lactobacillus amylovorus</i> -like	AF371469	22	0	98
2	<i>Lactobacillus reuteri</i> (DSM 20016 T)	X76328	11	2	98
3	<i>Lactobacillus acidophilus</i> subsp. <i>johnsonii</i>	M99704	1	19	99
4	<i>Lactobacillus</i> sp. oral clone CX036	AY005048	7	0	98
5	<i>Lactobacillus</i> sp. strain CLE-4	AY017059	4	0	99
6	<i>Lactobacillus mucosae</i>	AY445125	2	1	97
7	<i>Lactobacillus gallinarum</i>	AJ242968	3	1	97
8	<i>Sarcina ventriculi</i> (DSM 286)	X76649	0	12	98
9	<i>Salmonella</i> sp	AF130955	1	5	95
10	<i>Pasteurella aerogenes</i>	M75048	1	4	93
11	<i>Clostridium lituseburense</i>	M59107	1	3	97
12	<i>Haemophilus</i> sp.	M75077	1	3	96
13	<i>Moraxella lacunata</i>	AF005161	1	2	97
14	<i>Veillonella atypical</i>	X84007	1	2	98
15	<i>Leptotrichia</i> sp. (oral clone)	AY349385	1	2	96
16	<i>Ruminococcus albus</i>	AF104843	1	1	91
17	<i>Ruminococcus callidus</i>	X85100	0	1	95
18	<i>Holdemania filiformis</i>	Y11466	0	1	91
19	<i>Calonectris diomedea</i>	Y16359	1	1	96
20	<i>Veillonella parvula</i>	AF439640	1	0	90
Total number of clones			60	60	

^aClones were retrieved from ileal lumen samples of four HF-fed piglets and four LF-fed piglets at 5 weeks of age. The clones are listed according to their abundance in the eight different 16S rDNA gene clone libraries.

Nucleotide sequence accession numbers

The sequences reported in this study were deposited in Genbank under the following accession numbers: AY493201- AY493245.

Results

Animal observations

Animals remain healthy throughout the experimental period. The average weaning weight of piglets was 7.5 kg. At the end of the experiment, no significant difference in body weight gain was observed between the dietary treatments.

Effects of fermentable carbohydrates on the bacterial diversity in the ileum and colon

A comparative 16S rRNA gene-targeted DGGE fingerprinting analysis of bacterial communities was performed for ileal- and colonic- lumen samples of piglets that were fed two different diets. Samples from piglets that were sacrificed at the day of weaning ($n=12$), 4 days ($n=48$) and 10 days after weaning ($n=48$), were analyzed. The number of DGGE bands and Shannon index of general diversity were assessed for each sample and subjected to statistical analysis according to GI tract location across all time periods. For all samples, a comparison between the two locations along the GI tract revealed a statistically higher number of DGGE bands (24.2 ± 5.5 ; $p<0.05$) in the colonic compared to the ileal lumen (9.7 ± 4.2). The Shannon index of diversity in the colon (1.43 ± 0.3 ; $p<0.05$) was also higher compared with the ileum (0.86 ± 0.26). No significant differences in the number of DGGE bands or diversity were detected at Day 4 in the ileum, or in the colon of piglets fed the different diets. However, by day 10 after weaning, the diversity was significantly higher in the colonic samples of HF piglets, as evidenced by the number of bands (27.5 ± 5.6 ; $p<0.05$) compared with the LF group (20.5 ± 6.3). There was no statistical difference between the number of DGGE bands and the diversity index in the ileal samples by day 10.

The influence of the diet on the bacterial community structure in the ileal and colonic lumen of HF and LF group piglets at days 4 and 10 was further elucidated. By day 4, there were no DGGE bands detected in one dietary group, which were completely absent in the other (data not shown). In contrast, a simple visual comparison of the DGGE banding patterns by day 10 of the experiment revealed a marked difference between the ileal samples of the two dietary groups. A representative DGGE analysis of the PCR fragments generated with primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 is shown in Figure 1A. Two particularly strong bands (A and B) were present in the ileal samples from piglets fed with HF diet at day 10. Band B was also detected in 18 out of 24 of the colonic lumen samples of the HF diet at day 10 and was absent in pigs fed with LF diet (data not shown). The ileal samples of the LF group, on the other hand, were dominated by another band located at the uppermost part of the DGGE gels (Figure 1A band C). Band C was not present in the samples of the HF diet. To obtain an objective interpretation of the electrophoretic patterns of the HF and LF ileum, the samples were subjected to a numerical analysis based on the Dice similarity coefficient followed by cluster analysis. The similarity was visualized using the unweighted pair group method with averaging algorithm (Figure 2). Cluster analysis revealed that all 24 samples of HF diet fed piglets formed a coherent cluster with similarity indices above 60 %. Within this cluster, 20 out of 24 samples grouped together, with similarity indices higher than 75

%. The low similarities between HF and LF samples confirmed the visual differences in their DGGE fingerprints (Figure 1). The average similarity index of the HF colon samples was 45% (data not shown). Taken together, the results obtained after DGGE analysis demonstrate that bacterial composition in the ileum and colon of piglets was modulated by the HF diet by day 10.

Identification of cloned 16S rRNA gene sequences in DGGE patterns

In order to identify changes in the bacterial diversity detected by DGGE analysis, the 16S rRNA gene from the ileal lumen samples of four HF-fed piglets (10 days) and four LF-fed piglets was amplified and cloned into *E. coli*, and 15 clones per sample were partially sequenced (Table 4). To identify the dominant bands A and B that appeared in 90% of samples 10 days after starting the HF diet, together with a third distinct band (C) in the LF diet (Figure 1A), V6 to V8 regions of the 16S rRNA gene were amplified from the cell lysates of a total of 120 transformants. The mobilities of these amplicons during DGGE was compared to those obtained from rRNA gene sequences retrieved from samples of the piglets fed 10 days with the HF and LF diets. Sixty-one percent of the clones were assigned to one of the dominant bands in the DGGE profiles, while 39% did not match any of the detectable bands. The clones from the eight different clone libraries corresponding to bands A, B and C were completely sequenced. The 16S rRNA gene sequences of the clones representing band A were identified as *Lactobacillus reuteri*-like or OTU173, while band B showed similarity to OTU171 or *Lactobacillus amylovorus*-like.⁷³ Clones matching the position of band C were 98% similar to *Sarcinia ventriculi* (Figure 1A).

Although the amplification of the V6 to V8 regions using general bacterial primers allowed for the visualization of the major differences between the HF and LF samples, and to screen the 16S rRNA gene clone libraries, it yielded a poor resolution of *Lactobacillus* populations. Therefore, specific amplification of the *Lactobacillus* GI-tract bacterial community was used to screen the clones matching to one of the dominant bands after V6-V8 16S region DGGE analysis. *Lactobacillus*-specific amplification in combination with DGGE analysis confirmed the predominance of one particular phylotype related to *L. amylovorus*, while a band related to *L. reuteri* was not consistently found in the samples of the piglets fed with the HF diet (Figure 1B). *L. acidophilus* was also present in samples of HF and LF irrespective of diet.

To confirm the visual match between the 16S rRNA gene clones and the DGGE bands, the HF diet-specific bands were excised from DGGE gels after *Lactobacillus*-specific PCR, reamplified and sequenced. Sequencing analysis of the bands (A and B) confirmed their identities as *L. reuteri* and *L. amylovorus*-like.⁷³ Interestingly, of the four HF diet samples, an average of 4 other phylotypes related to *L. mucosae* (97%), *L. galinarum* (97%), and two different *L. species* clones: one oral (98%, accession no. AY005048) and one isolated from swine production facilities (99%, accession no. AY017059) were found (Table 4). However, their sequences did not match to any visible DGGE bands. In comparison, the *Lactobacillus* diversity in LF was predominated by *L. acidophilus*-like related sequences. The results of the clone libraries showed increased *lactobacilli* diversity in the HF samples, while DGGE

analysis suggested a specific outgrowth of *L. amylovorus*-like phlotypes in the terminal ileum of weaning piglets.

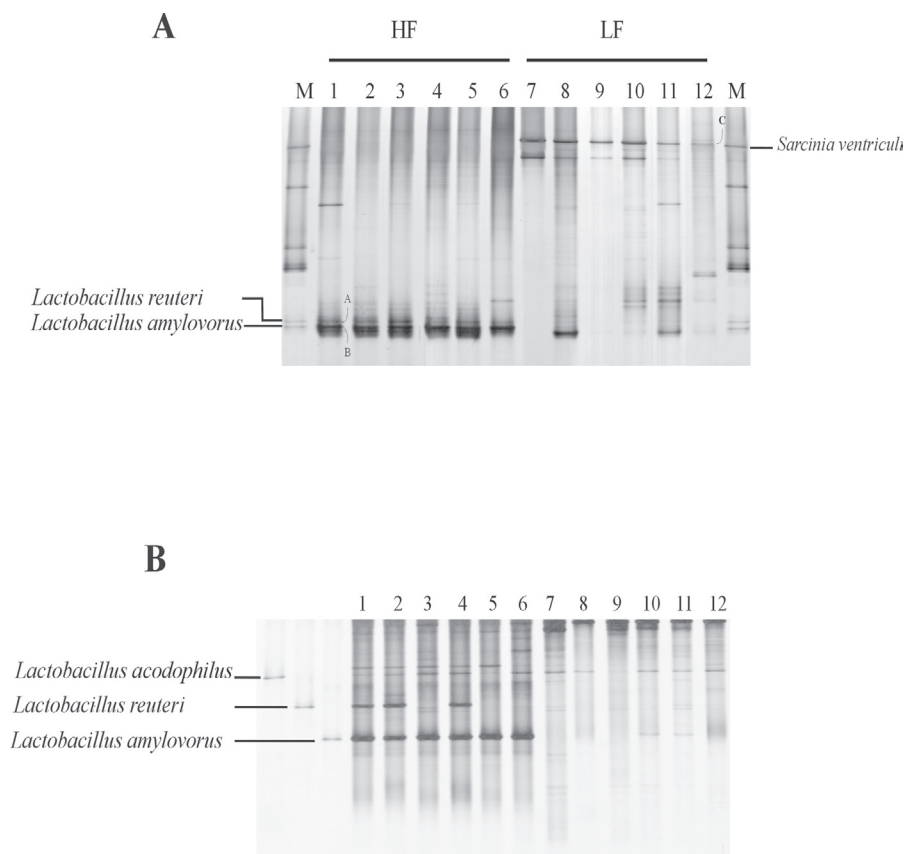


Figure 1. Effect of the fermentable carbohydrates containing diet on the ileum bacterial community by day 10 of the experiment. (A) DGGE of PCR products of V6 to V8 regions of 16S rRNA gene sequences retrieved from lumen samples at 10 day after weaning. 1 to 6, piglets on HF diet. 7-12, piglets on LF diet. M, marker. Fragments that are indicated by A, B, C were identified from 16S rRNA gene clone libraries. (B) Monitoring of the *Lactobacillus*-like community of piglets. DGGE analysis of amplicons generated by nested PCR with primers S-G-Lab-0159-a-S-20 and S-* -Univ-0515-a-A-24-GC, originating from: 1 to 6 – piglets on HF diet, 7-12 piglets on LF diet. The dominant fragments in *Lactobacillus*-like patterns were identified by the clones corresponding to *L. acodophilus*, *L. reuteri* and *L. amylovorus*.

Development and evaluation of FISH probes specific for *Lactobacillus amylovorus* and *Lactobacillus reuteri*- like isolates

Potential probes were identified based on the alignment of the complete 16S rRNA sequences of the clones matching DGGE bands A and B (Figure 1A) and related *Lactobacillus spp.* (Table 3).

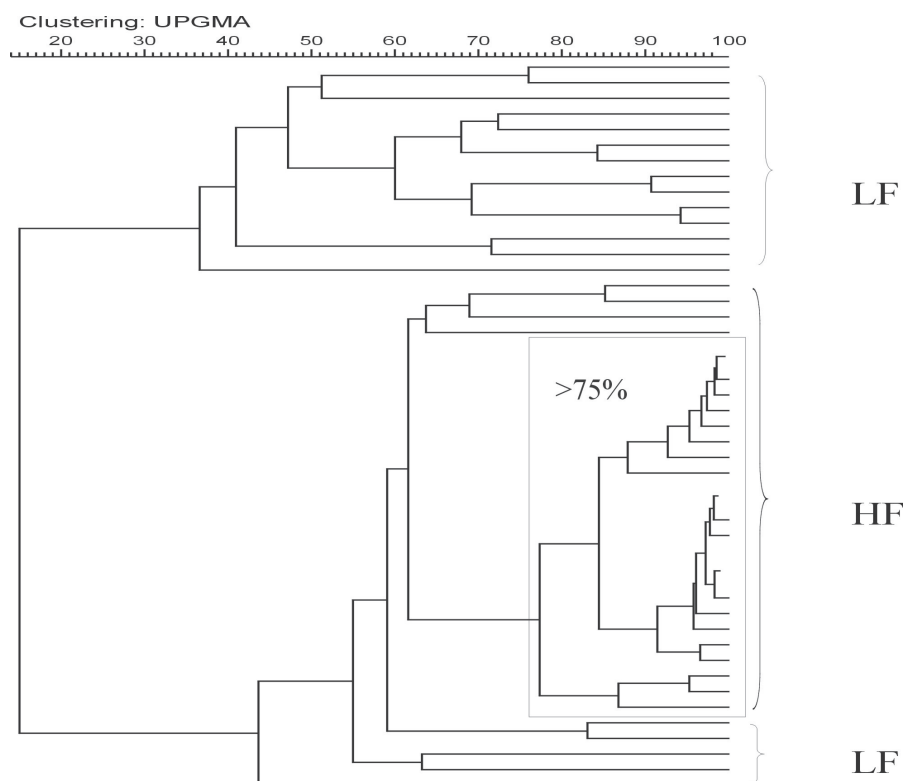


Figure 2. Similarity index of DGGE profiles obtained from ileal lumen microbiota of 48 piglets fed either HF or LF diet for 10 days. The normalization of the DGGE gels was done with respect to the reference standards included in three gels containing the ileal lumen samples of HF and LF from the three replicate experiments. The Dice coefficient of similarity between banding patterns of different gels was calculated. This allowed the generation of a dendrogram and the samples were grouped according to the similarity of their community profiles.

The probes were experimentally validated by performing FISH analysis on a range of *Lactobacillus* species and other bacteria that are commonly found in large numbers in the pig GI tract. 68,106 A constant temperature of 50°C for 16 h. and 0 % (vol:vol) of formamide in the hybridization buffer were used, resulting specific hybridization only with the respective target strains (Table 3). Subsequently, the validated probes were used to enumerate target bacteria in individual ileal and colonic samples from piglets fed the different diets for 10 days. To evaluate whether the microbiota was affected by the diet, the total cell counts, and the total bacterial and lactobacilli-enterococci counts were compared between the HF and LF diet (Table 5). Within the HF, the lactobacilli/enterococci were significantly higher ($p < 0.05$) than in the LF. The hybridization with L⁻-OTU171-0088-a-A-18 probe detected the OTU171 phylotype in 83 % of the ileal samples and 75 % of the piglet colonic samples of the HF diet, while no hybridization positive cells were obtained for the LF diet (Table 5).

In comparison, *L. reuteri*-like related population was detected in 75 % of the ileal lumen samples and 41 % for the colonic lumen from the HF diet using the L-^{*}-OTU173-0085-a-A-18 probe.

Lactic acid concentration in ileum lumen

Lactic acid was measured in the lumen samples from the terminal ileum of all piglets from days 1, 4 and 10 after the introduction of the diet (Figure 3). By days 4 and 10, a significantly higher lactic acid concentration was recovered in the samples of HF diet compared with LF. As lactic acid is a common end-product of fermentation of lactobacilli, these results were in agreement with the outgrowth of lactobacilli in the terminal ileum as demonstrated by 16S rRNA gene based DGGE and FISH analysis, and suggests that the *lactobacilli* were not only present, but also metabolically active.

Discussion

The bacteriological results reported here, indicate that the addition of specific fermentable carbohydrates to the diet can lead to a shift in both the composition and activity of the microbial community of the small and large intestine of weaning piglets. Two particular phylotypes related to *Lactobacillus amylovorus* and *L. reuteri* were the most prevalent populations in the ileum of piglets fed the HF diet for ten days, as demonstrated by DGGE analysis and a phylotype-specific 16S rRNA targeted FISH analysis. In addition, bacterial diversity was increased by day 10 in the colon of the HF group, as evidenced by the higher number of DGGE bands and the Shannon index of diversity in the corresponding samples. Given current global concern of in-feed antibiotics replacement these results show that careful design of the diet can indeed stimulate supposedly beneficial bacteria. They also indicate that other species can be suppressed. These findings therefore, are not only interesting for piglet microbiology and nutrition at the time of weaning, but also provide new insights into the specific effect of prebiotics on the indigenous *Lactobacillus* communities of piglets.

The availability of fast sequencing techniques offers an unprecedented opportunity to conduct comprehensive surveys of pig microbial communities^{71,73}. Results based on comparative sequence analysis of the 16S rRNA and chaperonin-60 genes documented the complexity of the intestinal microbial community and suggested that the majority of the bacterial species colonizing the GI tract in pigs have not yet been characterized. However, cloning and sequencing is time-consuming and may limit the number of samples that can be processed.

Thus, the high sample throughput required to determine community responses to experimental treatments such as introduction of prebiotics or probiotics needs yet to be achieved by the analysis of multiple clone libraries. Alternatively, denaturing gradient gel electrophoresis (DGGE) and a similar technique called temperature gradient gel electrophoresis (TGGE) have been introduced into microbial ecology^{90,91,107} as one attempt to obtain an overview of the structural diversity of microbial communities. As reported previously, DGGE and TGGE

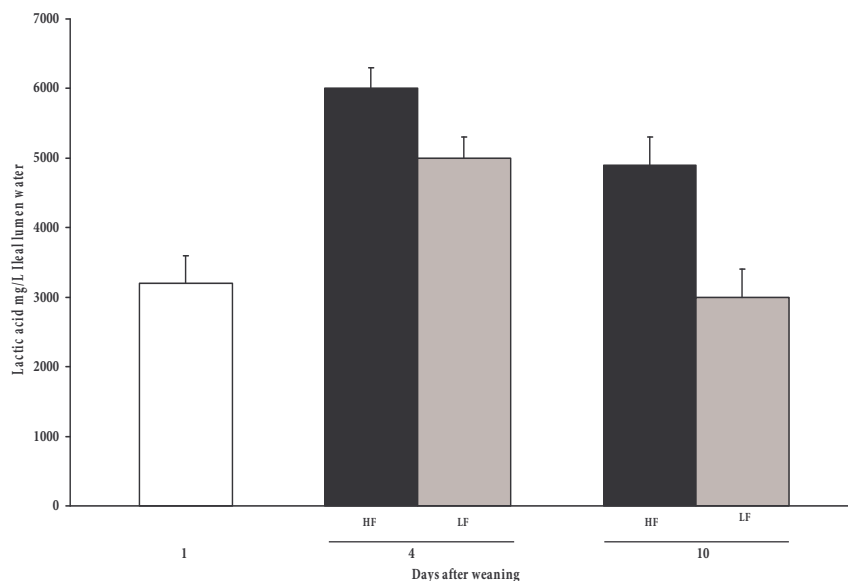


Figure 3. Luminal lactic acid concentration (mg/l) in the terminal ileum of piglets. The data are expressed as a mean value plus standard error mean for all samples. * Significantly different concentration of lactic acid ($p < 0.05$).

are sensitive enough to detect bacteria that constitute 1% of the total bacterial community.¹⁰⁸ The PCR-DGGE detection limit has been also estimated previously by dilution series of pure cultures¹⁰⁹. In addition, the primer pair used in the current study (S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17) was found to amplify with the same efficiency 16S rRNA genes from the complex soil bacterial communities¹¹⁰. Individual DGGE bands can be assigned to cultured organisms or retrieved ribosomal sequences^{24,111}. This is usually not possible in activated sludge, sediments, soil, and other highly diverse microbial systems because the banding patterns are too complex⁹⁶. However, the number, precise position, and intensity of the bands reflect the number and relative abundance of dominant rRNA sequence types in the sample and thus allow a comparison to be made of microbial communities with each other. By applying this approach to piglet GI-tract lumen samples, a distinct diversity value for each sample was obtained and changes in community diversity over time in different experiments were observed. In agreement with previous analyses of 16S rRNA gene libraries obtained from pig ileum and colon samples⁷³, the results reported in this study showed a significantly lower diversity in the ileum compared to the colon. Further elucidation of the diet effect by using 16S rRNA gene PCR-DGGE analysis unveiled the impact of the ileum microbiota in the utilization of the fermentable carbohydrates. Marked differences in the bacterial communities, composition were demonstrated by Day 10 of the experiment (Figure 1 A, B) between ileal samples from piglets fed with HF or LF diets. While this has not been previously demonstrated by culture-independent approaches, there are numerous

studies showing that the ileum of pigs harbors a diverse and active bacterial population³² and reviewed by Verstegen *et al.*⁶⁶ Furthermore, the increased diversity in the colon of piglets fed the HF diet as demonstrated by DGGE is in agreement with our earlier data²⁴. Such a strong effect of the diet on the porcine colon and faecal bacterial populations has also been demonstrated when the animals were fed different diets¹⁰⁶ containing fermentable carbohydrates and after introduction of an exogenous *Lactobacillus* strain⁸².

