In vitro fermentation capacity of hindgut microbiota in pigs in relation to dietary fibre

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Thesis

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Those three grim ogres of distress,
Hunger cold and shabby dress,
Which most men fear, he smiled upon
And never wished them to be gone,
Saying: "From all that comfort brings
But little inspiration springs."

Taken from "The Artist" by Edgar A. Guest, 1931

Dedicated to all pigs.

Abstract

As pigs compete with humans for high quality feed ingredients such as grains, alternative feed sources need to be found. These alternative feed ingredients are usually high in indigestible carbohydrates, consisting mainly of non-starch polysaccharides (NSP) and are less suitable for human consumption. In order to find the most suitable feed ingredients for pig nutrition, they need to be well characterized regarding their nutritional value. This is possible with *in vitro* methods which mimic the hindgut degradation of these mainly indigestible feed ingredients. These *in vitro* methods either use enzymes or faecal inocula to incubate the feed ingredient to be tested. However, there is a lack of information on some general aspects of *in vitro* methods with pigs, such as the number of animals to use for inocula preparation, influence of enzymatic pre-digestion of feed ingredients, repeatability of fermentation results, adaptation time of donor animals ect.

The studies described in this thesis describe a number of experiments which were designed to test some of these above mentioned aspects in order to improve the *in vitro* procedure and therefore the predictability of feeding values of new feed ingredients (in the following called substrates). The first study compared two *in vitro* methods; one using enzymes and using inocula for fermentation and a combination of the two. The results showed a similar ranking order. Nonetheless, a combination of enzymatic pre-digestion with subsequent fermentation seems most suitable to best predict the nutrient availability of the tested substrate. The second study determined if 19 days of adaptation of donor animals is adequate to adapt them to a new diet, either high or low in NSP. The adaptation to the new diet was not completed and had the biggest influence on the slow fermentable substrate cellulose. The third study revealed that the results of a single fermentation run can be representative for the fermentation capacity of the donor animals. However, the optimal number of donor animals remains to be determined in order to receive results with a low variation coefficient. The last study compared the fermentation capacity of pigs raised on different organic farms. The results showed that animals across organic farms had a relatively similar and high fermentation capacity despite the different rations fed on single farms and varying farm management. This might be caused by the animals' lifelong exposure to a high variety of fibre.

The results of this thesis show that *in vitro* fermentation methods can be further improved and need further attention regarding the optimal number of donor animals and their nutrition. It would be of special interest to compare the differences of fermentation capacity and energy yield between animals that are fed diets either high or low in NSP. Pig diets based on an improved *in vitro* methodology have the potential to increase economic profit for feed industry and farmers, but also to increase animal health and welfare.

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Chapter 1

General introduction

General introduction

Feed for pig production in the western world is mainly based on grains which contain high amounts of dietary carbohydrates, mainly starch. Grains can be considered as high quality feed ingredients, as starch is highly digestible and the main energy source for maintenance and growth of commercially farmed pigs. However, as grains are also suitable for human consumption, pigs are more and more regarded as competitors to humans for high-quality food/feed ingredients. Therefore, the need to find possible alternative feed ingredients for pig nutrition increases and is expected to further increase in the future. The investigation into alternative feed ingredients for pig production is the topic of this thesis. Some pig production systems, such as sow husbandry and organic pig farming, already use several alternative feed sources which contain relatively high concentrations of indigestible carbohydrates. These feed ingredients are in most cases unsuitable for human consumption (e.g. straw, grass silage or by-products from food and bio-fuel industries) and as such are not in competition with human foods.

Classification of dietary carbohydrates

Dietary carbohydrates can be divided into a lignified, an indigestible and a digestible fraction (Figure 1). The lignified fraction is virtually indigestible and as such is unchanged during transit through the digestive tract of pigs (Graham *et al.*, 1986). The indigestible fraction is fermentable by microbes. Some indigestible fractions are partly lignified as well, and can be considered as slowly fermentable (Bach Knudsen *et al.*, 2001).

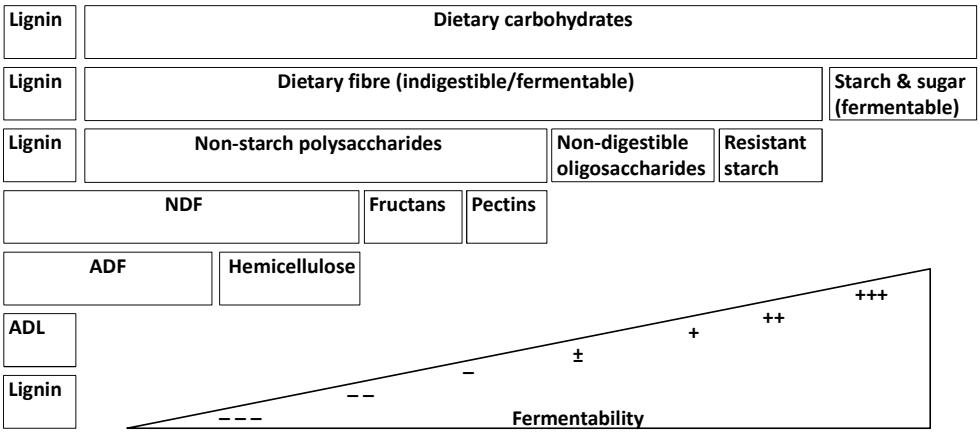

Figure 1. Classification of dietary carbohydrates (modified after the Van Soest analysis, 1991). NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin.

The (site of) degradation of carbohydrates depends largely on the way their different molecules are organized and linked. Starch and sugars are digestible, because their molecules are mainly linked by α-glycosidic bondages which can be broken down by endogenous enzymes. The indigestible carbohydrate fraction (i.e. dietary fibre) consists primarily of non-starch polysaccharides (NSP, see Figure 1) and other complex carbohydrates such as non-digestible oligosaccharides and resistant starch. Their molecules are mainly linked by ß-glycosidic bondages which are resistant to enzymatic degradation by endogenous enzymes and can potentionally be broken down by microbial enzymes. Sometimes digestible starch escapes enzymatic digestion (approx. 4%, due to encapsulation within plant cells, crystalline nature, etc.) and can be fermented in the large intestine of animals (Bach Knudsen, 2011). The fermentation of readily fermentable NSP and other indigestible carbohydrates (e.g. oligosaccharides) already starts by the microbiota in the small intestine, but most NSP are fermented by microbes residing in the large intestine of pigs (Bach Knudsen & Jørgensen, 2001).