The combination of 16S rRNA gene directed DGGE, cloning and sequencing in this study has identified the phylogenetic changes in the piglets microbiota and highlighted the outgrowth of *L. amylovorus*-like populations in the ileum of HF fed group piglets. However, because these approaches are all based on PCR amplification methods, the results cannot be converted to actual bacterial numbers. FISH, in combination with microscopic analysis has provided a powerful tool in detecting and quantifying various bacterial genera including *Lactobacillus* in human faeces.^{94,104} Sequences related to *L. amylovorus* or phylotype OTU 171 were recovered from the colonic wall and lumen of a pig⁷² and were found to be the most abundant *Lactobacillus* phylotype in the GI tract of Danish pigs of different ages and feeding regimes.⁷³ The same phylotype was detected independently to predominate in the small intestinal microbiota of weaning piglets based on 16S rRNA gene-sequence analyses and DGGE.¹¹² Since the current results suggest a significant stimulation of this bacterium in the presence of the HF diet, a DNA oligonucleotide probe targeting this phylotype was developed and validated for FISH analysis. After validation, the probe was used to quantify the number of hybridized cells in the ileal and colonic lumen of piglets fed 10 days with HF or LF diet. The FISH results showed that the size of OTU 171-related populations varied from 0.64×10^8 to 2.4×10^8 /g of ileal lumen of 20 out of 24 piglets (Table 5). The results were in agreement with the DGGE analysis where the phylotype was detected as a dominant DGGE band in 80% of the analyzed piglets fed 10 days with HF diet.

Various studies on the effect of prebiotic oligosaccharides on the colonic microbiota in humans have reported a stimulation of *lactobacilli* by inulin and lactulose¹¹³ and reviewed by Rastall¹¹⁴. However, in many of the *in vitro* and *in vivo* experiments further characterization beyond the genus level has not been achieved¹¹⁴. Populations of *lactobacilli* related to *L. amylovorus* and *L. reuteri* have been identified as a common inhabitant of the human and animal intestine. The properties of the type strains are also well established. They are known for their ability to degrade starch. In addition, both strains produce bacteriocins, potentially suppressing other populations within the intestinal microbiota¹¹⁵. However, the extrapolation of functional properties from well-characterized cultured strains to the related phylotypes *L. amylovorus*-like and *L. reuteri*-like may not be justified. In particular, a high level of 16S rRNA gene relatedness (>97.5%) was found between the type strains of *L. amylovorus*, *L. crispatus*, *L. galinarum*, *L. kitastonis*¹⁰³ and the most abundant *L. amylovorus*-like phylotype detected in the study. Therefore, a study on the physiological and genomic properties of a large collection of *L. amylovorus*-like populations isolated from the pig intestine is underway in our laboratory.

In the current study, sugar beet pulp (SBP) with significant fermentable carbohydrate

Table 5. FISH results

Parameter	Value ^b			
	HF		LF	
	Ileum (n=24)	Colon (n=24)	Ileum (n=24)	Colon (n=24)
Total cell count ^a	2.1±1.1 X 10 ⁸	3.1±2.4 X 10 ¹⁰	1.2±1.2 X 10 ⁸	2.3±1.4 X 10 ¹⁰
Total bacterial count ^a	1.89±1.4 X 10 ⁸	2.7± 1.5 X 10 ¹⁰	1.17±1.1 X 10 ⁸	1.94±0.9 X 10 ¹⁰
Lactobacillus-enterococci ^a	1.5±0.6 X 10 ^{8d}	3.8± 2.1 X 10 ⁸	0. 4±0.3 X 10 ⁷	6.3±3.1 X 10 ⁸
<i>L. amylovorus</i> -like (Probe, L-S-OTU171-a-A-0088) ^c				
No of piglets colonized (%)	20 (83)	18(75)	ND	ND
Median count	1.3 X 10 ⁸	3.7 X 10 ⁹	ND	ND
Range	0.64-2.4 X 10 ⁸	1.3-5.6 X 10 ⁹	ND	ND
<i>L. reuteri</i> -like (Probe, L-S-OTU173-a-A-0085) ^c				
No of piglets colonized (%)	18 (75)	10 (41.2)	ND	9 (37.5)
Median count	7.7 x10 ⁷	5.8 x 10 ⁷	ND	5.5 x 10 ⁷
Range	4.4 –12 x10 ⁷	5.3-6.2 x10 ⁷	ND	5.1-6.3 x10 ⁷

^a Results for total cell counts (DAPI staining), total bacterial counts (S-D-Bact-0338-a-A-17), and lactobacillus-enterococcus counts (S-G-Lab-0158-a-A-20).

^b Mean _ standard deviation (cells per gram). ND, no bacteria detected.

^c Specific counts for the probes L-S-OTU171-a-A-0088 (*L. amylovorus*-like) and L-S-OTU173-a-A-0085 (*L. reuteri*-like) for ileal- and colonic-lumen samples from piglets fed for 10 days on HF or LF diet.

^d Significant differences (P < 0.05) from the values compared (bold face).

(including cell walls) content was also included. The cell wall component of the diet has previously been found to affect not only the microbial fermentation in the GI tract of pigs³², but also to play a role in stimulation or inhibition of certain pathogens in the intestine¹¹⁶. The addition of the SBP to the diet of pigs was reported to reduce the population of coliforms¹¹⁷, while others suggested an increased proliferation of the pathogenic *Escherichia coli* if the piglets were fed with fibre-enriched diet¹¹⁸. The effect of dietary fibre on the development of swine dysentery is currently also under discussion. As shown by some reports, diets with low fibre and resistant starches prevent the pigs from infection with *Brachyspira hyodysenteriae*¹¹⁹⁻¹²¹, while others were not able to confirm these findings^{106,122}. Our results suggest that the

combination of fermentable dietary fibre and oligosaccharides may specifically stimulate the *L. amylovorus*-like population along the gut of weaning piglets.

Acknowledgements

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Chapter 4

Difference in *in vitro* fermentability of four carbohydrates and two diets, using ileal and faecal inocula from unweaned piglets

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Abstract

An experiment was conducted to examine differences in *in vitro* fermentability of four carbohydrate-rich feed ingredients, and two weaning piglet diets with and without these ingredients, using both the ileal contents and the faeces of unweaned piglets as inocula. In the first part of experiment, cumulative gas production was measured in time, using faecal inocula mixed from nine specially-raised crossbred piglets (no creep feed or antibiotics) at three weeks of age. Inulin, lactulose, unmolassed sugar beet pulp, wheat starch and the two diets, were used as substrates and fermented *in vitro* for 72 hrs. Gas production was measured as an indicator of the kinetics of fermentation. Fermentation end-products including VFA, and ammonia, and organic matter loss, were also measured. For the fermentations of feed ingredients, samples were also collected for PCR / DGGE analyses initially, and after the fermentation process, to study changes in the composition of the bacterial community. This procedure was repeated one week later, using ileal contents from the same piglets as inoculum. There were significant differences between the inocula, both in terms of overall fermentation characteristics and composition, and between the substrates. There was also a significant interaction between inocula and substrates, which suggests that there were potentially important differences in the microbial activity occurring in these two areas of the gastro-intestinal tract (GIT). For the two diets, one with and one without addition of these fermentable ingredients, there were significant differences in terms of the kinetics, but less so in terms of the end-products of fermentation. It was concluded, that inocula from both the small intestine and large intestine should be used, to obtain a more accurate assessment of potential feed ingredients which will stimulate fermentation in piglet GIT.

Introduction

Exposure to a solid diet at weaning can lead to sudden changes in the activity and composition of the gastro-intestinal tract (GIT) microflora, which can ultimately affect the health of the animal. Such abrupt changes both in the diet and the environment at weaning, are thought to predispose piglets to gastrointestinal diseases. Given the impending EU ban on the use of antimicrobial growth promoters in animal feeds, there is now an urgent imperative in Europe to examine alternative ways of maintaining piglet health by stimulation of the autochthonous gastro-intestinal microflora³¹.

The mammalian GIT contains a complex, dynamic, and diverse community of usually non-pathogenic bacteria⁶⁵. Lactic acid bacteria have been shown to be the first to colonize a young piglet's GIT⁶⁷. During the suckling period, the microflora of the piglet remains fairly stable in terms of species, for as long as the piglets receives sow milk³¹.

The microbial population itself is, to a large extent, dependent upon the host diet as the main source of substrates for metabolism. As a result, changes in dietary composition or nutrient density can have a significant effect on the GIT microbial populations, which in turn, may affect the ability of an animal to digest and absorb nutrients²⁹. The autochthonous microflora is also considered to act as one of the body's natural defences, and consists of the population of mostly non-pathogenic bacteria normally residing in the GIT⁶⁷. This population is considered to play an important role in the development of "colonization resistance" against potential pathogens¹. Stimulation of this autochthonous microflora, and thereby potentially of colonization resistance, is being investigated by use of probiotics, prebiotics and synbiotics¹²³. Manipulation of the diet by the use of prebiotics is considered to be a comparatively easy way to influence the composition and activity of the microflora. Fermentable carbohydrates are therefore considered to be an ideal ingredient of diets, to encourage growth of beneficial bacteria, and thereby colonization resistance to potential pathogens²¹⁻²³. However, before choosing such ingredients for inclusion in a diet, it is helpful to first assess a number of them for their *in vitro* fermentability to assist in the selection process¹²⁴.

Most often, tests for fermentability of potential dietary ingredients are carried out using faeces as an inoculum and while it is understood that fermentation in the small and large intestines are likely to differ, assumptions are made from results which would suggest that the results from such tests may also be relevant for small intestinal fermentation. Therefore, it would be helpful to examine differences in microbial activity at different sites in the GIT more closely. Very little is known about the fermentative activity of the microflora from the different sites of the GIT in unweaned piglets, though a few studies have been carried out with growing pigs^{32,124}. Adjunct to an *in vivo* experiment, it was decided to examine both the four fermentable ingredients chosen for the test diet, and the two complete diets (control and test) for their *in vitro* fermentability, using both ileal digesta and faeces as inocula.

Therefore, there were two aims for this study: i) to evaluate four carbohydrate-rich feed ingredients for their fermentability, using the two inocula. This allowed an assessment both of the substrates and of the inocula, and any interaction between them. ii) to assess

the two weaning diets, with and without those ingredients, for differences in their *in vitro* fermentability with the same inocula.

Materials and Methods

Experimental design

The study was conducted as two separate fermentation runs, one week apart, using the Automated Pressure Evaluation System (APES)¹²⁵. All procedures involving animals were conducted in accordance with the Dutch law on experimental animals and had been approved by the Wageningen University Animal Experimental Committee (Dier Experimenten Commissie).

Animals

The first run was done using faeces from unweaned piglets as inoculum. Nine three-week old piglets from three litters were used as faecal donors, in order to have both a representative microbial population, and sufficient material as inoculum. After one week (at four weeks of age), these nine piglets were slaughtered to collect ileal contents as inoculum for the second experiment. The piglets had free access to sow's milk and did not receive any creep feed nor antibiotics. During the complete experimental period, piglets remained with their sow and littermates on the farm.

Substrates

The same four carbohydrate-rich ingredients and two diets were used as substrates for both runs. The ingredients tested were inulin, lactulose, unmolassed sugar beet pulp (SBP) and wheat starch (WST). All substrates were obtained from commercial suppliers. The air-dried substrates were ground to pass a 1-mm sieve. Diets had been formulated which were appropriate for newly weaning piglets, for an *in vivo* experiment reported elsewhere. The main ingredients for both diets included corn starch, fishmeal, dextrose and soya oil. However, the test diet contained all four carbohydrate-rich ingredients (lactulose: 2%; inulin: 0.75 %; sugarbeet pulp: 5%; and wheat starch: 5%) , while the control diet was a semi-purified diet containing as few fermentable carbohydrates as possible (See Table 1, Chapter 1).

Inoculum

Faeces were collected per rectum with a gloved finger on the farm. The faeces were immediately placed in small containers filled with CO₂. In the laboratory, equal amounts of faeces from each animal were combined (by wet weight), and diluted 1:15 with sterile anaerobic saline (0.9% NaCl- neutral pH), which had been pre-warmed to 39°C. The large dilution was necessary due to the very small quantity of material obtained. This diluted mixture was then homogenised for 60s to ensure proper mixing, and was then filtered through a double piece of clean cheesecloth (16 threads/ cm each direction). The resultant

filtrate was used as the inoculum. All procedures were carried out under a constant stream of CO₂ to maintain anaerobic conditions. This faecal material was assumed to provide a microbial population which would be representative of the large intestinal microflora.^{126,127}

One week later, the same nine piglets were slaughtered. The piglets were slaughtered according to a prescribed procedure. First, Ketamin was used as a pre-anaesthetic, and 30 min later, the piglet was euthanatised by intra-cardiac injection of T61 (Hoechst Roussel Vet). The gastro-intestinal tract was tied off at regular intervals to avoid mixing of digesta, and ileal contents collected (~ 1 metre back from the ileo-caecal junction). This inoculum was prepared as for faecal contents, except that the dilution with saline was 1:9.

Four ml of inoculum was injected into each fermentation bottle (three replicates per substrate) and the bottle immediately attached to the Automated Pressure Evaluation System (APES)¹²⁵. Each bottle contained ~0.5 g of substrate and 82 ml of a modified semi-defined medium¹²⁸. Blanks contained only medium and inoculum. Bottles were incubated at 39°C for 72 hours.

Analyses

All substrates were analysed for their dry matter (DM), and ash contents to allow correction of all gas data to per gram OM. The pH (pH meter – Hanna Instruments) and dry matter content were also determined for both inocula. At the end of the incubation period, the pH of each bottle was recorded and samples of fermentation fluid collected for the measurement of DM, ash, VFA, and ammonia. Samples were also collected for PCR/DGGE analysis, before and after fermentation of the feed ingredients, to allow an examination of changes in bacterial composition of microflora following fermentation of these substrates.

Dry matter was determined by drying to a constant weight at 103°C³⁴ and ash by combustion at 550°C³⁵. Volatile fatty acid (VFA) concentrations in the fermentation liquids were analysed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101, as carrier gas N₂ saturated with methanoic acid, at 190°C and using iso-caproic acid as internal standard. Ammonia was determined according to the method described by Searle.³⁶

Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE)

A DNA “fingerprint” of the microbial population of the inoculum and of the populations after fermentation of the carbohydrate substrates was made using the PCR/DGGE method described by Zoetendal.¹²⁹ For this analysis, only two of the four bottles were analysed following fermentation of the ingredients only.

First, the DNA of the samples was isolated using the FastDNA[®] SPIN Kit for Soil (BIO 101, Carlsbad, CA 92008, USA). This kit has been used previously for faecal and colonic microflora of pigs and humans, and has shown good results.⁴⁸

PCR was performed with primer 8f [5' CAC GGA TCC AGA GTT TGA T(C/T)(A/C) TGG CTC

AG] by using the Taq DNA polymerase kit from Life Technologies to amplify the bacterial 16S rDNA¹⁰⁸. PCR was performed under the following conditions: 94°C for 3 min; 35 cycles of 94°C for 30s, 52°C for 30s, and 68°C for 1.5 min; and finally 68°C for 7 min. The amount and integrity of the nucleic acids was determined visually after electrophoresis on a 1.2 % agarose gel containing ethidium bromide. PCR amplicons were separated by electrophoresis at a constant voltage of 85 V and a temperature of 60° C for 16 h. The DNA fragments were made visible by AgNO₃ staining, after which the gels were developed. Polyacrylamide gels (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) in 0.5 x Tris-acetic acid-EDTA buffer with a denaturing gradient were prepared using a gradient mixer and Econo-pump (Bio-Rad) and solutions containing 45 and 55% denaturant. A 100% denaturant corresponds to 7M urea and 40% (vol/vol) formamide.

The gels were then scanned, and inter-sample comparisons of the bands on the gel were performed using the program Molecular Analyst (Molecular Analyst Software, fingerprinting, Biorad). Both the correlations calculated and the clustering schemes made by the program are based on the number of bands and the position of the bands in the gel.

Statistics

For curve-fitting, the gas profiles (as ml of gas produced per g OM in time) were fitted to the monophasic version of the multiphasic model described by Groot *et al.*¹³⁰ as follows:

$$G = A / (1 + (C/t)^B) \dots \dots \dots (1)$$

Where G = total gas produced; A = asymptotic gas production; B = switching characteristic of the curve; C = time at which half of the asymptote has been reached (T1/2); t = time (h)

The maximum rate of gas production (Rmax) and the time at which it occurs (Tmax), were calculated according to following equations¹³¹:

$$Rmax = (A * (C^B) * B * (Tmax^{-(B-1)})) / (1 + (C^B) * (Tmax^{-(B)}))^2 \dots \dots \dots (2)$$

$$Tmax = C * (((B - 1) / (B + 1))^{(1/B)}) \dots \dots \dots (3)$$

The four ingredients were statistically tested separately from the two diets. For both sets of data, differences between the substrates, inocula and the interactions between them were tested for significance using two-way ANOVA. The statistical model was as follows:

$$Y = \mu + Si + Ij + (S^*I)ij + \epsilon_{ijk} \dots \dots \dots (4)$$

Where Y is the parameter to be tested, μ the mean, Si effect of the substrate i, Ij effect of the inoculum j, (S*I)ij the interaction between substrate and inoculum, and ϵ_{ijk} is the error term. The effect of replicate bottles was tested separately, and was not significant for any of the parameters. It was therefore excluded from any further statistical analysis and was therefore part of the error term.

All statistical analyses were performed using the Proc GLM procedure of the statistical program SAS³⁸.

Results

Dry matter and Ash

The values for DM for the faecal and ileal inocula were 418 g/kg and 190 g/kg respectively, while the pH values were 5.78 and 5.6. The DM and ash contents of the different substrates were as follows: inulin: 946.1 g/kg DM and 1.9 g/kg ash; lactulose: 703.5 g/kg DM and 0.3 g/kg ash; SBP: 902.4 g/kg DM and 67.9 g/kg ash; WST: 849.8 g/kg DM and 2.4 g/kg ash; Control diet: 845.1 g/kg DM and 50.4 g/kg ash; and Test diet: 816.7 g/kg DM and 47.6 g/kg ash.

Gas production kinetics

The mean values for parameters of fermentation kinetics after 72 hours are shown in Table 1. The mean values show the effect of substrate, inoculum, and the interaction between substrate and inoculum. The data for ingredients and diets are shown separately. For both ingredients and diets, most parameters showed a significant interaction. Figure 1 shows representative examples of the gas profiles that compare the two inocula for each of the four ingredients.

For the ingredients, the total gas produced (OMCV) was significantly higher for all substrates ($p < 0.001$) fermented with faeces compared to ileal contents. Of these, lactulose produced the most gas (OMCV). For the diets, there was an interaction between substrate and inoculum, so the results for OMCV were not consistent for substrate (Test diet higher for faecal inoculum, and Control diet higher for ileal inoculum). For the ingredients, there was an overall tendency for the T1/2 to occur earlier for the faecal compared with the ileal inoculum. In the case of lactulose and inulin, this difference between inocula was significant. However, all four ingredients differed significantly for T1/2 ($p < 0.001$), whereby T1/2 occurred later in the order: lactulose, inulin, WST and SBP. For diets, on the other hand, the T1/2 occurred consistently later for the Test than for the Control and for the faecal inoculum than for the ileal inoculum.

For the ingredients, Tmax was also significantly different between the two inocula (except for lactulose). Generally, the faecal inoculum, within the respective substrate-inoculum combinations, had an earlier Tmax than the ileal inoculum. Substrate also exerted a significant effect on Tmax. Irrespective of inoculum, lactulose had the earliest Tmax. The diets had consistent results for inoculum, whereby again, the Tmax occurred earlier for the faecal inoculum. Again, there was an interaction between substrate and inoculum, so that the diets were not consistent between inocula.

Rmax was also significantly different for both inocula and substrates ($P < 0.001$) for the ingredients. Generally, the faecal inoculum had a faster Rmax compared with the ileal inoculum. A comparison of ingredients as substrates showed that lactulose had the fastest while SBP had the slowest Rmax. For the diets as substrates, the ileal was much faster than the faecal inoculum, but there was no difference between substrates.

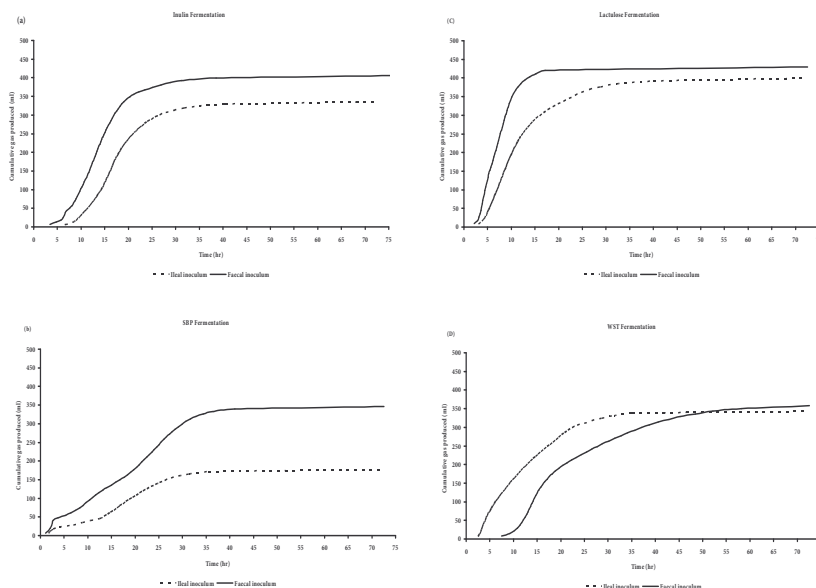


Figure 1. Gas production profiles of individual representative bottles to demonstrate the fermentation kinetics of four carbohydrates, by the ileal and faecal inocula: (a) Inulin; (b) SBP; (c) Lactulose and (d) WST.

Organic matter loss (OML) was much lower ($p < 0.001$) for SBP compared with the other substrates, though it must be remembered that both inulin and lactulose are highly soluble, so these results do not necessarily reflect amount of material fermented. There was no effect of inoculum observed for OML, except for SBP, which had a lower OML for the ileal inoculum. There was also no significant difference between the diets for this parameter, and the inocula differed in terms of slightly greater losses for faecal compared with ileal inocula.

Fermentation end-products

There were no significant differences between the ingredients in terms of total VFA (Table 2). For lactulose and inulin, total VFA were higher when fermented by ileal rather than by the faecal inoculum, while in the case of SBP and WST, there were more total VFA for the faecal compared with ileal inoculum. There was no significant interaction between substrate and inoculum for total VFA production, or for the individual acids (Table 2). For the diets, there was only a significant difference between inocula, where ileal contents had a consistently

higher total VFA value compared with faeces.

For the ingredients, there was a significant effect on butyric acid production ($P < 0.05$), and a tendency to significance for acetic ($P = 0.06$) and propionic acids ($P = 0.07$). Acetic acid production differed significantly between inocula, while propionic and butyric acid also showed significant differences between inocula, except for SBP (Table 2). For all ingredients, both acetic and butyric acid were higher for the faecal compared with the ileal inoculum, whereas propionic acid production was higher for the ileal inoculum. For the diets, there was no significant difference between substrates at all, except for butyric acid, which was higher for the Control compared with the Test diet. However, there were significant differences between inocula for all the individual acids, except for butyric. For these diets, the ileal contents consistently produced higher amounts of post-fermentative acetic and propionic acids, which accounted for the higher total VFA for both diets.

Table 1. Gas production parameters for fermentation of four carbohydrates and two diets using ileal and faecal inocula from suckling piglets. (n=3 per substrate inoculum combination)

Substrate	Inoculum	OMCV ¹	T1/2 ¹	Tmax ¹	Rmax ¹	OML ¹
Inulin	Faeces	419.2	11.8	10.5	38.0	98.4
	Ileal contents	361.4	16.6	15.1	24.5	99.6
Lactulose	Faeces	446.2	6.5	5.3	61.4	97.3
	Ileal contents	400.6	10.2	7.9	31.0	99.6
SBP	Faeces	407.6	21.6	11.1	13.2	87.5
	Ileal contents	266.2	22.2	16.2	8.1	73.6
WST	Faeces	394.6	13.7	11.4	25.4	97.4
	Ileal contents	368.5	16.7	6.6	17.1	99.3
Main Effects	Substrate	<0.001	<0.001	<0.001	<0.001	<0.001
	Inoculum	<0.001	0.078	0.022	<0.001	0.421
	Sub*Inoc	0.002	0.775	<0.001	0.007	0.112
	MSD ²	35.2, 18.2	6.2, 3.2	2.8, 1.4	7.7, 4.0	10.3, 5.3
Control Diet	Faeces	338.4	3.29	0.54	75.9	86.62
	Ileal contents	407.6	2.02	1.80	215.8	84.74
Test Diet	Faeces	367.3	4.07	0.30	75.2	87.17
	Ileal contents	389.4	2.26	2.12	229.06	85.14
Main Effects	Substrate	0.002	<0.001	0.721	0.482	0.536
	Inoculum	<0.001	<0.001	<0.001	<0.001	0.027
	Sub*Inoc	<0.001	0.016	0.018	0.439	0.924
	MSD ²	3.39, 3.45	0.19, 0.2	0.21, 0.2	18.6, 19.1	1.61, 1.65

¹OMCV = Cumulative gas production (ml/g organic mater weighed in); T1/2 = Half time of asymptotic gas production (h); Rmax= Maximal rate of gas production (ml/h); Tmax = Time occurrence of Rm (h); OML = Organic matter lost during the fermentation (% of the organic matter incubated).

²MSD = Minimum significant difference ($p < 0.05$). Where there are two values, the first refers to the substrate, the second to the inoculum.

There were significant differences between ingredients and inocula, in terms of ammonia concentration at the end of fermentation (Table 2). Interestingly, the ileal inoculum led to significantly more ammonia compared with the faecal inoculum after fermentation of the three substrates: lactulose, SBP, WST. Lactulose had the highest ammonia concentrations of

Table 2. End-products of fermentation of four carbohydrates and two diets using ileal and faecal inocula of suckling piglets (n=3 per substrate inoculum combination).

Substrate	Inoculum	Tot VFA ¹	Acetic ¹	Propionic ¹	Butyric ¹	NH3 ¹	pH
Inulin	Faeces	4.97	3.08	0.94	0.74	52.4	6.35
	Ileal contents	5.36	2.39	1.97	0.53	52.1	6.25
Lactulose	Faeces	4.88	3.25	0.53	0.85	76.2	6.51
	Ileal contents	4.99	2.44	1.78	0.57	77.3	6.48
SBP	Faeces	5.18	3.54	0.97	0.39	60.5	6.4
	Ileal contents	5.02	3.22	1.19	0.38	76.5	6.46
WST	Faeces	6.14	3.34	1.51	0.83	52.2	6.25
	Ileal contents	5.62	2.37	2.16	0.49	70.6	6.35
	Substrate	0.175	0.068	0.072	0.038	<0.001	<0.001
Main Effects	Inoculum	0.886	0.002	0.003	0.015	<0.001	0.637
	Sub*Inoc	0.715	0.567	0.364	0.421	<0.001	0.032
	MSD2	1.2, 0.62	0.69, 0.35	0.82, 0.42	0.29, 0.15	5.61, 3.16	0.09, 0.04
Control Diet	Faeces	4.78	2.9	0.51	1.07	87.9	6.52
	Ileal contents	5.71	3.4	0.91	1.06	104.2	6.55
Test	Faeces	4.72	2.86	0.54	0.96	89.9	6.57
Diet	Ileal contents	5.47	3.32	0.94	0.88	105.1	6.57
	Substrate	0.532	0.657	0.599	0.039	0.116	0.131
Main Effects	Inoculum	0.007	0.008	<0.001	0.463	<0.001	0.476
	Sub*Inoc	0.715	0.843	0.918	0.564	0.491	0.545
	MSD2	0.53, 0.54	0.31, 0.32	0.15, 0.15	0.13, 0.13	1.85, 1.89	0.04, 0.04

¹ Tot VFA = Total volatile fatty acids (mmol per g⁻¹ OM incubated); Acetic = Acetic acid; Propionic = Propionic acid; Butyric = Butyric acid; NH3 = ammonia (mg per g⁻¹ OM).

²MSD = Minimum significant difference (p < 0.05). Where there are two values, the first is referring to the substrate, the second one to the Inoculum

all the substrates, for both inocula. For the diets, the situation was the same: there was no significant difference in ammonia concentrations between substrates, but the concentrations

were higher for ileal compared with faecal inocula. There was no interaction.

Overall, there was no difference between inocula for the pH values measured at the end of fermentation, for any of the ingredients as substrates, even though there was a significant inoculum-substrate interaction. For inulin, the pH was lower for ileal inoculum, while for SBP and WST, the pH was higher for the ileal inoculum. There were significant differences in pH between the ingredients ($p < 0.001$). For the diets, there were no significant differences for substrates, or inoculum, and there was no interaction between the two for pH.

PCR- DGGE analysis

The similarity indices of the DGGE profiles of different substrates after fermentation with faecal and ileal inoculum were calculated and these are presented in Figure 2. The average similarity indices of replicates on the DGGE gel, between faecal and ileal inoculum after fermentation with different substrates were as follows;

Inulin – faecal	-	38.6 %	-	Inulin – ileal
Lactulose – faecal	-	22.65%	-	Lactulose – ileal
SBP- faecal	-	49.8%	-	SBP – ileal
WST – faecal	-	27.15%	-	WST – ileal

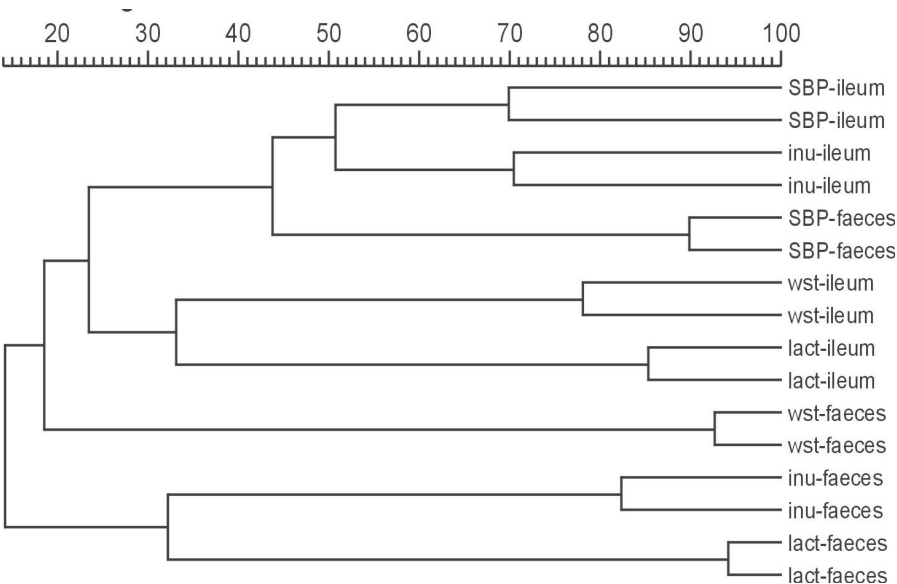


Figure 2. Similarity index of DGGE profiles obtained from fermentation liquids after fermentation of four fermentable carbohydrates (inulin, lactulose, SBP and WST) with faecal and ileal inoculum from unweaned piglets.

In Figure 2, it can be clearly seen that bands from the ileal and faecal inocula tended to cluster separately, even after fermentation of the same ingredient, except in the case of SBP.

Microbial communities at the end of fermentation of SBP were approximately 50% similar, unlike the other ingredients all of which had less than 40% similarity between their microbial communities when comparing the ileal and faecal inocula.

Figure 3 shows the DGGE bands of the different ingredients after fermentation with faecal and ileal inocula. Overall, for all carbohydrate ingredients, there were more bands after fermentation by faeces, compared with fermentation by ileal digesta. Also, there were some distinct bands, which were common to all faecal inoculated samples from all substrates, which were absent from the ileally fermented samples of the same substrates. Some of the most distinct bands which were common for all substrates are indicated in Figure 3 (letters A and B).

Discussion

The four carbohydrate-rich feed ingredients had been chosen as a result of earlier *in vitro* tests using only faeces as the inoculum (Williams *et al.*, submitted) and were to be incorporated into diets for an *in vivo* experiment examining the relation between nutrition and gut fermentation. The choice had been made partly due to the kinetics of fermentation, assuming that ingredients which were more rapidly fermentable by faeces, were more likely to be fermented earlier in the tract, and the more slowly fermentable ingredients would be fermented more distally. However, while it has been shown that the faeces is indeed representative of microbial activity which occurs within the large intestine,¹³² it is less likely to be representative of the small intestine, which was why there was interest in comparing microbial activity from the two sites.

For the work reported here, there were significant differences between the ileal and faecal inocula in relation to the four feed ingredients. Overall, there was more total gas produced, an earlier T1/2, a faster maximum rate of gas production (Rmax), and an earlier time at which this maximum rate occurred, Tmax (except WST), for the faecal compared to ileal inoculum. Therefore, one can conclude that the faeces fermented the ingredients faster and to a greater extent, compared with the ileal material. On the face of it, this is in agreement with the widely-held view, that most fermentation takes place in the large intestine. However, this was an *in vitro* test with the same fermentation times, so these results make it clear that the microbial activity was significantly less for the small compared with the large intestinal inocula. Coupled with this lower activity, it is also known that there is a faster small intestinal transit time of the digesta which would reduce the fermentative activity even further. Even so, the overall ready fermentability of these essentially complex carbohydrates (particularly the fibrous ingredient- SBP), came as something of a surprise, given that the piglets had not been exposed to any starch or fibrous materials before, let alone of these particular ingredients. Again, this should not be so surprising given that the small intestine of pigs contains a significant microbial population^{73,133}, in contrast to the human tract which has a very low number of bacteria¹³⁴. A study by McVeagh and Miller¹³⁵ has suggested that the oligosaccharides present in human breast milk can act as a soluble fibre for human babies. Although hardly any work has been reported concerning oligosaccharides in sows' milk, a

similar mechanism could also be present in pigs, which may explain the ready fermentation of these complex carbohydrates.

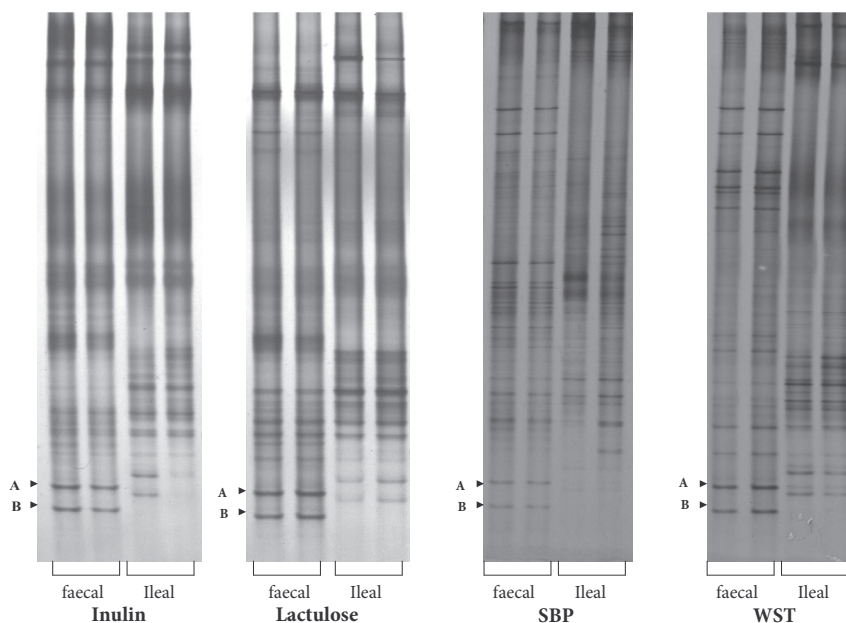


Figure 3. DGGE of amplicons obtained from fermentation liquids after fermentation of four fermentable carbohydrates (inulin, lactulose, SBP and WST) with faecal and ileal inoculum from unweaned piglets.

Taking into account assumptions about transit time, these results, using both ileal and faecal contents as inocula, confirmed that in terms of their kinetics at least, lactulose and inulin are more likely to be fermented in the small intestine, and sugarbeet pulp in the large intestine, which is consistent with the conclusions reached for studies using only faeces. However, the result for WST was unexpected; studies (Williams *et al.* submitted) using only faeces, suggested that fermentation would be much slower, and that WST was more likely to be fermented in the large intestine. Use of an ileal inoculum however, showed that substantial fermentation of starch could also occur in the small intestine, so less of this ingredient would reach the large intestine than originally thought, when tested only faecal inoculum.

For the diets, however, this was essentially reversed, whereby the ileal contents produced more gas more rapidly than the faecal contents. However, it must be remembered that these diets were semi-purified, and that there were only comparatively small amounts of the fermentable ingredients added (lactulose: 2%; inulin: 0.75%; sugarbeet pulp: 5%; wheat starch: 5%). Also, there was no pre-treatment of these diets with digestive enzymes. Work reported by Bauer *et al.*¹³⁶ comparing enzyme pre-treatment of a diet with actual ileal chyme from the same diet, suggested that the pre-treatment led to significant losses of fermentable material, and indicated that significant fermentation occurs along the entire small intestine.

Therefore, to obtain results describing the fermentability of the whole material, any enzymatic pre-treatment was avoided in this work reported here. The results suggest that in terms of fermentation, the microflora present in the ileum had no difficulty fermenting starch, given that this was the main ingredient present in both diets. It had been expected that these piglets would be totally unadapted to utilization of starch, both in terms of their bacterial populations, and their digestive enzymes⁶, so this ready fermentation of starch, and of the diets was quite unexpected.

The DGGE profiles of samples after fermentation, suggest that the bacteria present in the inocula, differed in terms of species present. Overall, there were more bands detected in the faecal inoculated samples, than for the ileal inoculated samples for all substrates (see Figure 3). This would suggest that the ileal inoculum had fewer bacterial species present compared with the faecal inoculum, which is in agreement with work done in this area using both conventional and molecular techniques.^{48,73}

There were a few distinct bands consistently present in all faecal inoculated samples after fermentation, which were not necessarily detected in the equivalent ileal samples. This was found for all ingredients tested. However, it is also interesting to note that for each ingredient, there were some bands which were common for both ileal and faecal samples. It could be speculated that some of the bacteria present in small intestine begin the fermentation process there, and move to the large intestine either with normal flow, or because of attachment to substrate, and continue their activity in the large intestine, where transit time is slower, and fermentation can continue. This *in vitro* finding would suggest that there are probably more bacterial species in common between the two areas, which may be consistently selected for in both regions if the appropriate substrate is available.

In the case of rapidly fermentable ingredients, fermentation may already be complete within the small intestine. For example Houdijk *et al.*,¹³⁷ found that in growing piglets of ~55d old (45 kg) non-digestible fructo-oligosaccharides (FOS- Tmax = 10.4 hr) added to the diet had disappeared by the terminal ileum. However, the greater bacterial diversity of the faeces may have led to faster adaptation and better fermentation of most of the selected ingredients (see Figure 1), though this was not the case for the diets. In the case of the diets, there would be many ingredients present *in vitro* which would normally be assumed to be digested by the piglet enzymes. However, in a newly weaned piglet, the digestive enzymes are not always well developed⁶, so perhaps the ileal commensal bacteria had more fermentable material available to them.

In a review by Fooks *et al.*,¹³⁸ it was reported that in adult humans, the total bacterial count at the terminal ileum is 106- 107 per ml of contents, compared with 1011- 1012 per gram of content in the large intestine. As this is likely to be similar if not greater for pigs, it would appear that the bacterial populations in the large intestine are qualitatively (more diverse population), quantitatively (higher numbers), and logistically (more time available due to decreased transit time) better able to ferment ingredients with a more complex structure. If one considered only total gas production, it would appear that all ingredients chosen were

sufficiently complex, that fermentation by the faecal inoculum was better, given that the total gas production was greater for all four ingredients. However, that is why the kinetics of fermentation is also so important. To compare the two extremes for the ileal inoculum in this work: lactulose with a comparatively simple structure had an R_{max} of 31 ml/h which occurred at 8 hours, while sugarbeet pulp with a complex structure had an R_{max} of 8.1 ml/h which occurred at 16 hours.