In comparison to ruminants, research into the contribution of NSP to the energy supply of pigs has been neglected for a long time, because their fermentation was considered of minor value (Cummings, 1983). However, over the past 30 years there has been increasing interest in feeding monogastric animals diets high in NSP as it has been shown that NSP can significantly contribute to the energy supply, health and welfare of animals (Varel & Yen, 1997; Yen *et al.*, 1991; Imoto & Namioka, 1978).

Inclusion of non-starch polysaccharides in diets for pigs

Diets for pigs are usually grain-based and therefore contain relatively large amounts of enzymatically degradable starch. In terms of energy conversion, fermentation of NSP is less efficient than enzymatic digestion of starch. However, recent insights on feed formulation have led to proposals to include more NSP-rich components in the diet of pigs. The reason for this is that a shift towards feed ingredients high in NSP is considered beneficial for a number of reasons:

1) Food-competition: Pigs compete with humans for food and (bio-) energy (mainly grains) and as a result, grain in pig diets should be substituted for other ingredients which are not usable as human food (Le Gall *et al.*, 2009). Suitable ingredients are grass-based roughages or straw and by-products from the food- and ethanol industry (e.g. sugar beet pulp or distillers grains, Le Goff *et al.*, 2003) .

2) Ammonia emission: An increased inclusion-level of NSP in diets for pigs has been shown to reduce the pH of manure and results in a reduction in ammonia (NH_3) emissions. As N-excretion shifts from unstable volatile urine-urea to more

stable faecal N bound in microbial protein, less ammonia is produced (Bindelle *et al.*, 2008).
3) Energy supply: Fermentation of NSP can contribute up to 30% of the maintenance energy supply of pigs (Yen *et al.*, 1991; Imoto & Namioka, 1978).
4) Animal health and welfare: Ingestion of diets high in NSP can improve animal health and welfare. The improved health is a result of a reduction in gastric ulcers and a reduction of incidences in constipation; both are associated with an increased fibre intake. Diets high in NSP improve welfare and can reduce abnormal stereotypic behaviour, as animals spent more time on feed related behaviour, feel satiated longer and have a decreased physical activity (Williams *et al.*, 2001).
5) Organic pig production: One of the main principles in organic farming is the provision of roughage as is stipulated by EU-regulations (EEC No 834/2007). Examples of roughage are: fresh or dried fodder, such as grass, silage or straw, which all contain high levels of NSP. The difference between diets used in conventional and organic farming is mainly the source and type of dietary NSP. Diets for organic pigs contain more diverse sources of NSP in the form of roughage and by-products from the food/feed industry, while diets for conventional pigs contain often only one NSP source from by-products of the food/feed industry. Although the proportion of roughage provided in organic farming is not specified, organic pig diets usually contain more NSP compared to pigs kept under conventional conditions (20% vs. 13-15%, respectively; Högberg & Lindberg, 2004).

For many NSP it is unknown how much actual energy they provide to the animal. This unknown energy contribution together with the fact that the market for organic pig meat is increasing (Andersen *et al.*, 2005), makes that a) development of knowledge on the capacity of organic pigs to utilize NSP and b) to predict the potential of the NSP sources to contribute to the animal's energy supply becomes more important.

In contrast to the earlier mentioned beneficial effects of NSP in pig diets, dietary NSP can also cause a decrease in nutrient digestibility. In a study using 75-kg female pigs receiving either a basal diet (15% NSP) or a basal diet where 33% of dry matter (DM) was substituted with wheat bran (22% NSP) or beet pulp (34% NSP), Graham *et al.* (1986) concluded that increased NSP inclusion in the diet had a negative impact on the nutrient density and apparent ileal and faecal digestibility of dry matter. More recent studies by Owusu-Asiedu *et al.* (2006) and Högberg *et al.*, (2004) confirm these findings. Reasons for a decreased nutrient digestibility associated with an increased NSP-intake can be:

1) An increased passage rate of digesta which reduces the time available for nutrient absorption in the gastrointestinal tract (Potkins *et al.*, 2007).

2) Encapsulation of dietary macronutrients (such as proteins, sugars) which is known to decrease the digestion of these macronutrients (Bach Knudsen *et al.*, 1993).
3) Increased energy expenditure during fermentation in the form of heat and gas production compared to enzymatic degradation of starch (Kirchgeßner *et al.*, 1989).

An additional problem which may be observed when pigs have to ferment increased amounts of fibrous ingredients in their diets can be a too high concentration of (soluble) NSP in the diet. When the amount of carbohydrates entering the colon increases, carbohydrate-induced diarrhoea can occur (Soergel, 1994). In addition, if a reduced energy digestibility associated with an increased NSP level of the diet exceeds the animal's capability to compensate this deficit through a higher feed intake (which is the case below 14.5 MJ DE/kg, depending on weight of the pig; NRC, 1987) then the daily weight gain of the animal will decrease. A decreased daily weight gain is often associated with a decreased profitability for the farmer. However, increased inclusion of NSP in commercially available concentrate feeds can reduce feeding costs for the farmer, as NSP components are usually less expensive compared to highly digestible components. In order to select the most beneficial NSP sources for inclusion in pig diets, it is important to know the fermentation characteristics of the different types of NSP.

Non-starch polysaccharides are highly variable in their properties and can have different fermentation characteristics. It is therefore unwise to add NSP to the diet of pigs without understanding the effects on overall digestibility and health. In order to compose a diet which best benefits the pig in terms of health and performance, it is essential to characterize NSP sources in terms of their fermentability and interaction with other feed ingredients. In addition, the time a pig requires adjusting to a newly implemented diet enriched in NSP needs to be taken into account. It is important to determine the length of this adaptation period as this can vary for the different NSP sources (Pluske *et al.*, 1999).

Influence of the diet on the fermentation of non-starch polysaccharides

The degree of fermentation/degradation of NSP depends on the source and type of NSP as well as on the fermentation capacity of the animal ingesting it. As mentioned earlier, fermentation starts already in the small intestine of pigs for readily fermentable NSP (Bach Knudsen & Jørgensen, 2001). The non-readily, slower fermentable NSP are mainly fermented in the large intestine.

Factors influencing the degree of NSP degradation are the impact of NSP on physico-chemical properties of the diet (i.e. water holding capacity, viscosity and

transit time of digesta) as well as on the composition and activity of the large intestinal microbiota of the animal (Bindelle *et al.*, 2008). The microbiota in turn are highly dependent on substrate available for fermentation, but are also affected by the genotype and the age/weight of the pig (Noblet & Le Goff, 2001). The content (digesta) of the large intestine is known to contain 10^{10} g microbes per g organic matter (OM). About 4 to 6% of the total microbiota composition in pigs is cellulolytic and able to degrade NSP (Varel & Pond, 1985). So far, it is largely unknown how substrate-microbiota-host interrelations affect gut health, fermentation, and the resulting feeding value of NSP sources (Williams *et al.*, 2001; Coles *et al.*, 2005).

Compared to the vast number of studies concerning the digestibility of feedstuffs rich in starch, relatively little research has been conducted to characterize NSP sources. As such, no standardized values exist for the fermentability of NSP in pig nutrition. Possibilities to study fermentability of NSP are *in vivo* measurements. *In vivo* measurements of nutrient digestion in animals require that animals are surgically prepared with cannulas in the intestines. The use of invasive surgical procedures has become more difficult to justify due to increased public scrutiny. Further, *in vivo* measurements are labour intensive, time consuming and subject to errors in terms of inherent animal variation (Stern *et al.*, 1997). Alternatively, *in vitro* measurements of nutrient digestibility/fermentability in pig nutrition research can be conducted which is a cheap and relatively non-invasive alternative procedure (Babinszky *et al.*, 1990). A relatively new methodology to determine the fermentability of NSP is to indirectly measure the microbial activity *in vitro* by the use of faecal microbiota or enzymes. Some of the available *in vitro* methods will be explained in more detail later in this introduction (see paragraph *in vitro* methods for NSP characterization). Several of the *in vitro* fermentation methods measure gas production and fermentation end products which contribute to characterize NSP fermentation in more detail. Figure 2 provides a general overview of components in digesta (indigestible carbohydrates/ NSP in particular) which can be fermented and what the end products of fermentation are. The most beneficial fermentation end products for the animal in terms of energy contribution and health are the short chain fatty acids (SCFA): acetate, propionate and butyrate (Williams *et al.*, 2001). Although amino acids are also formed in the large intestine as a result of microbial metabolism, in mammals there is no significant capacity in the large intestine to absorb these synthesised, endogenous or dietary amino acids and as such are of no nutritional value to the animal (Hendriks *et al.*, 2012).

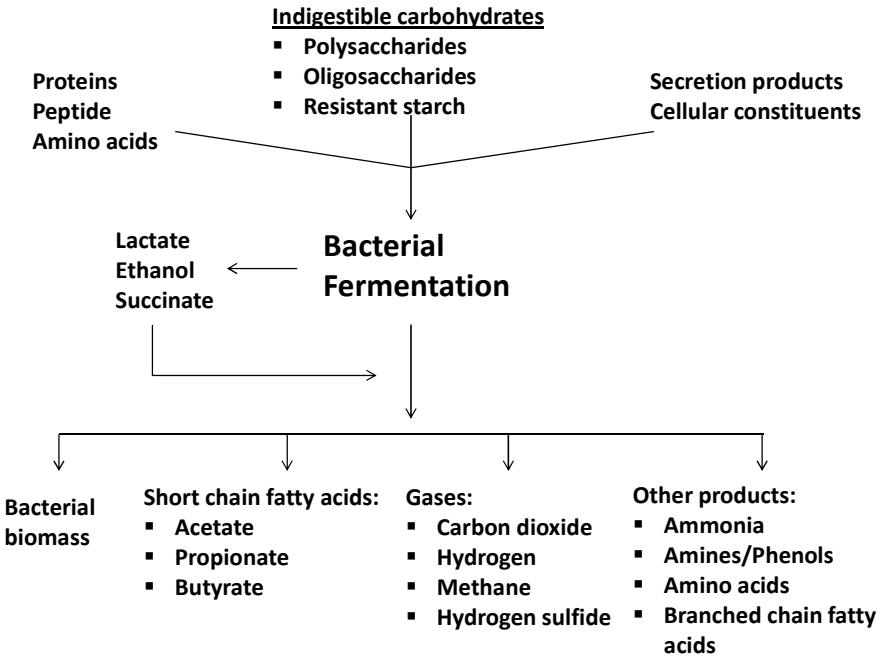

Figure 2. Major fermentation products formed by microbial fermentation in the gastro-intestinal tract of pigs (modified after Jensen, 2001).

Contrary to amino acids, SCFA are largely taken up from the large intestine via passive diffusion and are an important indicator for potential feeding value of NSP. Acetate reaches the liver via the blood and is used, mainly for muscle and fat metabolism. Butyrate is mainly absorbed by the colonic mucosa as it is an essential energy source for colonocytes. Propionate can be used in the liver for gluconeogenesis (Williams *et al.*, 2001; Bergman, 1990). Other end products of fermentation in the large intestine are ammonia (NH_3), and the gasses carbon dioxide (CO_2), methane (CH_4) and hydrogen (H_2). In order to expand the range of possible available feed ingredients for diet formulation further, potential NSP sources need to be evaluated. The data of these evaluation studies can be used to assist researchers, the feed industry and farmers to choose the most beneficial NSP sources or combination of NSP sources for feed formulation in terms of animal health and welfare, but also in terms of economic profitability.