Differences in patterns of individual VFA are more likely to reflect differences in the bacterial species which are most active in relation to the substrate being offered to the whole population. Ileal contents have been shown to have a higher proportion of lactate-producing bacteria. These species primarily produce lactic acid, which can then be rapidly metabolised further to propionic acid by other species.⁴⁵ In this study, lactic acid concentration was not measured, as after 72 h it was assumed that lactic acid would no longer be detectable. Lactate is considered to be an intermediate in GIT fermentation, and is metabolised to VFA to varying extents, by cross-feeding species in the ecosystem. It is therefore considered unlikely to accumulate to a substantial extent in the bowel.⁴⁴

In terms of VFA, there were some interesting interactions between the substrates and inoculum for the feed ingredients. For example, SBP and WST produced more total VFA when fermented with faecal inoculum, while lactulose and inulin produced more total VFA when fermented with the ileal inoculum. This difference is most likely due to differences in microbial composition between the two inocula, which is in agreement with the results shown by the DGGE. Generally speaking, there was much less difference between the inocula for the fermentation of the two diets. The most important question is, therefore, which *in vitro* result is more representative of what will happen in the piglet GIT? The *in vitro* results of the ingredients suggest animals fed a diet containing those ingredients will have higher concentrations of VFA in the digesta from different parts of the digestive tract. The results for the diets, suggest the contrary. An animal experiment examining this subject has been carried out, and the results will be reported shortly.

Conclusion

It was concluded that both the kinetics and end-products of fermentation of these feed ingredients varied according to the the origin of the inoculum. From the kinetic results, it seems that inulin and lactulose are more likely to be fermented before the end of the ileum, while SBP is more likely to be fermented in the large intestine. WST appeared to be well fermented by inocula from both sites, with a comparatively early T_{max} (6.6h) suggesting that in animals where digestion of starch is poor, as in newly weaned piglets with low amylase activity, a significant proportion of WST could be fermented in the small intestine.

When designing diets which will encourage some fermentation in the weaner piglet GIT, differences in fermentation kinetics of different ingredients *in vitro*, combined with end-product information, may provide information to assist in the choice of the best ingredients to stimulate colonization resistance. However, while use of faeces can apparently give an approximation of what may occur in the large intestine, it would be better if possible to use

an ileal inoculum to estimate small intestinal microbial activity.

The PCR-DGGE profiles measured after *in vitro* fermentation, demonstrated the kind of shifts which occurred in the microbial population when exposed to a single substrate. Though it would be very time-consuming and expensive to sequence all the bands found in DGGE profile, a selection of bands, may be able to provide useful information in terms of which specific species may be stimulated under the influence of a certain substrate. Indeed, these molecular techniques, if used in combination with probes of specific species of interest, might in the future, also be useful to highlight bacteria of interest (e.g. *Lactobacillus spp.*), and to thereby design diets exhibiting a prebiotic effect.^{48,139}

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Chapter 5

Effect of Substrate Adaptation on the Microbial Fermentation and Microbial Composition of Faecal Microbiota of Weaning Piglets Studied *in vitro*

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Abstract

The *in vitro* cumulative gas production technique can be used to assess microbial activity of a complex community, in relation to fermentation of a particular energy source. Therefore, in combination with an *in vivo* study to examine the effects of two different diets for weaning piglets, microbial activities of faeces were compared from animals on the two different diets. The two diets were: CHO diet (containing added fermentable carbohydrates – including sugarbeet pulp (SBP) and wheat starch (WST)), and Control diet without any added fermentable carbohydrates. Neither diet contained antibiotics nor extra added copper. Twenty-four piglets were selected from 12 litters (2 per litter), weaned at 4 weeks of age (neither creep feeding nor any antibiotic treatment before and during the study), and introduced to one of the two diets. After nine days on the diet, faecal samples were collected from selected animals, and tested for their activity in terms of gas production kinetics, and end-products such as volatile fatty acids (VFA), ammonia, and dry matter (DM) disappearance of two test substrates SBP and WST. The bacterial diversity was also analyzed before and after *in vitro* fermentation using denaturing gradient gel electrophoresis (DGGE) analysis of amplified 16S rRNA genes. There were differences both in kinetics and end-products of the substrates. More interestingly, significant differences were detected between inocula, though mainly in terms of fermentation kinetics of the two substrates. With the CHO inoculum, SBP was fermented faster, than with the Control, while this effect was reversed for WST. Significantly higher diversity, as measured by DGGE fingerprint analysis, was detected in the microbial community enrichment on SBP as compared to WST at the end of fermentation. The difference between the kinetics of SBP and WST fermentation by faecal microbiota from the CHO diet fed piglets, suggests better adaptation of the same to SBP fermentation than to WST fermentation. The WST fermentation was more unexpected, given that a significant amount of starch is known to be fermentable by the small intestinal microbiota. It was concluded that the microbial community composition and activity in the GIT may be changed in response to diet, and that this change can be detected *in vitro*.

Introduction

Intensification of the pig industry has led to increased risk of both clinical and sub-clinical enteric diseases. A major problem of the intensive pig industry is the occurrence of diarrhoea in weaning piglets associated with increased mortality³⁰. In order to control this disease problem, there has been widespread use of in-feed antibiotics at both a therapeutic and sub-therapeutic level. However, as a result of fears related to buildup of antibiotic resistance in the environment, there is increasing pressure to reduce such use of antibiotics. This has led to increased interest in alternatives to enhance both animal performance and disease resistance²³.

Given the impending EU ban on the use of antimicrobial growth promoters in animal feeds, there is now an urgent imperative to examine alternative ways to improve pig health by stimulating the autochthonous GIT microbiota.

At weaning, exposure to a solid diet combined with other stresses, can lead to dramatic changes in the gastro-intestinal tract (GIT) microbiota, leaving piglets potentially susceptible to proliferation of pathogens and thus to the well-described “post-weaning syndrome”³⁰. The autochthonous GIT microbiota is considered to be one of the body’s natural defence mechanisms, and consists of a complex assembly of mostly non-pathogenic bacteria residing normally in the GIT⁶⁷. It plays an important role in the development of colonization resistance against pathogens¹. Stimulation of this autochthonous microbiota can be done by the use of probiotics, prebiotics and synbiotics¹²³.

The microbial community is itself dependent upon the animal diet as the main source of substrate for its own metabolism. As a result, changes in dietary composition or nutrient density can have dramatic effects on the intestinal microbial community, which can, in turn, influence the ability of an animal to digest and absorb nutrients²⁹. Manipulation of the diet by the use of prebiotics is considered a comparatively easy way to influence the composition and activity of the microbiota²¹. Being potential substrates for bacteria, fermentable versus digestible carbohydrates, can be effective, in terms of influencing gut microbiota beneficially²¹⁻²³. The addition of non-digestible but fermentable carbohydrates can lead to a higher bacterial diversity and more rapid stabilisation of the microbial community²⁴.

The aim of this study was to determine whether the prior dietary exposure to fermentable carbohydrates by their addition to the host diet, influence *in vitro* fermentation of the added carbohydrates by the faecal microbiota of the host.

Materials and Methods

Experimental design

The study was conducted in three identical but independent gas runs, using the Automated Pressure Evaluation System (APES)¹²⁵ for which twenty-four piglets (four weeks old) from 12 litters, were used as faecal donors. Therefore, for each gas run, eight piglets from four litters were used (two per litter). The two piglets per litter each had one of the diets: CHO

or Control. For the inoculum, faeces were collected after nine days of exposure to the diet. All the procedures involving animals were conducted in accordance with the Dutch law on experimental animals and had been approved by the Wageningen University Animal Experimental Committee (Dier Experimenten Commissie).

Animals

Twenty-four crossbred piglets from 12 different litters (two from each litter) were used. The piglets received sow's milk only, during the pre-weaning period. They received neither creep feed nor antibiotics prior to the start of the study. During the experimental period of nine days, the piglets had free access to their diet and clean drinking water. Piglets from the same litter were kept together to prevent cross-contamination between piglets and to avoid social stresses. However, the piglets were separated by a wire mesh, so that they could have visual contact with their littermate, but no interference in dietary treatment. Two piglets from the same litter were each assigned to one of the two dietary treatments.

Dietary treatments

Both diets were based on the same ingredients, except that the CHO diet (CHO) contained added fermentable carbohydrate ingredients (unmolassed SBP, WST, lactulose and inulin); while the Control diet (CON) contained none of these. Neither diet contained antibiotics or extra added copper. The diets were composed in such a way that total energy and protein contents were comparable. The detailed composition of the diets is given in Table 1 in Chapter 1.

Cumulative gas production

Substrates

The two substrates (fermentable carbohydrates) used *in vitro* were: sugar beet pulp (SBP) and wheat starch (WST). Both the substrates were obtained from commercial sources, and were from the same batch as that added to the diets. The air-dried substrates were ground to pass a 1-mm sieve.

Inocula

Faeces from twenty-four piglets (8 pigs per gas run) were collected per rectum with a gloved finger. The faeces were immediately placed in small containers filled with CO₂. Then, in the laboratory, faeces from each animal were diluted with sterile anaerobic saline (0.9% NaCl), which had been pre-warmed to 39° C. This diluted sample of faeces from each piglet was then homogenised for one minute to ensure proper mixing, before being filtered through a double piece of clean cheesecloth (16 threads per cm in each direction). The resultant filtrate was used as the inoculum. All procedures were carried out under a constant stream of CO₂.

to maintain anaerobic conditions. It was considered appropriate to use faeces as inoculum since this microbiota has previously shown to be representative of the large intestinal microbiota.^{126,127}

Incubation

Four ml of inoculum was injected into each fermentation bottle, which was immediately attached to the Automated Pressure Evaluation System [(2 substrates + 1 blank) x 3 replicates]. Each bottle contained 0.5 g of substrate and 82 ml of a semi-defined medium¹²⁸. Bottles were incubated at 39°C for 72 hours.

Sampling and Analyses

All substrates were analysed for their dry matter (DM) and ash contents. The pH (pH meter – Hanna Instruments) and dry matter content were determined for both inocula. At the end of the incubation period, the pH of each bottle was recorded and samples of post-fermentative fluid were collected for the measurement of DM, ash, VFA, and ammonia. Samples were also collected for PCR/DGGE analysis to allow an examination of changes in bacterial composition of microbiota at the end of fermentation.

Dry matter was determined by drying to a constant weight at 103 °C³⁴, and ash by combustion at 550 °C³⁵. Volatile fatty acid (VFA) concentration in the post-fermentation samples were analysed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101, as carrier gas N₂ saturated with methanoic acid, at 190°C and using iso-caproic acid as internal standard. Ammonia was determined according to the method described by Searle³⁶.

Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE)

A DNA “fingerprint” of the microbial community of the inoculum and of the community after fermentation of a number of the substrates was made using the PCR/DGGE method described by Konstantinov *et al.*⁴⁸

First, the total genomic DNA of the samples was isolated using the FastDNA® SPIN Kit for Soil (BIO 101, Carlsbad, CA 92008, USA). This kit has previously been validated and used for faecal and colonic microbiota of pigs and humans, and shown good results.⁴⁸

PCR amplification was done as previously described.⁴⁸ Briefly, primers S-D-Bact-0968-a-S-GC (5'-CGC CCG GGC CGC GCC CCG GGC GGC GGC GCA CGG GAA CGC GAA GAA CCT TAC-3') and S-D-Bact-1401-a-A-17 (5'-CGG TGT GTA CAA GAC CC-3') were used to amplify the V6–V8 regions of 16S rRNA gene. PCR was performed using the Taq DNA polymerase kit from Life Technologies (Gaithersburg, MD). PCR mixtures contained approximately 1 ng/μl concentration of the genomic DNA. The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) and the cycling consisted of 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 68°C for 40 s, and 68°C for 7 min

final extension. Aliquots of 5 μ l were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing ethidium bromide to check for product size and quantity.

The amplicons were separated by DGGE using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was performed in an 8% polyacrylamide gel of 37.5:1 acrylamide–bisacrylamide (dimensions 200 \times 200 \times 1 mm) using a 38–48% denaturing gradient for separation of PCR products obtained with primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17. The gels were electrophoresed for 16 h at 85 V in 0.5 \times TAE buffer at a constant temperature of 60°C and subsequently stained with AgNO₃.

Analysis of all DGGE samples was done as described previously.⁴⁸ In short, all gels were scanned at 400 dpi and analysed using the software of Molecular Analyst/PC (version 1.12, Bio-Rad, Hercules, CA). Further, the number of DGGE bands was assessed per lane using the bands searching algorithm within the program. The DGGE fragments constituting more than 1% of the total area of all bands were included in the further statistical analysis.

Calculations

Branched chain proportion (BCP)

As an indicator of the protein fermentation, BCP was calculated as follows;

$$\text{BCP (\%)} = [(\text{isoButyric} + \text{isoValeric}) / \text{Total VFA}] \times 100$$

Gas production kinetics

The data for cumulative gas production (as ml of gas produced per g DM with time) was fitted to the mono-phasic model described by Groot *et al.*¹³⁰ as follows:

$$G = A / (1 + (C / t)^B) \dots \dots \dots (1)$$

Where G = total gas produced; A = asymptotic gas production; B = switching characteristic of the curve; C = time at which half of the asymptote has been reached (T1/2); t = time (h)

The maximum rate of gas production (Rmax) and the time at which it occurs (Tmax), were calculated according to the following equations¹³¹:

$$\text{Rmax} = (A * (C^B) * B * (\text{Tmax}^{-(B-1)}) / (1 + (C^B) * (\text{Tmax}^{(-B)}))^2 \dots \dots \dots (2)$$

$$\text{Tmax} = C * (((B - 1) / (B + 1))^{(1/B)}) \dots \dots \dots (3)$$

Statistics

Differences between the substrates, inocula and the interactions between them, were tested for significance using two-way ANOVA. The statistical model was as follows:

$$Y = \mu + S_i + I_j + (S * I)_{ij} + \epsilon_{ijk} \dots \dots \dots (4)$$

Where Y is the parameter to be tested, μ the mean, S_i effect of the substrate i, I_j effect of the

inoculum j , $(S \times I)_{ij}$ denotes the interaction between substrate and inoculum and ϵ_{ijk} is the error term. The effect of replicate bottles was tested separately, and was not significant for any of the parameters. It was therefore excluded from the statistical model. All statistical analyses were performed using Proc GLM procedure of statistical program SAS.³⁸

Results

pH, DM and Ash

The average values ($n=12$) for DM for the CON and CHO inocula were $214(\pm 9.45)$ g/Kg and $180(\pm 7.35)$ g/Kg respectively, while the mean pH ($n=12$) was $6.02(\pm 0.012)$ for CON and $5.99(\pm 0.015)$ for CHO inocula. The DM and ash contents of the substrates were 911 g/kg and 82.8 g/kg respectively for SBP and 863 g/kg and 2.4 g/kg for WST.

Fermentation end-products

The least square means of fermentation end-products such as VFA and ammonia, as well as pH of the post-fermentative samples are shown in Table 1. There was a significant difference between the substrates for the total VFA production per gm DM incubated. However, there was no effect of inoculum, or of inoculum substrate interaction. The total VFA production was significantly higher for WST, for both inocula, compared with SBP fermentation. ($p<0.001$).

Substrate also had a significant effect ($p<0.001$) on individual VFA production. Except for valeric acid, there was no inoculum effect for individual VFA production, although there was an interaction effect for acetic, propionic and valeric acids. For both inocula, acetic acid production was significantly higher for SBP, while propionic, butyric and valeric acids were higher for WST (Table 1). BCP (calculated as total BCFA production out of total VFA production) was higher for SBP than for WST for both inocula, while inocula or inoculum substrate interaction had no effect on BCP.

Ammonia concentration was significantly ($p<0.001$) higher post-fermentation for SBP compared with WST, for both inocula. There was no effect of inoculum or of inoculum substrate interaction for ammonia concentration. (see Table 1). Similarly, there was a significant difference in pH of the post fermentative samples according to substrate, but not for inoculum, or the interaction. The pH was significantly higher for SBP for both inocula.

Gas production kinetics

The least square mean values of the fermentation kinetic parameters are shown in Table 2. The table shows the effect of inoculum, substrate and the interaction between inoculum and substrate. Figures 1 and 2, show the cumulative gas production profiles comparing the two substrates using CON and CHO inocula.

From Table 2, it can be seen that most of the kinetic parameters were significantly different ($p<0.001$) for substrate, except $T_{1/2}$. However, only T_{max} was significantly different for

Table 1. End product profile after the fermentation of SBP and WST using faeces of the piglets fed with CHO and control diet.

Substrate	Inoculum	TotVFA ¹	Acet ¹	Prop ¹	But ¹	Val ¹	NH ₃ ¹	BCP ¹	pH ¹
SBP	CON	8.78 ^a	5.49 ^a	1.97 ^a	0.79 ^a	0.24 ^a	60.10 ^{a,b}	3.20 ^{a,b}	6.59 ^a
	CHO	9.03 ^a	5.82 ^a	1.83 ^a	0.84 ^a	0.24 ^a	60.58 ^b	3.28 ^b	6.58 ^a
WST	CON	9.74 ^b	4.69 ^b	2.33 ^b	1.66 ^b	0.77 ^b	58.03 ^{a,c}	2.99 ^a	6.48 ^b
	CHO	9.50 ^b	4.45 ^b	2.61 ^c	1.54 ^b	0.60 ^c	55.97 ^c	3.11 ^{a,b}	6.48 ^b
SEM ²		0.128	0.12	0.092	0.047	0.039	0.866	0.091	0.012
Substrate		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.038	<0.001
Main effects	Inoculum	0.985	0.736	0.438	0.440	0.032	0.364	0.288	0.945
	Sub*Ino	0.066	0.024	0.025	0.099	0.032	0.148	0.812	0.567

¹TotVFA = Total volatile fatty acids (mmol per g -1 DM incubated); Acet = Acetic acid; Prop = Propionic acid; But = Butyric acid; Val= Valeric acid; NH₃ = ammonia (mg per g-1 DM incubated); BCP= branched chain proportion (%);

²SEM= Standard error mean;

^{a,b,c} the LSmeans within a column, with different superscripts are significantly different $p < 0.05$.

inoculum. Most of the parameters, except DMCV, were also significant for the interaction of inoculum and substrate. Both T_{1/2} and T_{max}, occurred earlier for CHO with SBP, and earlier for CON with WST. For DMLoss, the values were higher for CHO with SBP, and higher for CON inoculum with WST. These differences indicate that for SBP as an *in vitro* substrate, the CHO inoculum had a higher R_{max} which occurred earlier, compared with the CON inoculum. For WST, this was completely reversed: CON had the higher R_{max} which occurred earlier. (Table 2)

PCR - DGGE Analysis

Changes in the DGGE profile were observed between pre- (inocula) and post- fermentative samples (Figure 3). The substrate played a major role in determining the post-fermentative DGGE profile, as suggested by the greater number of bands present after fermentation of SBP (~13 bands) compared with that of WST (~9 bands), for both inocula.

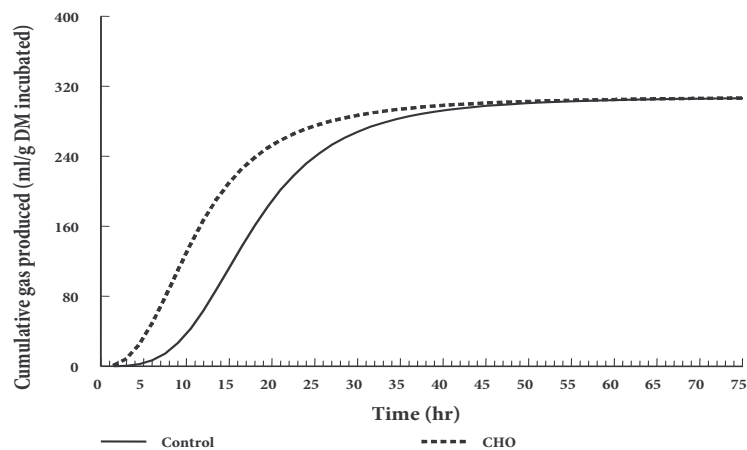


Figure 1. Cumulative gas production profiles for fermentation of SBP, by using faeces of the piglets fed with CHO and control diet as inoculum.