In vitro methods for the characterization of non-starch polysaccharides

The earlier mentioned *in vitro* methods are relatively cheap and rapid to characterize NSP compared to *in vivo* studies. Boisen and Fernandez (1997) used a three-step enzymatic incubation procedure where step one and two mimic the pre-

caecal enzymatic digestion, while step three simulates microbial enzymatic degradation in the large intestine. Several other *in vitro* fermentation methods exist for the assessment of NSP substrates (i.e. Bindelle *et al.*, 2007a; Williams *et al.*, 2005a) using faecal inocula to incubate NSP substrates and measure the gas production during incubation. The faecal inoculum used in the method of Williams *et al.* (2005a) is a medium mixed from nutrients and faeces and represents the available microbiota and their activity in the hindgut. During incubation of the substrate, the microbes in the inoculum will grow and reproduce (Bindelle *et al.*, 2007a; Williams *et al.*, 2005a; Bauer *et al.*, 2004). Measurements of gas-production can be used to characterize fermentative capacities of NSP. Even if the results do not provide absolute values in terms of *in vivo* digestibility, they allow a relative comparison of substrates and ranking between NSP sources based on their possible fermentability (Coles *et al.*, 2005). Thus, a high ranked NSP substrate of an *in vitro* study would indicate a relatively higher energy yield *in vivo* for the animal compared to a lower ranked substrate.

In this thesis, faecal inocula were prepared according to the protocol of Williams *et al.* (2005a). The substrate tested is incubated with the faecal inoculum and cumulative gas production is measured for 72 h by the use of the "automated gas production system" of Cone *et al.* (1996). A pooled faecal sample of three to four animals is taken as an inoculum to reduce between-animal variation and obtain an average result. With the gas production output, fermentation kinetics such as fermentation speed and maximal rate of gas production can be calculated. The fermentation fluid can be analysed for end products such as SCFA and NH_3 at specific time points within the fermentation process.

Although many studies use *in vitro* fermentation methods to assess the fermentation capacity of NSP substrates for pigs, to the author's knowledge, no studies have been undertaken to establish a relation between *in vitro* and *in vivo* digestibility as has been done in ruminant nutrition. The challenge in pig nutrition is first to establish a standardized *in vitro* fermentation method. Most *in vitro* fermentation studies do not include a step to mimic digestion in the upper part of the digestive tract. Therefore, recent studies use combinations of enzymatic pre-digestion steps with a subsequent fermentation of the pre-digested material to improve accuracy of the output parameters during fermentation (Bauer *et al.*, 2003; Bindelle *et al.*, 2007b). Further, few studies have been undertaken to validate the different *in vitro* fermentation methodologies. For example, Bauer *et al.* (2004) arbitrarily used digesta from three sections of the porcine large intestine for *in vitro* fermentation in order to evaluate if the use of faeces is valid for *in vitro* assessment of large intestinal fermentation. In addition, digesta from single animals were used in this study and results led to the conclusion that pooling samples from several animals is important. The number of animals to obtain faecal samples was not

provided by the author. Subsequently many other researchers have used pooled samples of three to four animals (Bauer *et al.*, 2003; Bindelle *et al.*, 2007c; Le Goff *et al.*, 2003). In addition, the adaptation period of faecal donor animals to attain a stable microbial population whose fermentation capacity is representative for the diet fed is largely unknown. One to two weeks are usually used to allow for adaptation of the animals to experimental diets (Bauer *et al.*, 2004; Martín-Peláez *et al.*, 2009; Anguita *et al.*, 2006). Most studies have "optimised/validated" the *in vitro* assay using commercial available diets which are relatively low in NSP content compared to e.g diets for pigs raised under organic conditions which are enriched with roughage (Bauer *et al.*, 2003; Bauer *et al.*, 2001). It is known that increased ingestion of NSP leads to changes in the microbial community and possibly an increased adaptation time of microbiota to NSP in the large intestine compared to animals ingesting diets which are relatively low in NSP. Therefore, the characterization of NSP sources in many studies might not be truly representative for animals receiving diets high in fibre/NSP for a long period of time. From the above it is apparent that there is a lack of information on the precise assay conditions to use for *in vitro* fermentation to yield representative and repeatable results which simulate the fermentation of NSP sources in diets for pigs.

Scope of this thesis

This thesis describes studies which evaluate *in vitro* conditions to determine NSP fermentability by pigs including pre-treatment of substrates, diet-adaptation period of pigs fed NSP rich diets, influence of different inocula on fermentation outcome, repeatability of the method and finally, the variation of fermentation capacity between animals on different organic farms. The main emphasis in this thesis lies on the nutrition and adaptation of the donor animals, as nutrition is the main tool to influence the microbial population and activity in the large intestine of pigs. The results of this thesis can assist in improving methods to predict feeding values of NSP sources and provide a first insight into differences in fermentation of diets on different organic farms, where pigs are exposed to diets varying highly in NSP sources.

Thesis outline

This thesis describes the results of four *in vitro* experiments. In **Chapter 2** organic matter loss of enzymatically pre-digested substrates and their undigested counterparts was evaluated *in vitro*. Seven different NSP sources (grown under organic conditions, such as grass silage, rye grass etc.) were used as substrates in a three-step enzymatic *in vitro* incubation assay, *in vitro* gas production assay and a combination of both. The effects of the three methods on the ranking of the

substrates based on organic matter loss were investigated. For *in vitro* gas production two inocula were used, one inoculum originating from animals receiving concentrate and one inoculum originating from animals receiving roughage addition to a reduced concentrate feeding regime.

Chapter 3 describes a study which determined the dietary adaptation of animals as expressed by fermentation kinetics and microbial screening. The aim of the study was to determine changes in microbial composition and activity for two groups of sows in relation to a diet change. The diet of sows was changed from a high to a low NSP level and vice versa. Faecal inoculum was used for *in vitro* fermentation which was conducted every three days for a period of 19 days to monitor effects of the diet change. The microbial composition of the sows' large intestinal microbiota was investigated using a high throughput microarray and were related to the fermentation results.

In **Chapter 4**, the influence of time on repeatability of fermentation results was investigated. Three inocula from the same sets of sows were used at successive time points (repeated use of inocula of the same origin) to determine if consistent fermentation results are obtained. The variation in fermentation results between the sets of animals kept under equal conditions was determined to assess variability between different inocula.

The final research chapter (**Chapter 5**) reports a study which investigated the variation in fermentation kinetics using finishing pigs as donors from ten different organic farms. Three inocula sources were used per farm to incubate three substrates which provided information on variability. The results were used to provide an overview of the fermentative capacity of finishing pigs kept under organic conditions. In addition, it was determined if the composition of diets of individual farms could be related to individual fermentation results.

Chapter 6 consists of a general discussion in which all chapters are discussed and conclusions are formulated.