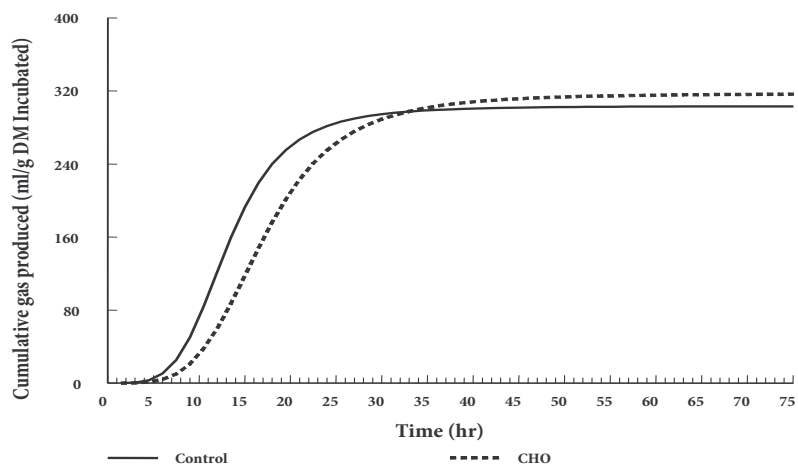


Figure 2. Cumulative gas production profiles for fermentation of WST, by using faeces of the piglets fed with CHO and control diet, as inoculum.

Table 2. Gas production parameters for fermentation of SBP and WST using faeces of the piglets fed with CHO and control diet.

Substrate	Inoculum	DML ¹	DMCV ¹	Thalf ¹	Tmax ¹	Rmax ¹
SBP	CON	88.09 ^a	277.81 ^a	17.52 ^a	13.82 ^a	15.76 ^a
	CHO	86.89 ^b	287.57 ^a	11.28 ^c	7.65 ^c	22.38 ^c
WST	CON	98.09 ^c	316.08 ^b	13.16 ^b	11.56 ^b	27.56 ^b
	CHO	98.53 ^c	321.7 ^b	17.07 ^a	14.64 ^a	22.22 ^c
SEM2		0.342	6.68	0.61	0.48	1.24
Main effects	Substrate	<0.001	<0.001	0.255	<0.001	<0.001
	Inoculum	0.251	0.256	0.062	0.002	0.613
	Sub*Ino	0.015	0.7591	<0.001	<0.001	<0.001

¹DML = Dry matter lost during the fermentation (% of the DM incubated). DMCV = Cumulative gas production (ml/g DM incubated); T1/2 = Half time of asymptotic gas production (h); Tmax = Time occurrence of Rm (h); Rmax = Maximal rate of gas production (ml/h);

²SEM = Standard error mean;

^{a,b,c}the LSmeans within a column, with different superscripts are significantly different $p < 0.05$.

Discussion

The aim of these *in vitro* measurements was to examine the differences in *in vitro* microbial activity of faeces from piglets fed two different diets. In this study, the two diets with SBP and WST (CHO) and without SBP and WST (CON) were fed to newly weaned piglets, and their faeces were then tested *in vitro*, using the SBP and WST as substrates.

It was expected that the CHO inocula (from animals fed diets containing both substrates tested) would ferment both substrates better than the CON inocula, due to the prior exposure of the microbial community to these substrates in the diet. For SBP, this was indeed the case. However, in case of WST, the CON inoculum showed a better fermentation kinetics (faster, with more VFA production) than that with the CHO inoculum.

The *in vitro* fermentation end-products as measured here, strongly suggest that it may be the structure and availability of the substrate, which determines the fermentation end-products, rather than the microbial community present. However, there are several important points to be remembered. Firstly, the inoculum used here, came from piglets of the same age and environment. Bauer *et al.*¹³¹ showed that for a wide range of different substrates, a comparison between unweaned and adult pigs revealed some dramatic differences between some, though not all, substrates tested *in vitro*. Secondly, the post-fermentative samples were collected after 72 hrs fermentation. This was apparently, enough time for both inocula to adapt to

the fermentation of both SBP and WST, which seems to have led to no effect of inocula on the end-product profile. Perhaps if samples for end-product analysis had been taken earlier in the fermentation, differences which matched the fermentation kinetics could have been found.

Of the two substrates used, there was more acetic acid produced by SBP fermentation compared to WST fermentation, while the opposite occurred for propionic and butyric acids. This is likely to be related to the differences in fermentability of two substrates, both in terms of the actual molecular structure of the substrates, but also to those bacterial species which are able to utilize those substrates. In this case, SBP was more slowly fermentable, so the efficiency of ATP formation may be maximised by converting pyruvate to acetyl phosphate instead of lactate, which would then result in more acetate being formed.⁴⁰ On the other hand, in the case of WST, comparatively more butyric acid and propionic acid would be formed. WST is a comparatively fast fermenting carbohydrate, which makes the substrate availability higher and in this case microbes maintain the acetic acid production to lower extent and use lactate as an electron sink⁴⁴. Higher butyric acid production associated with fermentation of starch has been reported in an *in vivo* study with human volunteers⁴¹.

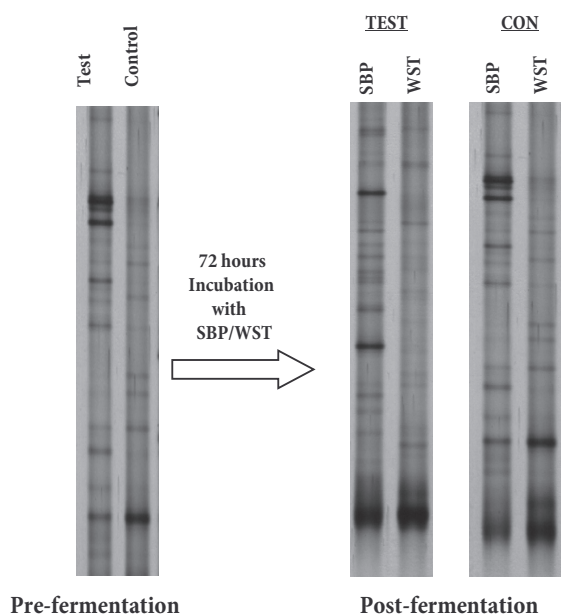


Figure 3. Differences in DGGE profile of the microbial communities before and after the fermentation SBP and WST, by using faeces of the piglets fed with CHO and control diet, as inoculum.

The BCP and ammonia concentration, which are indicators of protein fermentation, were higher with SBP fermentation. This could have been result of SBP being a more complex⁴² and slowly fermentable carbohydrate; and the availability of the fermentable part of carbohydrate substrate is lower, which might lead to utilization of protein present in the medium, as a source of energy by microbiota. Moreover, the *in vivo* effect of the dietary addition of fermentable carbohydrates (sugar beet pulp and fructooligo-saccharides) has been reported recently.²⁴ A significant stimulation of species belonging to the *Clostridium* coccoides group was found in faeces of pigs on the diet containing sugar beet pulp and fructooligosaccharides, compared to pigs on the diet without sugar beet pulp. Many of the intestinal clostridial species are known to be both saccharolytic and strongly proteolytic.

As shown in Table 2, the CON inoculum showed faster T1/2, Tmax and higher Rmax for WST fermentation compared with SBP. In previous work using both ileal contents and faeces from unweaned piglets (Chapter 4), it was shown that WST was surprisingly well fermented by the ileal inoculum, so that it was unlikely in an *in vivo* situation, that this ingredient in the diet would reach the end of colon in significant quantities. The same work showed that SBP was fermented faster by the faecal inoculum. For the piglets in this study, it may be that the WST had been fully utilized by the microbial community in the ileal or caecal part of GIT in the CHO piglets, before reaching the end of the large intestine. It is likely that the microbial community from CHO inocula was better adapted to utilization of SBP, while they were novice for WST similar to the microbial community from CON inocula. However, for the CON animals on the other hand, it appears that the microbes at the end of the colon were better adapted to fermentation of starch, which had been present in the diets in higher amounts in the form of corn starch in the CON diet, rather than WST. It is therefore possible, that even if all the starch had been fermented by the end of the colon for the CON animals, previous activity earlier in the tract still meant that the starch-fermenting species were still the most active.

16S rRNA gene-targeted PCR-DGGE profiling of microbial composition before and after fermentation, gives some indication of microbial diversity within the bacterial community. This will be of specific value when there are likely to be a large number of bacterial species present, which are difficult to cultivate by classical plating techniques¹³⁹, DNA fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) have been used to describe the microbial diversity of gut microbial ecosystems⁹⁹.

In the present study, the DGGE profiles of the pre-fermentation samples showed that the presence of fermentable carbohydrates in the animal diet resulted in higher diversity of faecal microbial community. This was in agreement with *in vivo* work by Konstantinov *et al.*²⁴ where addition of fructo-oligosaccharides and SBP in the diet led to higher bacterial diversity and more rapid stabilisation of the faecal microbial community.

After 72 hrs of fermentation, the post -fermentative DGGE profiles showed some variation between inocula, and substrates. The effect of substrate was more pronounced than that of inocula. The fermentation of SBP showed greater number of bands overall after fermentation,

compared with the profile before fermentation, while the fermentation of WST had very little effect on DGGE profile in terms of the number of bands present. This is in agreement with results of Zhu *et al.*¹⁴² in an *in vitro* study. Where, the faeces of weaned piglets were used as inocula and incubated with SBP as the energy source.

This substrate effect after 72 hours of fermentation strengthens the hypothesis that exposure of microbes to a certain energy source for a sufficient time, can shift the microbial activity of the whole community as it adapts to fermentation of that particular energy source. In this case, 72 hrs was apparently sufficient time to show such an effect in terms of the dominant species present. Analysis of samples during the fermentation process might provide insight in the succession of microbiota composition and activity. The studies examining time effect are currently under way in our laboratories.

Conclusion

From the present study, it was concluded that microbial activity as measured by *in vitro* kinetics, can be influenced by prior exposure of microbiota to specific fermentable carbohydrates present in the diet.

This study also revealed that the fermentability of a certain fermentable carbohydrate ingredient should be taken into account while designing the diet, to have a desired effect along the GIT or in a particular part of GIT.

Acknowledgements

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Chapter 6

Use of *in vitro* Cumulative Gas Production Technique for Pigs: an Examination of Alterations in Fermentation Products and Substrate Losses at Various Time Points

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Abstract

An experiment was conducted to examine changes in volatile fatty acids (VFA) and ammonia concentrations at different time points, using four fermentable carbohydrate-rich feed ingredients as substrates and faeces of unweaned piglets as inoculum. Faecal inoculum was from a mix from nine specially-raised crossbred piglets (no creep feed or antibiotics) at three weeks of age. Inulin, lactulose, unmolassed sugar beet pulp, wheat starch, were used as substrates and fermented *in vitro* for 72 hrs (three replicates per substrates). Cumulative gas production was measured as an indicator of the kinetics of fermentation. In addition, three bottles per substrate per time point with similar contents (amounts of substrates, inoculum and medium) were also incubated at 39°C. These incubation time points were every hour between 1 to 24 hrs and 48 hrs. These bottles were taken out at different time points and fermentation fluid was sampled. Fermentation end-products including VFA, and ammonia, and organic matter loss, were measured. Dry matter and ash was analysed. The pH of contents from these bottles was also recorded. The correlation between fermentation end products and cumulative gas produced in time was studied. It was shown that, the prolongation of fermentation to 72 hr, specially in case of fast fermenting inulin and lactulose may lead to different end product profile compared to profile at the time at which most of the substrate is disappeared. The present study concludes that the fermentation product profile at the end of *in vitro* fermentation at a given specific time point can not be used to compare fermentability of carbohydrate sources with different fermentation kinetics in terms of the gas production.

Introduction

In recent years, the cumulative gas production techniques^{143,144} are gaining interests as a standard measurement of fermentability of the feedstuff or the microbial activity of source inoculum.

The basic principle of all the gas production techniques is that the *in vitro* fermentation of feeds by microorganisms is accompanied by the production of gas. The gas is formed; a) directly by microbial fermentation of the substrate, as well as b) indirectly by release of carbon dioxide due to production of VFA from the bicarbonate buffer which is often used in these techniques¹⁴⁵. Therefore, the amount of gas produced depends both on the amount of substrate fermented and the amount and molar proportions of the VFA produced¹²⁵. Although, it is very difficult to separate the direct and indirect gas production, based on the fact that both are directly related to the fermentation of a substrate, the gas production measured at each time point can be considered as an index for fermentation activity¹³⁰.

In ruminants, gas production technique is mainly used for forage evaluation¹⁴⁶. In monogastrics, however it is used as a tool to study the microbial activity. Few modified systems were proposed for the gas production studies such as, colon simulation technique (cositec) by Breves *et al.*,¹⁴⁷ or adaptation of Hohenheim gas test by Ahrens *et al.*¹⁴⁸ to simulate fermentation in large intestine.

The cumulative gas production technique of Theodorou *et al.*,¹⁴³ which was further automated by Davies *et al.*,¹⁴⁴ has been proposed to use in studies concerning monogastric fermentation by Williams *et al.*²³ and used for the purpose by Bauer *et al.*⁴⁶ In the standard technique used mainly for feed evaluation, the fermentation product profile (usually VFA and ammonia) is analyzed by sampling at the end of a standard fermentation time.

The present study, aims to study i) whether the fermentation product profile at the end of a specific fermentation time is really representative of the product profile present at the time when the substrate has been fully fermented; ii) the *in vitro* fermentation product profile pattern in time and its relation with cumulative gas produced.

Materials and Methods

Experimental design

The study was conducted using the Automated Pressure Evaluation System (APES)¹⁴⁴. Nine three-week old unweaned piglets from three litters were used as faecal donors and the faeces were mixed in order to have both a representative microbial population, and sufficient material as inoculum. All the procedures involving animals were conducted in accordance with the Dutch law on experimental animals and had been approved by the Wageningen University Animal Experimental Committee (Dier Experimenten Commissie).

Animals

The nine crossbred piglets from three different litters (three from each litter) had free access to sow's milk and did not receive any creep feed or antibiotics before the experiment. During the complete experimental period, piglets remained with their sow and littermates on the farm.

Substrates

Four carbohydrate-rich ingredients inulin, lactulose, unmolassed sugar beet pulp (SBP) and wheat starch (WST), were used as substrates. All substrates were obtained from commercial suppliers. The air-dried substrates were ground to pass a 1-mm sieve, before used for the *in vitro* experiment. The substrates were known to have different fermentation kinetics and chosen based on previous studies in our laboratory. (Awati *et al.* submitted)

Inoculum

Faeces were collected per rectum with a gloved finger at the farm. The faeces were immediately placed in small containers filled with CO₂. In the laboratory, equal amounts of faeces from each animal were combined (by wet weight), and diluted 1:15 with sterile anaerobic saline (0.9% NaCl), which had been pre-warmed to 39°C. The large dilution was necessary due to the very small quantity of material obtained. This diluted mixture was then homogenised for 60s to ensure proper mixing, and was then filtered through a double piece of clean cheesecloth (16 threads/ cm each direction). The resultant filtrate was used as the inoculum. All procedures were carried out under a constant stream of CO₂ to maintain anaerobic conditions. This faecal material was thought to have a microbial population which is representative of the large intestinal microflora.^{126,127,132}

Four ml of inoculum was injected into each fermentation bottle and then two different procedures were followed. i) The bottles were immediately attached to APES (three replicates per substrate). Each bottle contained ~0.5 g of substrate and 78 ml of a modified semi-defined medium.¹²⁸ Blanks contained only medium and inoculum. Bottles were incubated at 39°C for 72 hours. ii) In addition to the bottles attached to APES, three bottles per substrate per time interval with similar contents (amounts of substrates, inoculum and medium) were also incubated at 39°C. The fermentation was stopped at different time points at every hour between 1 to 24 hrs and at 48 hrs. The caps of these bottles were pierced since beginning with a needle to let gas go during the incubation. These bottles were taken sampled for VFA and ammonia analysis. pH of contents from these bottles was also recorded. Organic matter disappearance (OMloss) was also determined at each time point.

Analyses

All substrates were analysed for their dry matter (DM)³⁴ and ash³⁵ contents. The pH (pH meter – Hanna Instruments) and dry matter content of the post fermentative samples

were determined. Volatile fatty acid (VFA) concentrations in the fermentation liquids were analysed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101, as carrier gas N₂ saturated with methanoic acid, at 190°C and using iso-caproic acid as internal standard. Ammonia was determined according to the method described by Searle.³⁶

The lactic acid concentration of the digesta was analyzed according to the method described by Voragen *et al.*³⁷ using a Jasco HPLC unit fitted with Supelcogel HPLC column (C-610 H, 30cm x 7.8mm ID).

Gas production kinetics

The bottles attached to APES, were incubated for 72 hrs and gas production kinetics was studied. The data for cumulative gas production OMCV (organic matter cumulative volume, as ml of gas produced per gm organic matter weighed into the bottle) was fitted to the mono-phasic model described by Groot *et al.*¹³⁰ as follows:

$$G = A / (1 + (C/t)^B) \dots \dots \dots (1)$$

Where G = total gas produced (ml); A = asymptotic gas production; B = switching characteristic of the curve; C = time at which half of the asymptote has been reached (T_{1/2}); t = time (h)

The maximum rate of gas production (R_{max}) and the time at which it occurs (T_{max}), were calculated according to the following equations¹³¹:

$$R_{max} = (A * (C^B) * B * (T_{max}^{-(B-1)})) / (1 + (C^B) * (T_{max}^{(-B)})^2 \dots \dots \dots (2)$$

$$T_{max} = C * (((B - 1) / (B + 1))^{(1/B)}) \dots \dots \dots (3)$$

Statistics

Effects of the substrates, incubation time (specifically 24, 48 and 72 hours) on the fermentation kinetics, were tested for significance using two-way ANOVA using Proc GLM procedure of statistical program SAS³⁸.

The statistical model was as follows:

$$Y = \mu + S_i + T_j + (S*T)_{ij} + \epsilon_{ijk} \dots \dots \dots (4)$$

Where Y is the parameter to be tested, μ the mean, S_i effect of the substrate i, T_j effect of the incubation time j, (S*T)_{ij} denotes the interaction and ϵ_{ijk} is the error term. The effect of replicate bottles was tested separately, and was not significant for any of the parameters. It was therefore excluded from the statistical model.

The correlation between time, cumulative gas produced, pH and production of different fermentation end products was analysed by Proc CORR procedure of statistical program SAS³⁸. The existence of a quadratic component in this relationship was tested, but since it

failed to improve the statistical fit over the linear, the later one was preferred.

Results:

Dry Matter and Ash:

The DM and ash contents of the different substrates were inulin (946.1 g/kg and 1.9 g/kg); lactulose (703.5 g/kg and 0.3 g/kg); SBP (902.4 g/kg and 67.9 g/kg); WST (849.8 g/kg and 2.4 g/kg) respectively.

Cumulative gas production kinetics:

The gas production kinetics, during 72 hrs of incubation are shown graphically in fig 1. The mean values for fitted gas production parameters are shown in Table 1. There was no difference for OMCV between the substrates. However, T1/2, Tmax, and Rmax were significantly different ($p < 0.0001$) between the four substrates. Inulin showed the fastest fermentation among the four substrates with highest Rmax, earliest T1/2 and Tmax, while SBP showed the slowest fermentation with lowest Rmax, slowest T1/2 and Tmax. (see Table1).

Table 1. Gas production parameters for fermentation of four carbohydrates using faecal inoculum from suckling piglets.

Variable	Substrate				Main effect Substrate	
	Inulin	Lactulose	Sugar beet pulp	Wheat starch	MSD ²	p-value
T1/2 ¹	5.57	8.11	22.07	17.3	2.46	<0.001
Tmax ¹	4.69	7.13	14.74	14.51	1.78	<0.001
Rmax ¹	62.67	51.12	11.45	19.29	9.47	<0.001
OMCV ¹	370.66	387.07	359.02	348.32	55.38	0.218

¹ T1/2 = Half time of asymptotic gas production (h); Rmax= Maximal rate of gas production (ml/h); Tmax = Time occurrence of Rm (h);

² MSD = Minimum significant difference ($p < 0.05$).

The rate of gas production at different time points is shown graphically in Figure2. The rate of gas production for different substrates diminishes to zero at different time points, such as, for Inulin at 24 hrs, Lactulose at about 26 hrs, Wheat starch at about 60 hrs, and SBP even more than 72 hrs.