Chapter 2

Assessing fibre-rich feedstuffs in pig nutrition – Comparison of methods and their potential implications

Future trends in fibrous feed assessment in pig nutrition

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Abstract

Background In organic farming, roughage has to be added to pigs' daily rations for animal welfare reasons. Since little is known about how roughage affects pigs, seven carbohydrate-rich feedstuffs (corn silage, ryegrass, turnip leaf, turnip tuber, Jerusalem artichoke and two types of clover-ryegrass silage) were tested using two different *in vitro* methods: total tract digestibility (Boisen & Fernández, 1997) and the gas production technique (Williams *et al.*, 2005a) to determine apparent digestibility by observing organic matter loss. The aim was to determine whether the results of different methods and treatments lead to similar results or rankings.

Results The *in vitro* enzymatic incubation method showed 3% to 20% lower fermentabilities than the *in vitro* fermentation process. The pre-treatment of substrates with enzymes before fermentation led to similar fermentabilities but different gas production kinetics compared to the fermentation of untreated substrates. Using two different inocula generated no significant differences. When the feedstuffs were ranked by their fermentability, the rank order was relatively similar for both methods.

Conclusions Different methods and pre-treatments lead to different fermentabilities while a ranking of the results gives a similar order. Both methods seem to be appropriate tools for assessing feedstuffs. In order to determine which feedstuff is best utilized by the animal, further investigations of both digestibility and gas production kinetics are required.

Introduction

Several *in vitro* methods exist for predicting ileal and faecal digestibility of feedstuffs, the results of which can be used in the formulation of diets for fattening pigs. Most of the methods were based on diet ingredients commonly used that are generally low in fibre. The main focus until recently was laid on mimicking the enzymatic digestion occurring in the upper part of the digestive tract, while the hindgut was considered of little nutritional significance (Boisen & Fernández, 1997; Williams *et al.*, 2005; Coles *et al.*, 2005). Microbial fermentation in the hindgut, however, varies to a high degree and can contribute to maintenance energy needs from 7% up to 40% (Yen *et al.*, 1991).

In general, fibre-rich diets are avoided for fattening pigs due to their negative impact on energy digestibility (Bach Knudsen & Hansen, 1991). However, fibre can prevent metabolic disturbances and improve gut health (Williams *et al.*, 2001). In organic livestock production fresh, dried or ensiled roughage must be added to the daily rations of pigs with the aim to improve animal's welfare as stipulated by the EU Regulations (EEC No 834/2007). Inclusion of fibre-rich roughage sources in the diet of pigs will affect the animal's nutritional status, health and performance, and may be expected to decrease the benefit for the farmer. Therefore, it is essential to have proper knowledge on the feed value of the various fibrous feedstuffs that are available to the organic pig farmer. This will help farmers to decide which roughage sources to include and will help to implement act 843/2007 with success. Different methods have been developed for predicting nutrient digestibility of feedstuffs at the ileal and/or faecal level (Boisen & Eggum, 1991). Boisen and Fernández developed a three-step enzymatic incubation procedure for evaluating the total tract digestibility of pig diets. Comparing several studies using this method (Beames *et al.*, 1996; Chen, 1997; Spanghero, 1999), Coles *et al*. (2005) found a high repeatability between *in vivo* and *in vitro* values. However, this method only includes three successive enzymatic degradation steps and does not include a step that mimics the fermentation process in the hindgut. The extent and the rate of fermentation are important factors for determining the energy value of fibrous feedstuffs in pigs (Le Goff & Noblet, 2001; Rijnen, 2003). Methods assessing hindgut fermentation include, for example, the colon-simulation technique (COSITEC®; Dreyer, 1990) or the *in vitro* batch culture method (Williams *et al.*, 2005a), which in turn do not include the enzymatic degradation in the upper digestive tract.

To improve the predictability of nutrient availability in fibre-rich feedstuffs, a study was designed with the aim of comparing an enzymatic total tract digestibility method described by Boisen & Fernández (1997) with an *in vitro* batch culture method as described by Theodorou *et al.* (1994) and Williams *et al*. (2005a).

Furthermore, a method was designed that combines a modified enzymatic total tract digestibility method (mimicking enzymatic pre-digestion) with the *in vitro* batch culture method (mimicking hindgut fermentation). The objective of the trial was to compare the results of the modified pre-digestion and fermentation method with the results obtained with the enzymatic total tract digestibility and the batch culture method carried out in their original design. Moreover, the effect of inocula source was investigated, by obtaining inoculum from fattening pigs receiving either a standard commercially available concentrate or a concentrate and roughage diet.

Materials and methods

Substrates

Seven feedstuffs were used as substrates. They represent a wide range of field crops considered to be suitable for additional feed to a commercial standard diet in organic pig production. The substrates chosen were corn silage (**CS**), two varieties of clover-ryegrass silage (clover-ryegrass silage 1; **CGS1**: seed mix ratio: 85% red clover: 15% ryegrass; clover-ryegrass silage 2; **CGS2**: seed mix ratio: 25% white clover, 75% ryegrass; both sown in the previous summer), ryegrass (**RG**), Jerusalem artichoke tuber (**JA**), turnip leaf (**TL**) and turnip tuber (**TT**). These roughages were considered to be representative of the fermentable fibre sources that may be potentially used in pig diets. Representative samples were obtained from silage or from the field and dried at 70 ^{o}C in a forced-air stove for 48 h. After acclimatizing to ambient conditions, the air-dried substrates were ground to pass through a 1 mm mesh sieve.

Enzymatic digestion methods

The *in vitro* procedure simulating total tract digestibility processes in pigs by using mammalian enzymes was performed according to the Boisen & Fernández method (1997). In brief: a test sample is incubated with pepsin and pancreatin, followed by a third incubation step with Viscozyme®. No fermentation process is included. In addition, the Boisen & Fernández method (1997) was modified by omitting the incubation period with Viscozyme®, thus restricting the method to mimicking pre-caecal digestion only. The remaining residue was then isolated and used as a substrate for *in vitro* fermentation according to Williams *et al.* (2005a) to determine organic matter loss, cumulative gas production and fermentation kinetics in the hindgut. To obtain sufficient amounts of pre-digested residue per substrate for gas production measurements and further analysis, the pre-digestion procedure was scaled-up. Different quantities of the original feedstuffs (untreated = **UT**) were

pre-digested (pre-treated = **PT**). The amounts depended on their ileal/pre-caecal digestibility. The reagents used for pre-digestion were prepared following the Boisen & Fernández protocol (1997).

Details of the scaled-up pre-digestion treatment

Portions of 10 g of each substrate were weighed into 600 mL glass beakers. In the first incubation step, 250 mL of preheated (40 °C) Phosphate Buffer A and 100 mL of 0.2 mol L^{-1} hydrochloric acid (HCl) were added to each portion in its beaker. The solution was adjusted to pH 2.0 using either HCl or NaOH, and 10 mL of freshly prepared pepsin solution containing 250 mg pepsin (porcine, 2000 FIP-U/g, Merck No. 7190) was added. Deviating from the original protocol, no chloramphenicol was added to avoid a bactericidal effect on microbes of residual chloramphenicol remaining in the undigested material that could potentially affect the microbial degradation in the following *in vitro* fermentation step. Then the beakers were covered with a glass lid, placed in an incubator at 40 °C and incubated for 75 minutes with constant stirring.

In the second incubation step, 50 mL of 0.6 mol L^{-1} sodium hydroxide (NaOH) and 100 mL of Phosphate Buffer B were added to the glass beakers. The pH of the solution was adjusted to 6.8 using HCl or NaOH, and 10 mL of freshly prepared pancreatin solution containing 1 g pancreatin (porcine, grade IV, Sigma no. P-1750) was added to the solution. The beakers were again closed using a glass lid and incubated at 40 °C for 3.5 h with continuous stirring. After the second step, the beakers were removed from the incubator. The beaker contents were placed into 100 mL centrifuge tubes and the liquids were centrifuged at 4500 rpm for 10 minutes (Sigma centrifuge, type 2-15, Germany). The supernatant was discarded and the remaining residue was transferred to aluminium trays. It was assumed that in the supernatant, there was only enzymatically digested matter. The residues were freeze-dried (FTS, Dura-Dry programmable tray freeze drier, USA) to a constant weight to determine dry matter. The remaining residue was carefully homogenized and used for fermentation as PT substrates.

Cumulative gas production

The cumulative gas production was measured according to the *in vitro* fermentation method as described by Williams *et al.* (2005a) using two different types of faecal inocula; faeces from pigs receiving a concentrate diet (Control inoculum) or a concentrate-roughage diet (Roughage inoculum). In brief: 0.5 g of **PT** or **UT** substrate was incubated for 72 h with an 89-mL solution of a bicarbonate/phosphate buffer with one of the two faecal inocula. Cumulative gas production was measured using the 'automated gas production system' as

described by Cone *et al.* (1996). For each substrate-inoculum combination two replicate bottles were included. Further details are explained below.

Animals, feed and housing

As donors for the preparation of inoculum, twelve castrated finisher pigs (Dutch Landrace) were randomly distributed over two pens and group housed for one week in a barn and allowed to adapt to the new feed. In this initial feeding phase, the animals received a commercially available diet *ad libitum*. The composition of this diet is shown in Table 1. The diet contained 15.3% crude protein (CP), 6.6% crude fibre (CF), 20.7% neutral detergent fibre (NDF), 7.4% acid detergent fibre (ADF) and 12.4 MJ DE kg^{-1}, calculated according to the guidelines of the Dutch Commodity Board of Feed stuffs (CVB, 2003). At the start of the trial, the pigs had an average live weight of 78.8 ± 2.3 kg (mean ± SD). After one week both groups were moved to an outdoor system. The control group was fed the commercial concentrate diet once daily and had no access to any other feed. Mean daily feed intake was about 2.8 kg (average *ad libitum* intake in the initial feeding phase). The experimental group was fed restrictively 2.0 kg of the commercial diet once daily, but had free access to both pasture grass and a clover-grass silage mixture. The reduced feeding regime, envisaged at 70% of the control treatment on dry matter basis, was supposed to provoke a high roughage intake. Both groups received their dietary treatments 3 weeks prior to faecal sample collection to ensure adoption of the intestinal microbiota to either the concentrate or the roughage diet (Bauer *et al.*, 2004).

Table 1. Composition of the standard diet fed to pigs.

Ingredient	g kg^{-1}
Wheat bran	200
Rapeseed expeller	200
Tapioca	200
Barley	150
Maize meal	133
Wheat meal	50
Lime	5
Palm oil	16
Vinasse	40
Premix	6

Collection and preparation of inocula

Faeces was collected from the rectum, placed immediately in a plastic container, pre-flushed with CO2, placed on ice and transported to the laboratory. Within one hour after sampling, the faecal samples of each group were pooled proportionally. This pooling ensured an inoculum which was representative for pigs of the respective age and kept under the specified dietary conditions (McBurney & Thompson, 1989). Each pooled sample was diluted 1:10 with an autoclaved 0.9% NaCl–solution, pre-warmed to 40 °C. After homogenizing the pooled faeces dilutions with a blender, the fluid was filtered through a nylon cloth (pore size 40 µm, permeability 30%; PA 40/30, Nybolt, Switzerland), thus creating the inoculum. All handling while preparing the inoculum was carried out under a constant flow of CO_2 to ensure anaerobic conditions. Finally, the inoculum was added to a previously prepared medium, containing a phosphate and bicarbonate buffer with vitamins and a reducing agent, as described by Williams *et al.* (2005). The inoculum was added to the medium in the ratio 1:16.8 on a weight/volume basis.

Analyses

The PT and UT substrates were analysed for their dry matter (DM), organic matter (OM), ash, CF, CP and starch contents according to proximate analysisNaumann *et al.*, 1976). NDF, ADF and acid detergent lignin (ADL) were determined as described by Goering and Van Soest (1972). The sugar content was analysed using the Luff-Schoorl method (Matissek, 1992).

After the incubation procedure, fluid pH was recorded (Hanna Instruments pH meter) and samples were collected from each bottle and analysed for volatile fatty acids (VFA) and ammonia (NH_3). For VFA analyses 0.75 mL of the fermentation fluid was added to an Eppendorf vial containing 0.75 mL solution (composed of 300 mL of a 4-g/L 2-methyl valeric acid solution and 25 mL ortho-phosphoric acid 85% in 200 mL of Millipore). For NH_3 analyses, 0.75 mL of fluid was mixed with 0.75 mL 10% trichloroacetic acid in an Eppendorf vial.