Relationship among the production of different fermentation end products in time:

Correlation coefficients of fermentation kinetics parameters and end products with time (for 72 hrs) and among each other are shown in Tables 2a to 2d. The correlations among the end products and cumulative gas produced with incubation time are different for the different

substrates. The cumulative gas produced at different time points had significant positive relation with incubation time ($p < 0.001$). The coefficient of correlation for cumulative gas produced with time is much lower for the lactulose and inulin compared to wheat starch and sugar beet pulp. Amount of organic matter disappeared (OMloss) during the fermentation has significant influence on most of the parameters (except for lactulose). Total VFA concentration was positive related to cumulative gas produced. The correlation coefficient was increasing from inulin 0.77, lactulose 0.78, to WST 0.86 and SBP 0.95. It also has been observed that pH was decreased with incubation time but decrease was not significant for inulin and lactulose.

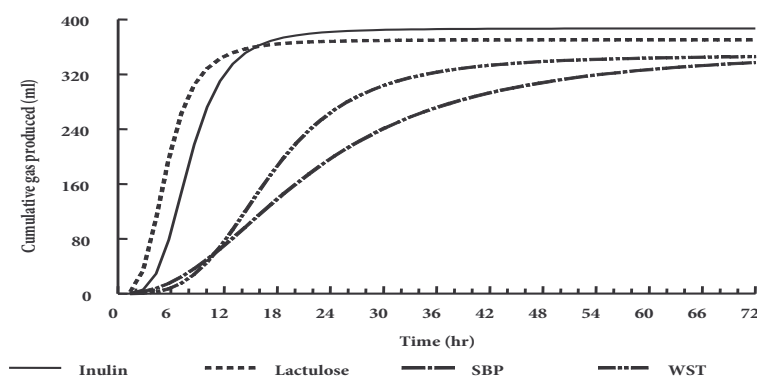


Figure 1. Gas production profiles after 72 hrs for fermentation of four carbohydrates, by faecal inoculum of suckling piglets.

End product profile comparison:

Table 3 shows fermentation end product profiles at three different time points 24hrs, 48hrs and 72 hrs. Mean values of fermentation end product concentrations and OMloss and pH, show the effect of incubation time and substrates and interaction. Substrate had a significant effect on fermentation end products (except on lactic acid), while incubation time also shown a significant differences in end product profile (except lactic acid and propionic acid). However in case of lactic acid, pH and Omloss, substrate-incubation time interaction had a significant effect. Lactic acid had disappeared in case of all substrate by 72hr.

SBP had the highest proportions of branched chain fatty acids, while wheat starch and inulin produced the highest proportions of propionic and butyric respectively. Incubation time and its interaction with substrate plays major role in branched chain proportions.

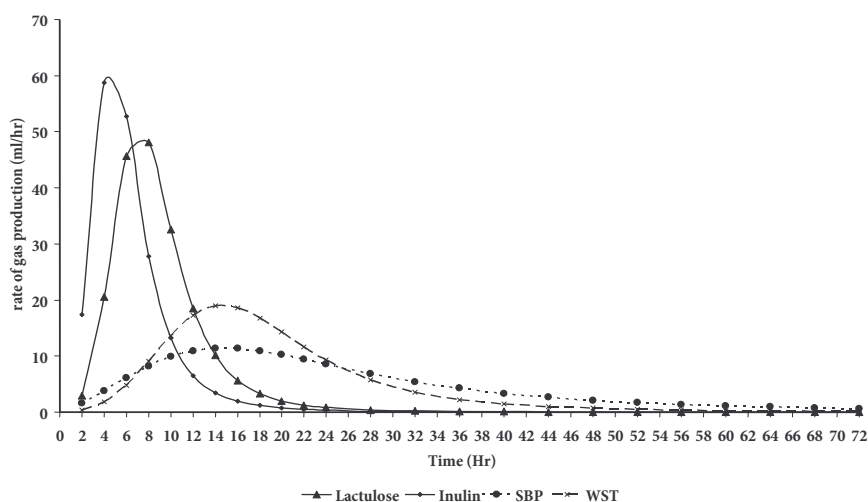


Figure 2. Rate of gas production at different time points during fermentation of four carbohydrates, by faecal inoculum of suckling piglets for 72 hrs.

Discussion:

The four carbohydrate substrates, used for the fermentation were already known to have different fermentation kinetics (Awati *et al.* submitted), which was confirmed in the present study (see Figure 1 and Table 1). Inulin had the fastest fermentation kinetics, and SBP the slowest. These differences were essential for an examination of end-product kinetics at different time points in relation to maximum substrate disappearance.

According to standard procedures the samples are collected for the fermentation end products at the end of the specific time interval to compare the in-vitro fermentability of the different substrates¹⁴⁹⁻¹⁵¹ or different sources of inoculum.^{131,132} This can vary quite considerably, though is usually between 24 and 72 hours. The present study evaluates the hypothesis that the fermentation end product profile will change once maximum substrate loss has occurred. Depending upon when that loss occurs, it may mean that comparing substrates with very different rates of fermentation at one fixed point is not valid.

Table 2 shows the correlation matrices for the four different carbohydrate sources. For the same inoculum, it is observed that the correlation coefficients between the parameters were different for different substrates. Specifically, the correlation between the incubation time and cumulative gas produced was significant for all the substrates but correlation coefficient was much lower for the lactulose (0.60) and inulin (0.52) compared to SBP (0.94) and WST (0.87) (see Table 2). Figure 2 shows that rate of gas production at different time points. In case

of lactulose and inulin, the rate of gas production dropped down to close to zero by 26 and 24 hr respectively. So it would appear that there was no further gas production with prolonged time of incubation. This may help explain the low correlation coefficients observed with lactulose and inulin compared to SBP and WST. However, when similar correlations between incubation time and cumulative gas produced were studied for the data upto 24 hrs for the inulin and lactulose, the coefficient of correlation was dramatically increased to 0.83 and 0.91 for inulin and lactulose respectively.

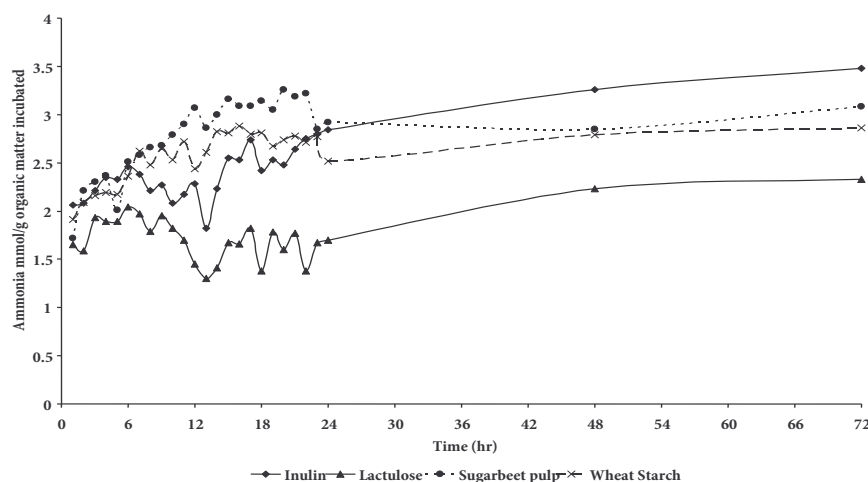


Figure 3. Ammonia production in time during fermentation of four carbohydrates, by faecal inoculum of suckling piglets for 72 hrs.

Similarly, drop in pH with incubation time become significant with coefficient of 0.8 for inulin and lactulose, when calculated for data of first 24 hrs instead of 72 hrs. on the other hand, it was insignificant decrease in pH for inulin and lactulose with incubation time when it was correlated with data up to 72hrs (Table 2). Concentration of VFA is responsible for lowering pH, but with substrate disappearance reaching to zero, production of VFA also lowers down. Furthermore VFA production also contributes to the gas production. 145 It can be clearly seen in table 2 that in case of inulin and lactulose the correlation coefficient between total VFA and cumulative gas produced is lower compared to SBP and WST. But when, it is calculated at 24 hrs, coefficient increase dramatically for inulin (0.89) and lactulose (0.87). This illustrates that for inulin and lactulose, fermentation until 24 hrs gives more realistic conclusions than at 72 hrs.

Furthermore, the time at which 99% of the substrate is disappeared is calculated by using the equation (equation 30) described by France *et al.*¹⁵² This resulted in 26 hr for lactulose, 21 hr for inulin, 65 hr for WST and 160 hr for SBP for the present study. This is very comparable

with time when rate of gas production reaches close to zero (i.e. asymptotic gas production is reached).

Table 2. Correlation matrices of the fermentation end product profiles with the time after fermentation of the four substrates (a) Inulin, (b) Lactulose, (c) Sugar beet pulp and (d) Wheat starch for 72 hr using faecal inoculum from suckling piglets.

(a) Inulin

Incubation time	1						
Cumulative gas	0.52 ***	1					
OMloss	0.92 ***	0.54 ***	1				
pH	-0.09 ns	-0.73 ***	-0.16 ns	1			
Ammonia	0.88 ***	0.45 ***	0.87 ***	-0.04 ns	1		
Lactic acid	-0.41 ***	0.06 ns	-0.56 ***	-0.16 ns	-0.47 ***	1	
Total VFA	0.78 ***	0.77 ***	0.84 ***	-0.47 ***	0.72 ***	-0.42 ***	1
	Incubation time	Cumulative gas	OMloss	pH	Ammonia	Lactic acid	Total VFA

(b) Lactulose

Incubation time	1						
Cumulative gas	0.60 ***	1					
OMloss	0.11 ns	-0.13 ns	1				
pH	-0.02 ns	-0.69 ***	0.15 ns	1			
Ammonia	0.51 ***	-0.08 ns	0.32 *	0.52 ***	1		
Lactic acid	-0.28 *	0.40 **	-0.05 ns	-0.80 ***	-0.54 ***	1	
Total VFA	0.90 ***	0.78 ***	-0.12 ns	-0.23 *	0.27 *	-0.13 ns	1
	Incubation time	Cumulative gas	OMloss	pH	Ammonia	Lactic acid	Total VFA

(c) Sugar beet pulp

Incubation time	1						
Cumulative gas	0.94	1					

OMloss	0.95	0.91	1				
	***	***					
pH	-0.28	-0.45	-0.17	1			
	*	***	ns				
Ammonia	0.44	0.55	0.29	-0.29	1		
	***	***	*	*			
Lactic acid	-0.54	-0.67	-0.42	0.34	-0.88	1	
	***	***	**	**	***		
Total VFA	0.92	0.95	0.95	-0.29	0.47	-0.61	1
	***	***	***	*	***	***	
	Incubation time	Cumulative gas	OMloss	pH	Ammonia	Lactic acid	Total VFA

(d) Wheat starch

Incubation time	1						
Cumulative gas	0.87	1					

OMloss	0.46	0.41	1				
	***	**					
pH	-0.58	-0.84	-0.31	1			
	***	***	*				
Ammonia	0.53	0.62	0.15	-0.50	1		
	***	***	ns	***			
Lactic acid	-0.14	0.18	-0.06	-0.51	0.19	1	
	ns	ns	ns	***	ns		
Total VFA	0.92	0.86	0.48	-0.60	0.38	-0.22	1
	***	***	***	***	**	ns	
	Incubation time	Cumulative gas	OMloss	pH	Ammonia	Lactic acid	Total VFA

¹ ns= non significant; * p<0.05; ** p< 0.01; *** p< 0.001

In Table 3, it is illustrated that difference in OMloss between 24 hrs and 72 hrs fermentation was much lower for Inulin and lactulose, compared to WST and especially SBP.

This suggests that when asymptotic cumulative gas production is reached, most of the

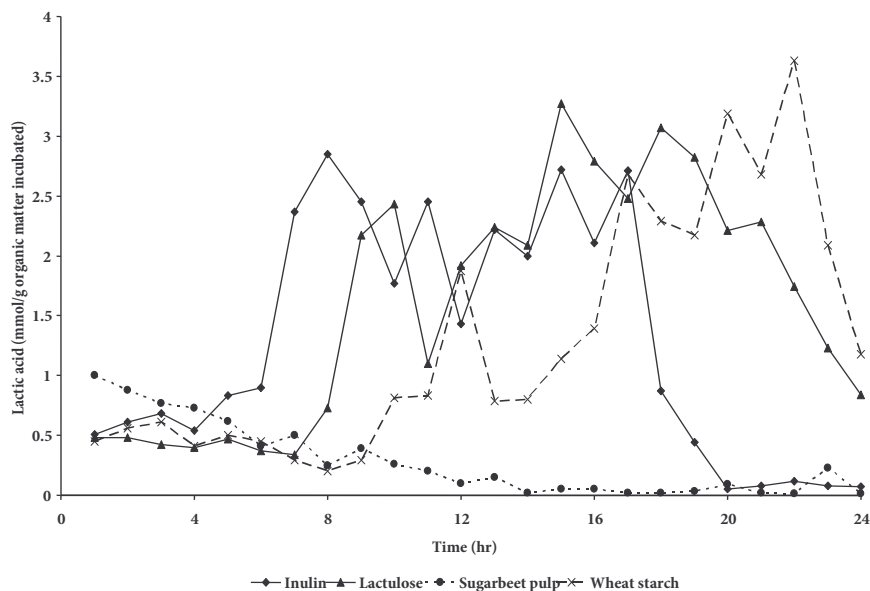


Figure 4. Lactic acid production in time during fermentation of four carbohydrates, by faecal inoculum of suckling piglets for 24 hrs.

substrate has been fermented and the fermentable portion is disappeared. This suggests that the fermentation end product profile at the time when asymptotic cumulative gas production is reached is the most appropriate indication of fermentability of a particular substrate in terms of *in vitro* fermentation end products.

This information in relation to end product profiles mentioned in Table 3, suggests that concentrations of end products at 24 hr are more appropriate indicators of fermentation end product profile of inulin and lactulose, while concentrations of end products at 72 hrs are more appropriate for WST and SBP. In the present study, incubation time has significantly influenced the ammonia, total VFA concentration and branched chain proportion. In particular, ammonia concentration and branched chain proportion, which are typical products of protein fermentation,⁵⁶ increase significantly from 24 hr to 48 and 72 hr, in case of lactulose and inulin. This change in ammonia concentration is also illustrated in Figure 3. Several other studies have shown similar effect of prolonged fermentation.^{153,154} Study reported by Cone *et al.*,¹⁵³ suggested that this rise in ammonia concentration is probable result of microbial turnover.

Table 3. End product profile for fermentation of four carbohydrates using faecal inoculum from suckling piglets at different incubation times.

Substrate	Incubation time ¹	Omloss ¹	pH	Ammo ¹	lactic ¹	Acet ¹	Prop ¹	But ¹	TotVFA ¹	BCP ¹
Inulin	24	91.4	6.27	2.91	0.061	4.299	1.419	0.789	6.678	0.011
	48	95.8	6.50	3.27	0.013	4.629	1.331	0.774	6.954	0.013
	72	97.5	6.48	3.48	0.000	4.746	1.260	0.756	6.995	0.015
Lactulose	24	98.9	6.36	1.85	0.128	3.103	1.104	0.850	5.129	0.005
	48	99.1	6.46	2.23	0.034	3.625	1.349	1.017	6.204	0.010
	72	99.2	6.41	2.33	0.000	4.145	1.495	1.088	6.992	0.009
SBP	24	53.2	6.37	2.93	0.088	3.435	1.067	0.303	5.127	0.039
	48	86.5	6.44	2.85	0.000	4.927	1.416	0.494	7.116	0.023
	72	93.1	6.48	3.08	0.000	4.753	1.354	0.484	6.897	0.024
WST	24	89.1	6.09	2.41	0.003	3.638	2.544	0.413	6.782	0.016
	48	98.6	6.25	2.79	0.000	4.020	2.921	0.681	8.059	0.011
	72	98.3	6.25	2.87	0.000	3.951	2.652	0.733	7.911	0.019
Main Effects										
Substrate	<0.001	<0.001	<0.001	<0.001	0.188	<0.001	<0.001	<0.001	<0.001	<0.001
Incubation Time	<0.001	<0.001	<0.001	<0.001	0.276	0.001	0.115	0.001	0.001	0.003
Substrate*Incubation Time	<0.001	0.032	0.032	0.069	0.039	0.237	0.359	0.1726	0.324	<0.001
MSD ²	2.88-2.29	0.05-0.04	0.16-0.13	0.06-0.05	0.54-0.45	0.29-0.23	0.12-0.10	0.83-0.69	0.003-0.002	

¹ Incubation time= Duration of the fermentation (hr); OMloss= Organic matter loss during the fermentation (%); Tot VFA = Total volatile fatty acids (mmol per g -1 OM incubated); Acet = Acetic acid; Prop = Propionic acid; But = Butyric acid; lactic= lactic acid (mmol per g -1 OM incubated); Ammo = ammonia (mmol per g-1 OM).

² MSD = Minimum significant difference (p <0.05). Where there are two values, the first is referring to the substrate, the second one to the incubation time.

It has been shown that higher availability of amount of fermentable substrates alters the end product profiles of fermentation from *in vitro* pure cultures.¹⁴⁰ This relates to the observations about lactic acid concentrations in the correlation matrices (see Table 2) in the present study. With time lactic acid concentration goes down, may be because of i) the lower availability of fermentable substrate with time in *in vitro* systems shift products of fermentation to acetic acid production; ii) the produced lactic acid can be converted gradually into acetic, propionic, butyric and longer chain fatty acids by some bacterial species like, *propionibacterium spp.*, *clostridium spp.*, etc.⁴⁵ Both of these factors explain why there was no lactic acid found after 72 hrs of fermentation with all the substrates (see Table 3). Furthermore, figure 4 also explains this phenomenon. In case of SBP, which is very slow fermenting carbohydrate, negligible amount of lactic acid is produced (even in first 24 hrs), while in case of other tested carbohydrate sources, lactic acid concentration increased with time until the rate of gas production was increasing and later on with depletion in the amount of substrate and conversion to other VFA, decreased the concentration of lactic acid.

The study has shown that the fermentation product profile at the end of *in vitro* fermentation at a specific time point, does not necessarily give a valid comparison between substrates which vary widely in the kinetics of fermentation.

Ideally, in future experiments, it would be best to group substrates together, with similar rates and end-points of fermentation, so that the procedure could be stopped as closely as possible at the time when all fermentable substrate has been used. However, from a practical point of view, this may be difficult. Usually, when gas production is being measured as a means of feed evaluation (both for ruminants and monogastrics) the kinetics are unknown, and it would be quite laborious to have to repeat the whole exercise grouping substrates into different end-point group. It might be possible however, to double the number of bottles used, and then remove half of the bottles for end-point measurements, at the moment when gas production has reached a point close to zero. More work will be required to solve this dilemma.

Acknowledgement

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General Discussion

Introduction

The aim of this thesis was to study the effects of inclusion of fermentable carbohydrates in weaning piglets' diet, on GIT fermentation and any changes in microbial community composition and activity. In this way, it was expected to examine the possibilities of using such feed ingredients as a replacement for in-feed anti-microbial growth promoters (AMGP). As part of this overall aim, the *in vitro* cumulative gas production technique was used to detect changes in the microbial activity of faeces from animals fed different diets, with and without the inclusion of fermentable carbohydrates. Some recommendations are also made in this regard based on the results of Chapter 6. The benefits of combining microbial community analysis based on fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) with nutritional analysis of fermentation end product profiles, *in vitro* fermentation kinetics are also discussed. The overall conclusions and recommendations for future studies in this regard, are stated.

Post weaning Anorexia

In natural conditions, weaning is not an event but a gradual process. This may take 15-20 weeks for pigs.¹⁵⁵ During this time, piglet gradually becomes independent by a gradual decrease in maternal contact and suckling frequency and increase in exploratory behavior and ingestion of solid feedstuffs. On the other hand, in practice for economical reason piglets are weaned abruptly at age of 3-4 weeks (In EU, it is not allowed to wean pig, younger than 3 weeks). This weaning practice brings a series of simultaneous stressors in piglet's life, in the form of change in the diet, to change in environment by removal from sow, in addition mixing with unknown piglets and transportation to new place.¹⁵⁶ One of the common responses from piglets to post weaning stress is dramatic reduction in feed intake. It might take 7-8 days post-weaning to attend the daily maintenance requirements for metabolizable energy.⁴⁷ This post-weaning anorexia may have detrimental effects on the morphology and function of the gastrointestinal tract.^{60,157} This upset of GIT function is related to so called, "Post-weaning diarrhoea".^{30,158} These profound changes in intestinal morphology and function due to brief period of starvation and then consumption of new solid feed concurrently result in changes to the mass, composition and complexity of the intestinal microflora³⁰. The study by Jensen¹⁵⁹, reported microbial activity in large intestine was not significantly increased until 20 days after weaning, whilst in the small intestine within one week bacterial population was established.