VFA was analysed using a GC (Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with a split/splitless injector operated in split mode (split ratio 1:10) and fitted to a flame ionization detector (FID), using a capillary column (Mega bore EC-1000, length 30 m, i.d. 0.53 mm, film thickness 1.00 µm, Alltech Associates, Inc., Deerfield, IL, USA) with Helium as the carrier gas (50 kPa pressure). The starting temperature of the column was set at 110 °C for 2 min followed by an 18 °C/ min increase to 200 °C that was maintained for 1 min. Iso-caproic acid was included as an internal standard. The NH_3 level was determined using the method described by Houdijk (1998). Supernatants were deproteinized with 100 g L^{-1}

trichloroacetic acid. NH_3 forms a blue complex with phenol and hypochlorite in an alkaline environment, which was measured colorimetrically at 623 nm using an UV-spectrophotometer (Beckmann-Coulter DU64, Fullerton, USA).

Statistics

Profiles of cumulative gas production (measured as mL of gas produced per g of OM unit in time) were fitted iteratively using a modified multiphasic Michaelis-Menten equation (Groot *et al.*, 1996):

$$G = \sum_{i=1}^{2} \frac{Ai}{1 + \left(Ci / ti\right)^{Bi}}$$

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 (T ½)
- t = time (h)
- i = number of phases included in the model.

The number of phases was determined according to the method described by Groot *et al.* (1996). The maximum rate of gas production (R_{max}) and the time at which it occurred (T_{Rmax}) were calculated with the following equations (Bauer *et al.*, 2001):

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

$$T_{Rmax} = C \times (((B - 1)/(B + 1))^{(1/B)})$$

All parameters were tested for differences within the inoculum type (control and roughage) by applying the GLM procedure SAS 9.1 (1989), using the following model:

$$Y = \mu + S_i + T_j + (S \times T)_{ij} + \varepsilon_{ijk},$$

where

- Y = the dependent variable tested
- μ = the mean
- S_i = effect of the substrate i
- T_j = the effect of the pre-digestion treatment j

- $(S \times T)_{ij}$ = interaction between substrate and pre-digestion treatment
- ε_{ijk} = error term.

An effect of replicate bottles was tested separately but did not contribute significantly to the model for any of the parameters, and was therefore excluded from the model main effects. Differences between main effect S_i and T_j were analysed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS.

Results

Proximate, fibre and starch analyses

The results of the proximate, fibre and sugar analyses of the UT and PT substrates are shown in Table 2. Analyses were not performed when it was considered that the content of the respective constituent would be negligible in the respective substrate (i.e. starch for ryegrass, turnip tuber, Jerusalem artichoke, clover-ryegrass silage 1 and 2). In comparison to the ash content of the UT, the PT material showed an accumulation of this component, resulting in a lower OM content as a result of the enzyme incubation of the PT substrates. The PT Jerusalem artichoke contained no sugar at all whilst the UT contained 60% sugar. CF and cell wall components (NDF, ADF and ADL) were on average increased in the PT substrates compared to UT. An exception was PT turnip leaf with a 44% CF-content after enzymatic incubation. The Jerusalem artichoke had the lowest CF and cell wall component amount in both substrates. For PT turnip leaf and both, UT and PT turnip tuber, the amount of NDF and ADF was almost alike, whilst for UT turnip leaf, ADF was even higher than NDF. Except for ryegrass, PT substrates resulted in similar or decreased CP contents in comparison with the UT substrates. The values for starch and sugar content showed a considerable decrease following the enzymatic pre-treatment step, except for the ryegrass.

Table 2. Proximate and fibre analysis of untreated and pre-treated substrates.

Substrate	Treat-ment	DM	Ash	CF	CP	Starch	Sugar	NDF	ADF	ADL	OM
		g kg $^{-1}$	g kg $^{-1}$ DM								
Corn silage	UT	907	42	180	75	325	-	362	220	82	958
	PT	899	174	299	59	30	-	579	370	84	826
Rye grass	UT	919	140	258	139	-	85	565	340	40	860
	PT	872	295	274	136	-	73	557	336	10	705
Turnip leaf	UT	924	236	201	285	14	29	204	352	24	764
	PT	940	412	113	141	0	0	390	375	26	588
Turnip tuber	UT	926	161	182	143	-	283	189	184	52	839
	PT	975	289	298	73	-	-	194	189	54	711
Jerusalem art.	UT	904	70	45	73	-	606	73	51	22	930
	PT	977	299	123	60	-	-	126	52	23	701
Clover grass sil.1	UT	889	106	242	182	-	89	408	245	46	894
	PT	888	265	295	126	-	34	502	279	130	736
Clover grass sil.2	UT	903	104	191	169	-	185	336	176	54	896
	PT	873	303	237	166	-	70	387	222	152	697

CF, crude fibre; CP, crude protein; OM, organic matter; PT, pre-treated; UT, untreated.

Organic matter loss

Table 3 shows the organic matter loss (OML) of the substrates measured after total tract digestibility and modified enzymatic incubation pre-treatment, after gas production with UT and PT substrates using two different inocula, and after combined gas-production and modified enzymatic pre-treatment. When comparing the results of the OML, a high variation between the different treatments became obvious. The complete Boisen & Fernández (B&F; 1997) method (Table 3) resulted in a 22.8% (corn silage) to 195.7% (turnip leaf) higher OML than the modification representing pre-caecal digestion only. Results of the *in vitro* fermentation (Table 3; GP) of the UT substrates showed a 4.9% (Jerusalem artichoke) to 53.8% (corn silage) higher OML than for the PT substrates. The OML of PT substrates after pre-treatment + gas production did not differ from the OML after gas production of UT substrates.

Table 3. Percentage of organic matter loss (OML) for different incubation steps and methods.