In the present study, experiments in chapter 1 and 2, describe the effect of enforced post weaning fasting by withholding feed from a group of piglets from the moment of weaning. Parameters examined, focused on fermentation end products in the different parts of GIT and faeces for 10 days after weaning. However, this study didn't find any difference in fermentation end products (Chapter1 and 2), or in microbial community analysis (unpublished data) or in cytokine production by intestinal epithelium (Pie *et al.* submitted), between fasted and non-fasted groups for both the diets with or without inclusion of fermentable carbohydrates. However, the present study was in agreement with most of the studies summarized by Brooks and Tsourgiannis¹⁶⁰ for the observed individual variation in feed intake in the beginning of the post-weaning period (~30%). This variation appears to be an individual characteristic of an animal, which suggests that may be in future similar study can be done by offering feed to all the pigs and then depending on their voluntary intake, they can be divided in groups of low, moderate or high feed intake, which probably will give better assessment of post-weaning anorexia and diet interaction.

Effects of fermentable carbohydrates on GIT microbiota

The term prebiotic was first coined by Gibson and Roberfroid²¹ and the formal definition is 'a non digestible food ingredient that beneficially affects the host by, selectively stimulating the growth and/or activity of one or limited number of bacteria in colon that can improve the host health.' So the sole purpose of prebiotic is to provide the necessary substrate for the potentially beneficial microflora. Most of the compounds investigated for this prebiotic properties, were some or the other form of dietary fibres.

Soluble fibres in general are better energy substrates for gastrointestinal micro-organisms than are insoluble fibres¹⁶¹. Different soluble fibre components and their sources are shown in Table 1. Most of these fibre components are very well fermented in GIT, but the rate of fermentation may vary depending on the source of the fibre component, and the host species.

Table 1. Different fibre component which are well fermented by mammalian GIT microflora and dietary source (Based on Tungland and Meyer¹⁶²)

Fibre Component	Source
β -glucans	grains (wheat, barley, oat, rye)
Pectins	fruits, vegetables, legumes, sugar beet, potato
Gums	leguminous seed plants (guar, locust bean), seaweed extracts (carrageenan, alginates), plant extracts (gum acacia, gum karaya, gum tragacanth), microbial gums (xanthan, gellan)
Inulin	chicory, Jerusalem artichoke, onions, wheat
Oligosaccharides/ analogues	various plants and synthetically produced (polydextrose, resistant maltodextrin, fructooligosaccharides, galactooligosaccharides, lactulose)
Animal origin	Chondroitin

In this thesis, chapter 3 dealt with the prebiotic effects of the fermentable carbohydrates used in these experiments. In this chapter, the modulation of the bacterial community in the ileum and colon of weaning piglets after the inclusion of these fermentable carbohydrates namely, lactulose, inulin, wheat starch and sugar beet pulp was studied. Comparative molecular microbiological analysis revealed that the principal microbiological difference between the ileal and colonic digesta was the occurrence of higher number of DGGE bands in the colon compared to ileum. The application of quantitative FISH (fluorescent in-situ hybridization) using a species specific probe revealed that the *Lactobacillus Amylovorus*-like population was the most prevalent in the ileal and colonic lumen samples of the piglets fed for 10 days with fermentable carbohydrates, compared to lumen samples of the piglets fed control diet with negligible amount of fermentable substrates. Inclusion of sugar beet pulp and FOS has previously been shown to develop higher bacterial diversity and rapid stabilisation faecal microbiota post weaning.²⁴ Inclusion of the fermentable carbohydrates decreased the ammonia concentration branched chain proportion in lumen digesta and faeces (Chapter 1 and 2). Moreover this lowering ammonia and branched chain proportion has been observed along the GIT by day 10 (Chapter 2). Inclusion of fermentable carbohydrates was also associated with observed increased Total VFA, especially short chain fatty acids concentrations, compared to the control diet. These effects of inclusion of fermentable carbohydrates were more pronounced on day 10 compared to day 4 (Chapter 1 and 3).

Although, health parameters were not investigated, in present study, none of the piglets showed any signs of diarrhoea or any other illness. This was not unexpected, given that

this was not an infection challenge study, and was conducted in an experimental facility where husbandry may be more fastidious than may be possible on a commercial farm. However, a parallel study conducted on the gut wall tissue samples from the same piglets during the present *in vivo* studies by a member group of EU project “Healthy piglet” (Pie *et al.* submitted), has revealed that inclusion of fermentable carbohydrates have modulated the cytokine responses post-weaning. Especially fermentation end product profiles like branched chain proportion have been observed positively correlated with the pro-inflammatory cytokines in the ileum. Chapter 1 and 2, clearly showed a reduction in protein fermentation along the GIT with inclusion of fermentable carbohydrates in diet. These findings of inclusion of the fermentable carbohydrates in weaning diet leads to growth of beneficial lactobacillus species along with reduction in pro-inflammatory cytokine and toxic products like ammonia, certainly suggests that these ingredients qualify as prebiotics. The use of fermentable carbohydrates as prebiotics in pig nutrition has also been discussed by several other authors.^{23,66,163}

Literature is very contradictory about the use of dietary fibre in diet for the health claims. Recent studies on rats,¹⁶⁴⁻¹⁶⁶ have shown that dietary inclusion of the FOS, inulin or lactulose led to increased translocation of Salmonella, while simultaneous decrease in the resistance to Salmonella. On the other hand studies on poultry,¹⁶⁷⁻¹⁶⁹ have shown inhibition of Salmonella by addition of complex carbohydrates. In pigs, certain studies believe in limiting the fermentable substrates in GIT and thereby controlling the proliferation of pathogenic bacteria^{119,170-172} while there are other studies who claim the positive effects of fermentable carbohydrates against the pathogens.^{173,174}

Verstegen and Williams⁶⁶ pointed out, that in the traditional feed characterization (e.g. proximate analysis), no differentiation is made between different classes of carbohydrates. Cereal grains for example, have at least 80% of the components as carbohydrates, of which 70-90% are composed of starch. Thus 10-30% are non- starch polysaccharides present in the grains. However, not necessarily, all the NSPs from different carbohydrate source are equally fermentable and by all the bacterial species. As reviewed by Williams *et al.*,²³ different NSPs have different chemical compositions and thus different effects on the microbial community. So, one can not generalize the effects of specific dietary complex carbohydrates on the host animal.

Bach Knudsen *et al.*¹⁷⁵ estimated that 40-60% of non-digestible oligosaccharides (NDO) and up to 20% of the other NSPs, are already fermented in small intestine of pigs. Similar excess of small intestinal fermentation was also shown in piglets by Houdijk.²⁷ In our study, (Chapter 1, 2, and 3) we also found the effect on fermentation and on number of bacteria and composition of bacterial community in small intestine. This confirms that there is extensive fermentation activity in small intestine of pigs, unlike human.¹⁷⁶ This means that the term “Prebiotic” might have to be interpreted differently, for pigs versus humans. Therefore, it is proposed to define a broader meaning for the term “Prebiotic”, which might be useful in most of the non human mammalian species;

“Prebiotic is a non digestible dietary ingredient that beneficially affects the host by stimulating the activity, in terms of fermentation end products and stability of the diverse comensal microbiota in different parts of gastrointestinal tract, depending on the fermentability of the dietary ingredient itself.”

Fermentable carbohydrates as potential alternatives to anti-microbial growth promoters (AMGP)

In last few decades, feeding pigs with inclusion of sub-therapeutic levels of antibiotics in diets has been done to achieve higher weight gain and feed efficiency. Though, exact mechanism behind this has not been well understood, effects were more pronounced on the farms with poor hygiene status. There are different opinions about the mechanisms;

- ▷ Inhibition of pathogenic bacteria^{177,178}
- ▷ Prevent irritation of the intestinal lining and may enhance uptake of nutrients from the intestine by thinning of the mucosal layer^{178,179}
- ▷ Some antibiotic agents have shown to increase levels of insulin like growth factor^{180,181}

Despite of all good reasons, the ban on the in-feed antibiotic in pig production systems is inevitable with increasing concern worldwide on antibiotic resistance in human and veterinary medicine¹⁸²⁻¹⁸⁵.

The ban on in feed antibiotics decreased the antibiotic resistance in pig production systems of Denmark and Sweden.¹⁸⁶⁻¹⁸⁸ However, Figure 1 shows that there was a sudden increase in use of therapeutic antibiotics in Denmark, while Swedish farmers managed to keep use of antibiotics to lower extent, by improving the husbandry practices and hygiene on the farm. However, this in turn would have increased the production cost of the pig meat.

This implies, an alternative to AMGP, should fill the gap by reducing the incidence of pathogenic infections and keep the economics of pig production sustainable.

Verstegen and Williams⁶⁶ and Konstantinov *et al.*¹⁸⁹ proposed that, “a stable and complex commensal bacterial community, which is lacking a pathogenic invasion, certainly is a prerequisite of healthy ecosystem.” In this sense, results reported in chapter 3, showed a great deal of increase in complexity by number of bacteria and diversity of composition in ileal as well as colonic microbiota with inclusion of fermentable carbohydrates in diet. This was also observed in other studies which showed an early stabilization of faecal microbiota by *in vivo* inclusion of SBP in the diet. In this study also, there was an increased microbial diversity after *in vitro* fermentation by using SBP as a substrate (chapter 4). This increased diversity of microbiota can help in maintaining intestinal health of the animal by mechanism of “Colonization resistance”.¹

It is clear that, inclusion of fermentable carbohydrates in the diet has shifted the fermentation more towards the carbohydrate fermentation. Generally, higher VFA concentrations lead to lowered pH. The anti-pathogenic effects^{190,191} and nutritional benefits of VFA^{67,192,193} as well as

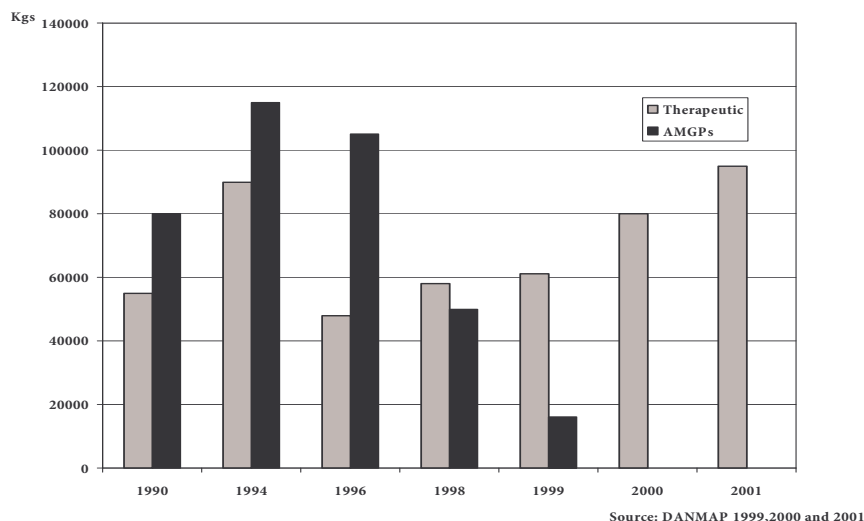


Figure 1. Effects of ban on AMGPs, on the use of antimicrobials for therapeutic purpose. (<http://www.ahi.org/mediacenter/mediakit/euexperiencewithdraw.asp>)

untoward health effects of protein fermentation have been confirmed by several authors^{58,194-197}

The health benefits of fermentable carbohydrates are well accepted, but their negative effects on the growth performance and on health of an animal have also been well documented.^{120,121,198-202} In the present study, growth performance was not assessed critically, as it was not the prime aim of the study. However, some sporadic studies have mentioned the increased performance in pigs fed with Jerusalem artichoke,²⁰³ while work of Rijnen *et al.*²⁰⁴⁻²⁰⁶ suggested that the decrease in the activity of the growing pigs as well as sows fed with fermentable carbohydrates, leading to indirect positive effect on their energy metabolism.

In present study, significant reduction in ammonia concentration has been observed in the lumen along the GIT with inclusion of fermentable carbohydrates (chapter 1, 2). This suggests that, with the presence of carbohydrate substrate the nitrogenous compounds present in the intestinal tract have been used for bacterial growth as a nitrogen source. Although in this study, urinary N concentration was not investigated, but one can assume that there must be shift in excretion of N from urine to the excretion of N in faecal biomass, which has been discussed as a positive environmental effect by Verstegen and Williams.⁶⁶ Similar results reported by a recent Canadian study.²⁰⁷ This group found a shift from urinary N excretion to faecal N excretion without any serious effects on feed efficiency, average daily gain or protein digestibility, after addition of sugar beet pulp in the diet of growing pigs.

Traditionally, performance of the pigs are always measured in terms of body weight gain, feed intake and feed conversion. An alternative approach by looking at overall production

cost of pig meat, might give more insight on future criteria on selection of an alternative to AMGP. Fermentable carbohydrates can be a potential alternative based on following;

- ▷ Reduction in veterinary cost involved (discussed by Houdijk)²⁷,
- ▷ Reduction in environmental cost by reduction in urinary N excretion (which is already a serious issue in western countries)
- ▷ Reduction in public defiance against pig farms by reduction in excretion of odor metabolites²⁰⁸

However more detailed production economics studies are needed in this regard.

Use of in-vitro cumulative gas production technique to study microbial activity

The four different fermentable carbohydrate sources (lactulose, inulin, wheat-starch, sugar beet pulp) used in the present study, were selected from an in-vitro gas production experiment^{125,143,144} conducted in our lab. This preliminary study investigated 45 different fermentable substrates for their *in-vitro* fermentability against faecal microflora. The four substrates were chosen based on their *in-vitro* fermentation kinetics after the fermentation for 72 hrs with faecal microflora of unweaned piglets..

As an adjunct to the *in vivo* experiment, it was decided to examine both the four fermentable ingredients chosen for inclusion in CHO diet, and the two complete diets (control and CHO) for their *in vitro* fermentability, using both ileal digesta and faeces as inocula. (Chapter 4). Williams *et al.*, (submitted- AFST special issue) suggested that one of the advantages of using the cumulative gas production technique in monogastric nutrition was to be able to relate kinetic parameters from fermentation *in vitro*, with transit time *in vivo*. In this way, an estimation could be made as to where in the GIT, the ingredient in question may be fermented. From the kinetic results, it was concluded that inulin and lactulose are more likely to be fermented before the end of the ileum, while SBP is more likely to be fermented in the large intestine. WST appeared to be well fermented by inocula from both sites, suggesting that in animals where digestion of starch is poor, as in newly weaned piglets with low amylase activity, a significant proportion of WST could be fermented in the small intestine. So the combination of these four carbohydrates was supposed to shift in fermentation towards carbohydrate fermentation along the GIT. This supposition was borne out by the results discussed earlier concerning the *in vivo* studies.

In chapter 5, results are described for an *in vitro* experiment carried out using inocula from piglets which had been eating diets with or without the above-mentioned fermentable carbohydrates for nine days. In this case, the faecal microflora was examined by incubating faeces *in vitro*, from both dietary groups using SBP and WST as the substrates. The piglets consuming the CHO diet showed a better adaptation to SBP compared with the piglets consuming the Control diet. For the WST, the opposite was true. While this was unexpected at the time, the fact that the earlier study had shown that the WST was readily fermented by the ileal microflora (Chapter 4) would suggest that if all the WST had been fermented before reaching the distal colon that the microflora was less well adapted to WST than those bacteria located more proximally.

In terms of ingredient characterization, the results of initial *in vitro* studies allowed a selection of ingredients to be made, which further helped in designing diets which would enhance carbohydrate fermentation in the GIT. Used for an assessment of differences between microbial populations, the technique also supported the hypothesis that diet would have an effect on microbial activity occurring in the GIT. Therefore, information derived from both of these uses of the technique can provide useful information for the relation between diet and the GIT microflora, from a functional point of view. The use of *in-vitro* cumulative gas production technique for microbial activity studies in monogastrics was proposed by Williams *et al.*²³ and studied extensively by Bauer *et al.*^{46,131,132,136} for pigs and Guo *et al.*¹⁵¹ for poultry.

However, this technique does have some shortcomings. Normally, the fermentation process is studied based on the fermentation end-product profiles. The study reported in chapter 6, emphasizes that the fermentation product profile at the end of *in vitro* fermentation at a given specific time point may actually deviate from that at 72hrs. This would suggest that the fermentation process should be terminated at another time, depending upon the rate of fermentation of an individual ingredient. Some aspects however can be similar when measured after 72 hrs or at the time point when asymptotic gas production is reached viz. cumulative gas produced, total VFA (Not molar proportions of VFA), organic matter loss during the fermentation. Further investigations and design improvements will be needed, if the technique is to be used more widely in the future.

Use of fingerprinting techniques for estimation of diversity of microbial community

In this thesis, a combination of PCR and fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), has been used to study the diversity of the microbial community (Chapter 3, 4, and 5). This methodological approach which examines molecular ecology of complex community, combined with nutritional parameters and fermentation end product profiles (*in-vivo*) certainly help to have more thorough insight in the dietary effect on the microbial community. The use and the principle of the technique for estimation of diversity of microbial community has been explained in detail by Konstantinov *et al.*¹¹¹ In the past most of the studies concern to microbial communities in pig intestinal tract were limited to easily cultivable commensal bacteria.^{67,68} However, many strict anaerobes in GIT are difficult to cultivate so remain undetectable using conventional technique^{69,70} though they might have important role in maintaining the diversity and stability of the GIT microbiota. Extra information on the changes in the microbial community due to change in dietary regime, might help to make the proper decisions on the dietary recommendations. Such cooperation between nutritionists and microbiologists will be critical in the future, to unravel the complexities between diet, the GIT bacteria, and health of the host animal.

General Conclusions

- ▷ This thesis leads to few specific conclusions which are listed below;
- ▷ Combination of fermentable carbohydrates with different fermentabilities was shown to exert a prebiotic effect along the gastrointestinal tract leading to a more diverse and stable microbial community.
- ▷ Combination of fast and slow fermentable carbohydrates in piglet nutrition can be an effective alternative for AMGP in “post in feed antibiotic era”.
- ▷ Fermentation kinetics obtained from *in vitro* cumulative gas production technique can be used as an indicator for possible fate of the fermentable substrate in the gastro-intestinal tract of the animal.

Recommendations for future studies

In retrospect with the discussed points in this thesis, following topics can be of interest for future research;

- ▷ More detailed studies on use of in-vitro gas production technique to study microbial activity of complex microbial communities.
- ▷ More studies combining expertise on immuno-histology, microbiology, and nutrition on same animal will help in defining a criteria for “healthy feed” for animals.
- ▷ A single correct definition and proper terminology for characterizing dietary fibre is necessary. This is particularly the case, in relation to the contribution (or not) of starch. Also, it must be recognized that not all monogastrics are the same, so definitions may have to vary according to the host animal /human in question.
- ▷ Development of microbiota during the suckling period and possible role of milk oligosaccharides in porcine milk

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Appendixes

Summary (English)

Samenvatting (Summary in Dutch)

Acknowledgement

Training and supervision plan

List of Publications

About the Author

Summary

In reference with forthcoming EU ban on use of antimicrobials as growth promoters (AMGP) has put forward a challenge in front of the nutritional research to find out an alternative, which will check expected increased disease risk while keeping the economic viability of the pig production system. There are several alternatives are being studied by researchers. As a part of a mega EU project “Healthy Piglet”, we are concentrating on the improvement of the fermentation within GIT, to indirectly reduce the risk of diarrhea in piglets. Especially weaning piglets, who are due to myriad of stressors during weaning process predisposed to post weaning diarrhea. In this regard understanding fermentation process, changes in the microbial communities during this period and how fermentable sources in the diet can skew the fermentation end product profile and composition of microbiota, are inevitable.

The generalized theory behind the carbohydrate to protein fermentation in the GIT is that in presence of fermentable carbohydrate substrate, microbes prefer to ferment carbohydrate source to derive energy and use the nitrogen available for their own growth. With this background information, it was hypothesized that inclusion of fermentable carbohydrates in the piglet diet will reduce the protein fermentation, which will be confirmed by reduced levels of ammonia and branched chain fatty acids in end product profile of the fermentation. Ammonia and branched chain fatty acids viz. iso-butyric and iso valeric acids are typical products of protein fermentation. Protein fermentation is considered detrimental to the gut health due to production of toxic substances as ammonia, amines, skatol, indole etc.

The aim of this thesis was to study the effects of inclusion of fermentable carbohydrates in weaning piglets’ diet, on GIT fermentation and any changes in microbial community composition and activity. In this way, it was expected to examine the possibilities of using such feed ingredients as a replacement for in-feed anti-microbial growth promoters (AMGP). As part of this overall aim, the *in vitro* cumulative gas production technique was used to study the fermentation of selected fermentable substrates, *in vitro* (Chapter 4, 5, 6). While these substrates namely lactulose, inulin, wheat starch and sugar beet pulp were included in test diet and their effect on GIT fermentation was studied *in vivo* (Chapter 1, 2, 3). The combination of microbial community analysis based on fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) with nutritional analysis of fermentation end product profiles, was used *in vivo* and *in vitro* studies (Chapter 3, 4, 5).

In this thesis, in *in vivo* trials, emphasis was given on using combination of slow fermenting carbohydrate sources such as, SBP and Wheat starch with fast fermenting lactulose and inulin. The hypothesis behind this approach was to induce carbohydrate fermentation along the GIT, by providing carbohydrate substrate for the microbiota in different parts of GIT. Especially by taking in to account the difference in the transit time of feed in the different parts of GIT, it was expected that fast fermenting lactulose and inulin would be fermented in small intestine while wheat starch somewhere in the beginning of the large intestine while, SBP will reach the distal part of colon. This hypothesis was formulated based on the *in vitro* rates of fermentation of these substrates. In Chapter 1 and 2, it was found that fermentation

along the GIT was improved or in other words skewed more towards the carbohydrate fermentation. It was observed *in vivo* that inclusion of fermentable carbohydrates in the diet reduces the protein fermentation in the GIT (Chapter 1 and 2) and ammonia concentration in end product profile was decreased by inclusion of fermentable carbohydrates. This decrease was observed along the GIT (Chapter 1) and in time in faecal fermentation end product profiles post weaning (Chapter 2).

Weaning process in an intensified pig production system brings many sudden changes in the environmental and physical factors in piglets' life. These sudden changes, especially in diet cause serious imbalance in the microbial community. Quicker stabilization and diversification of microbial community post weaning, is crucial in attending the gut health and reducing the risk of pathogenic infections by 'Colonization resistance'. In Chapter 3, microbial community analysis was done qualitatively by using fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and quantitatively by using florescent in situ hybridization (FISH) technique. It has been shown that inclusion of fermentable carbohydrates stabilized and diversified microbial community in the ileum as well as in the colon by day 10 post weaning. This way, the prebiotic effects of fermentable carbohydrates was evidenced.

The remarkable amount of *in vitro* fermentation was observed with inocula from the ileum along with the large intestine of piglets (Chapter 4). This led to a conclusion that the term prebiotic used for the human is not necessarily completely applicable in case of other monogastrics like pigs, where extensive fermentation was observed also in small intestine. So based on the observations of this thesis a broader definition of prebiotics for non-human mammals, was proposed:

"Prebiotic is a non digestible dietary ingredient that beneficially affects the host by stimulating the activity, in terms of fermentation end products and stability of the diverse comensal microbiota in different parts of gastro-intestinal tract, depending on the fermentability of the dietary ingredient itself."

Potential use of *in vitro* gas production technique (IVGPT), to study microbial activity and fermentation process has also been discussed in this thesis (Chapter 4, 5, 6). Chapter 5 emphasize that the substrate adaptation can be studied with IVGPT. The prior exposure of microbes to a certain substrate increases the ability of that microbiota to ferment that particular substrate and it can be studied *in vitro*. In this experiment, it has been clearly shown that different substrates with different fermentation rates are available in different parts of GIT. It has been observed that even after having wheat starch and SBP both in the diet of the piglet. Faecal microbiota showed better fermentability with SBP compared to wheat starch, although wheat starch is faster fermenting carbohydrate compared to SBP. This information can be very valuable understanding the fermentation fate of a particular substrate in different parts of GIT and thus understanding how diet composition can modulate the fermentation in different parts of GIT to have a desired effect.

Chapter 6, especially deals with the possibility of use of IVGPT to study fermentation *in vitro*

and what are the precautions one should take before concluding fermentation characteristics of the substrates with different fermentation rates, based on the end product profile at given specific time point. It was suggested that the end product profile at the time when gas production reaches to asymptote would be an ideal time point to collect the sample for end product profile and this time point can be different for different substrates depending on the fermentation rate.

Thus this thesis leads to few specific conclusions that;

- ▷ Combination of fermentable carbohydrates with different fermentabilities was shown to exert a prebiotic effect along the gastrointestinal tract leading to a more diverse and stable microbial community.
- ▷ Combination of fast and slow fermentable carbohydrates in piglet nutrition can be an effective alternative for AMGP in “post in feed antibiotic era”.
- ▷ Fermentation kinetics obtained from in vitro cumulative gas production technique can be used as an indicator for possible fate of the fermentable substrate in the gastro-intestinal tract of the animal.

In retrospect with the discussed points in this thesis, following topics can be of interest for future research;

More detailed studies on use of in-vitro gas production technique to study microbial activity of complex microbial communities.

- ▷ More studies combining expertise on immuno-histology, microbiology, and nutrition on same animal will help in defining a criteria for “healthy feed” for animals.
- ▷ A single correct definition and proper terminology for characterizing dietary fibre is necessary. This is particularly the case, in relation to the contribution (or not) of starch. Also, it must be recognized that not all monogastrics are the same, so definitions may have to vary according to the host animal /human in question.
- ▷ Development of microbiota during the suckling period and possible role of milk oligosaccharides in porcine milk

Samenvatting

Het aanstaande Europese verbod op antimicrobiële groeibevorderaars (AMGB) leidt zonder een andere voederstrategie tot minder groei. Dit vormt een uitdaging voor voedingsonderzoekers om alternatieven te vinden die de verwachte groeidepressie kunnen beperken en de economische levensvatbaarheid van de varkenshouderij kunnen handhaven. Diverse mogelijke alternatieven voor antibiotica worden onderzocht. Binnen het grote EU-project “Healthy Pigut” richten wij ons op verbetering van fermentatieprocessen in het maagdarmkanaal om het risico op het ontstaan van diarree bij biggen te verminderen. Vooral pas gespeende biggen worden blootgesteld aan diverse stressoren die kunnen leiden tot speendiarree. In dit perspectief is het essentieel om het fermentatieproces en de verandering van microbiële samenstelling tijdens spenen te begrijpen. Fermenteerbare voedercomponenten kunnen de microbiële samenstelling beïnvloeden en het profiel van fermentatie-eindproducten bepalen.

Fermenteerbare koolhydraten worden in het maagdarmkanaal gefermenteerd voor het verkrijgen van energie, terwijl het beschikbare eiwit en eiwiachtige bestanddelen worden gebruikt voor microbiële groei. Indien de voorziening van koolhydraten als energiebron voor de microflora echter onvoldoende is, kan ook eiwit gefermenteerd worden. Dit heeft als gevolg de productie van ammoniak en vertakt ketenige vetzuren (bijvoorbeeld iso-boterzuur en iso-valeriaan zuur) als gevolg. Eiwitfermentatie is nadelig voor de darmgezondheid vanwege het ontstaan van toxische stoffen, zoals ammoniak, amines, skatol en indol. Er werd verondersteld, dat gebruik van fermenteerbare koolhydraten in de voeding van pas gespeende biggen eiwitfermentatie vermindert. Dit zou kunnen bijdragen tot een goede darmgezondheid in biggen.

Het doel van dit promotieonderzoek was het bestuderen van de effecten van fermenteerbare koolhydraten op fermentatie in het maagdarmkanaal en de microbiële samenstelling en activiteit bij gespeende biggen. Wellicht kan door betere kennis van deze fermentatie het gebruik van degene met een optimale fermentatie als voedercomponent met werking als mogelijk alternatief voor AMGB worden onderzocht.

Voor het bestuderen van deze doelstelling werd gebruik gemaakt van de *in vitro* cumulatieve gasproductietechniek om de fermentatiekarakteristieken van geselecteerde substraten te meten (Hoofdstuk 4, 5, 6). Deze substraten (lactulose, inuline, tarwezetmeel en suikerbietenpulp) werden als voedercomponenten ook gebuikt in een proef met pas gespeende biggen om het effect op fermentatie in het maagdarmkanaal *in vivo* te testen (Hoofdstuk 1, 2, 3). Analyse van fermentatiekarakteristieken werd voor de *in vivo* and *in vitro* studies gecombineerd met microbiologische analyses gebaseerd op ‘fingerprinting techniques’ zoals denaturing gradient gel electrophoresis (DGGE) (Hoofdstuk 3, 4, 5).

In de *in vivo* proeven die in dit proefschrift worden beschreven ligt de nadruk op het gebruik van een combinatie van langzaam en snel fermenteerbare koolhydraatbronnen, zoals ‘langzame’ suikerbietenpulp en maiszetmeel en ‘snelle’ lactulose and inuline. De doelstelling van deze benadering was het creëren van fermentatie van koolhydraten over het

hele maagdarmkanaal vanaf eind van de dunne darm door het verstrekken van substraten voor de diverse delen van de darm. Door ook de passagesnelheid in beschouwing te nemen werd verwacht dat lactulose en inuline in de dunne darm, maiszetmeel in het proximale deel van de dikke darm, en suikerbietenpulp in het distale deel van de dikke darm zou worden gefermenteerd. Deze hypothese was gebaseerd op de *in vitro* fermentatiesnelheid van deze substraten. In Hoofdstuk 1 en 2 werd gevonden dat fermenteerbare koolhydraten in de voeding van gespeende biggen leidde tot een verschuiving van eiwitfermentatie naar koolhydraatfermentatie. Dit werd gekenmerkt door een lagere ammoniakconcentratie bij verstrekking van fermenteerbare koolhydraten in verschillende delen van het maagdarmkanaal (Hoofdstuk 1). De ammoniakconcentratie nam geleidelijk af in het eindproductenprofiel van fecale fermentatie naarmate de biggen langer gespeend waren (Hoofdstuk 2).

Spenen is een ingrijpend proces waarbij vele plotselinge veranderingen optreden in de omgeving van het varken, maar ook in de fysieke gesteldheid van het dier. Deze plotselinge veranderingen leiden tot een ernstige verstoring van de balans in de microbiële samenstelling in het maagdarmkanaal. Een snelle stabilisatie en diversificatie van de microbiële populatie na spenen is cruciaal voor een goede darmgezondheid en het beperken van het risico op pathogene infecties door kolonisatieresistentie. In Hoofdstuk 3 werd de microbiële samenstelling kwalitatief geanalyseerd door gebruik van 'fingerprinting techniques' zoals denaturing gradient gel electrophoresis (DGGE) en kwantitatief door fluorescent in situ hybridisatie (FISH). Aangetoond werd dat gebruik van fermenteerbare koolhydraten in de voeding van gespeende biggen zorgde voor een stabilisatie en diversificatie van de microbiële populatie in de dunne en dikke darm op de tiende dag na spenen. Dit vormde het bewijs van de prebiotische (voeding van gunstige microflora) werking van fermenteerbare koolhydraten in biggenvoeding.

Opmerkelijk was de omvangrijke fermentatie *in vitro* met inocula uit het ileum; deze was vergelijkbaar met de fermentatie in de dikke darm van dezelfde biggen (Hoofdstuk 4). Dit leidde tot de conclusie dat een humaan prebioticum niet noodzakelijkerwijs toepasbaar is voor andere eenmagigen (zoals varkens), waarbij ook substantiële fermentatie in de dunne darm plaatsvindt. Gebaseerd op de waarnemingen in dit proefschrift wordt een bredere definitie van prebiotica voor niet-humane zoogdieren voorgesteld:

"Een prebioticum is een onverteerbaar ingrediënt dat zorgt voor een positief effect op de darmgezondheid van de gastheer door stimulatie van de activiteit (door fermentatie-eindproducten) en stabiliteit van de verschillende commensale microflora in de diverse delen van het maagdarmkanaal, afhankelijk van de fermenteerbaarheid van het voedingsingrediënt"

Gebruik van de *in vitro* gasproductietechniek (IVGPT) om microbiële activiteit en fermentatieprocessen te bestuderen wordt bediscussieerd in dit proefschrift (Hoofdstuk 4, 5, 6). Hoofdstuk 5 benadrukt dat de adaptatie van microben aan diverse substraten kan worden bestudeerd met IVGPT. Een eerdere blootstelling van microben aan een bepaald

substraat verhoogde de capaciteit van de microflora om dit substraat *in vitro* te fermenteren. In deze proef werd aangetoond dat verschillende substraten met specifieke fermentatiekarakteristieken in bepaalde delen van het maagdarmkanaal beschikbaar zijn voor fermentatie. Fecale microflora van gespeende biggen, die tarwezetmeel en suikerbietenpulp in de voeding hadden, fermenteerden tarwezetmeel beter dan suikerbietenpulp, ondanks het snellere fermentatiepatroon van tarwezetmeel. Deze informatie kan erg waardevol zijn om de gevoeligheid voor fermentatie van een bepaald substraat in een specifiek deel van het maagdarmkanaal te kunnen begrijpen. Dit is noodzakelijk om te voorspellen hoe de ingrediëntensamenstelling van de voeding fermentatieprocessen in verschillende delen van het maagdarmkanaal kan beïnvloeden.

Hoofdstuk 6 beschrijft de mogelijkheden om fermentatie *in vitro* te bestuderen met de IVGPT. De keuze van het optimale tijdstip van bemonstering voor het fermentatie-eindproductenprofiel is essentieel en is verschillend voor afzonderlijke substraten. Voorgesteld werd dat het tijdstip waarop de gasproductie de asymptoot nadert het ideale moment is om een monster voor het eindproductenprofiel van fermentatie te nemen. Dit tijdstip kan verschillen voor verschillende substraten en is afhankelijk van de fermentatiesnelheid.

Dit proefschrift leidt tot de volgende conclusies:

- ▷ Een combinatie van fermenteerbare koolhydraten met verschillende fermentatiesnelheden resulteerde in een prebiotisch effect over het hele maagdarmkanaal en leidde tot een meer gevarieerde en stabielere microbiële populatie.
- ▷ Een combinatie van snel en langzaam fermenteerbare koolhydraten in biggenvoeding kan een effectief alternatief zijn voor AMGB in het “post-antibiotica tijdperk”.
- ▷ Een fermentatiekarakteristiek gemeten met de *in vitro* cumulatieve gasproductietechniek kan als indicator dienen voor het lot van het fermenteerbare substraat in het maagdarmkanaal

Naar aanleiding van het beschreven onderzoek in dit proefschrift kunnen enkele onderwerpen worden aanbevolen voor toekomstig onderzoek:

- ▷ Meer gedetailleerde studies naar het gebruik van de *in vitro* gasproductietechniek voor het bestuderen van microbiële activiteit van complexe microbiële populaties.
- ▷ Meer interdisciplinaire studies (immunohistologie, microbiologie en voeding) aan hetzelfde dier kan tot een betere definitie van criteria leiden voor gezonde diervoeding
- ▷ Een unieke, correcte definitie en passende terminologie is vereist voor het karakteriseren van voedingsvezels. Dit is ook het geval voor de bijdrage van zetmeel aan fermentatieprocessen. De karakterisering van voedingsvezels kan er toe leiden dat deze definities kunnen verschillen tussen monogastrische diersoorten (ook de mens).
- ▷ Ontwikkeling van microflora tijdens de zoogperiode en de mogelijke rol van oligosacchariden in moedermelk.

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Ajay..

Training and Supervision Plan		Graduate School WIAS	
Name	:Ajay Awati		
Group	:Animal Nutrition		
Period	:23-04-2001 untill 23-04-2005		
Supervisor	: Prof. Dr. Ir. Martin Verstegen		
Daily advisor	: Dr. B.A. Williams, Dr. M.W. Bosch		
The Basic Package		year	cp
WIAS Common Course (mandatory)		2001	2.0
WIAS Course Philosophy of Science and Ethics (mandatory)		2003	1.0
Course on Laboratory Animal Science		2002	3.0
SUBTOTAL			6.0
International Exposure		year	cp
International Conferences			
Beyond Antimicrobials-future of gut microbiology, 2002, Aberdeen, Scotland, UK		2002	0.8
Manipulating Pig Production IX, APSA, 2003, Fremantle, Western Australia		2003	0.8
"In vitro techniques" Second Healthy Pigut workshop, 2004, Paris, France		2004	0.4
Concerns and responses to food safety, health and environmental issues, Cl. Ferrand, France		2004	0.8
International debate conference for the feed and food chain, N. aan zee, The Netherlands		2005	0.4
WIAS Seminars Plus			
Stable Isotopes in studies nutrient dynamics related to Stress and Metabolic Adaptation.		2001	0.3
Other Seminars			
WIAS science day 2002, 2003, 2004, 2005		2002-5	0.8
PhD retreat 2004		2004	0.4
Presentations			
Beyond Antimicrobials-future of gut microbiology, 2002, Aberdeen, Scotland, UK (2 posters)		2002	1.0
Manipulating Pig Production IX, APSA, 2003, Fremantle, Western Australia (Oral)		2003	0.5
"In vitro techniques" Second Healthy Pigut workshop, 2004, Paris, France (Oral)		2004	0.5
Concerns and responses to food safety, health and environmental issues, France (Poster)		2004	0.5
WIAS science day 2005, (Oral)		2005	0.5
Nederlandse Dieren dag (2 Oral)		2002-3	1.0
SUBTOTAL			8.7
In-Depth Studies		year	cp
WIAS course 'Molecular Genetic Applications in Animal Sciences'		2001	1.0
WIAS course 'Stable Isotopes in Studies of Nutrient Dynamics'		2001	0.6
PHLO course 'New developments in feed evaluation'		2001	0.8
Larenstein course 'Safe handling with radioactive materials and sources (article 5B)		2002	1.0
VLAG/WIAS 'Advanced Course on Ecophysiology of the Gastro-Intestinal Tract'		2003	1.0
VLAG/WIAS 'Advanced course on Nutrition and Sports'		2004	1.0
SUBTOTAL			5.4
Professional Skills (support courses)		year	cp
WIAS Course 'Techniques for Writing and Presenting a Scientific Paper.'		2002	0.8
WGS course 'Career orientation and time planning'		2003	1.0
WGS course 'Time planning and Project management'		2004	1.0
SUBTOTAL			2.8
TOTAL			22.9
One credit point (cp) equals a study load of approximately 40 hours			

List of Publications

Peer- Reviewed Papers

Awati, A., Bosch M. W., Tagliapietra F., Williams B. A. and Verstegen M. W. A. (2005). Difference in *in vitro* fermentability of four carbohydrates and two diets, using ileal and faecal inocula from unweaned piglets. Journal of the Science of Food and Agriculture, accepted.

Awati A., Konstantinov S.R., Williams B.A., Akkermans A.D.L., Bosch M.W., Smidt H., and Verstegen M.W.A. (2005) Effect of substrate adaptation on the microbial fermentation and microbial composition of faecal microbiota of weaning piglets studied *in vitro*. Journal of the Science of Food and Agriculture, in press.

Awati, A., B. A. Williams, M. W. Bosch, W. J. J. Gerrits and M. W. A. Verstegen (2005). Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in faeces of weaning piglets. Journal of Animal Science, submitted.

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Smidt H., Konstantinov S.R., Awati A., Zhu W.Y., Williams B.A., Tamminga S., De Vos W.M., Akkermans A.D.L. (2004) In: Second Workshop, Healthypigut project, Paris, France, 5-6 February 2004.

Williams B.A., Bosch M.W.A., Awati A., Konstantinov S.R., Akkermans A.D.L., Verstegen M.W.A. (2004) *In vitro* assessment of GIT fermentation: fermentable substrates and microbial activity. In: Second Workshop, Healthypigut project, Paris, France, 5-6 February 2004.

Awati A., Tagliapietra F., Williams B.A., Bosch M.W., and Verstegen M.W.A. (2003)

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Tagliapietra F., Williams B.A., Awati A., Bonsembiante M., Schiavon S. and Verstegen

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About the Author

Ajay Annasaheb Awati,

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After working for a year as an independent veterinarian in India, in 1999 he continued his higher studies in Wageningen University and Research Centre, for Masters in Animal Science. In 2001, he accomplished his masters degree with cum laude (distinction), having major thesis in nutritional immunology. Since 2001 till date he has been employed in an EU project, which led to accomplishment of Ph.D. in 2005.

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