Substrate	B & F		GP			B & F + GP	
	T vs. M	%	Treatment	CI %	RI %	CI %	RI %
Corn silage	T	58.2	UT	67.0	68.6		
	M	47.4	PT	47.4	44.6	72.3	70.9
Rye grass	T	46.8	UT	67.7	68.4		
	M	22.6	PT	59.3	60.6	68.5	69.5
Turnip leaf	T	83.1	UT	81.1	80.7		
	M	28.1	PT	76.3	72.6	83.0	80.3
Turnip tuber	T	83.5	UT	88.5	88.4		
	M	34.4	PT	80.4	78.7	87.1	86.0
Jerusalem art.	T	95.3	UT	98.1	98.1		
	M	73.9	PT	93.6	93.5	98.3	98.3
Clover grass sil.1	T	62.7	UT	77.9	76.9		
	M	29.0	PT	71.2	69.7	79.5	78.5
Clover grass sil.2	T	71.4	UT	84.6	84.2		
	M	35.1	PT	77.3	77.4	85.3	85.4

B & F, Boisen and Fernández method (1997); CI, control inoculum; GP, *in vitro* gas production method; M, modified pre-caecal digestibility according to Boisen and Fernández (1997); PT, pre-treated substrate; RI, Roughage inoculum; T, total tract digestibility; UT, untreated substrate.

However, with respect to the OML as determined by the total digestibility method, the values of the gas production (GP) and the combined B&F + GP method were considerably higher in case of the more fibrous feedstuffs (i.e. corn silage, rye grass, and clover-grass silage). All OML values for the total gas production trial showed higher values than for the total B&F method. When the results of the different methods were ranked from highest to lowest degradation, the rank order differed only marginally:

1) OML (Boisen and Fernández method):
JA > TT > TL > CGS2 > CGS1 > CS > RG
2) OML (*In vitro* fermentation UT):
JA> TT > CGS2 > TL > CGS1 > CS/RG
3) OML (Pre-treatment & *in vitro* fermentation PT):
JA > TT > CGS2 > TL > CGS1 > CS > RG

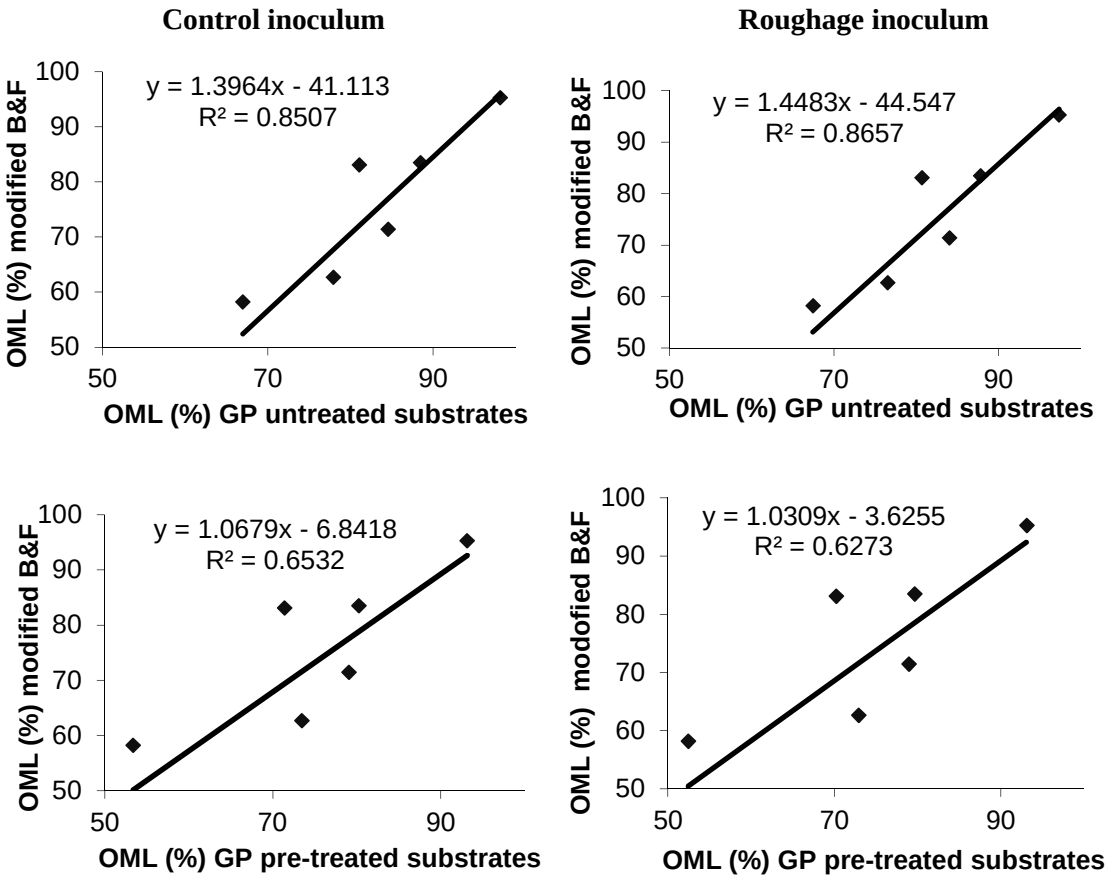


Figure 1. Relationship of organic matter loss between the modified Boisen and Fernández (B&F) method and the *in vitro* gas production (GP) method on untreated or pre-treated substrates, using control inoculum (graphs on left) and roughage inoculum (graphs on right).

Figure 1 shows the linear regression and the positive relationship between the OML with enzymatic incubation and the OML with fermentation for both inocula in this study.

Fermentation endpoint characteristics

The dry matter (DM) and ash content of the inoculum prepared from the control group were 18.8 and 11.0 g kg^{-1} DM, and 21.9 and 13.8 g kg^{-1} DM for the inoculum prepared from the roughage group. The pH of the inocula was 6.38 and 6.40 for the control inoculum and roughage inoculum, respectively. The mean values of the total gas production, volatile fatty acids (VFA) and NH_3 are shown in

Table 4. In general, the choice of substrate led to highly significant differences between the fermentability of the substrates ($P < 0.005$). A highly significant interaction between pre-treatment and substrate was detected for NH_3 production for both inocula ($P < 0.001$). The UT substrates resulted in higher gas production, except for the turnip tuber incubated with the roughage inoculum (UT = 203 mL g^{-1} OM vs. PT = 236 mL g^{-1} OM). The Jerusalem artichoke had the highest gas production with 373 mL g^{-1} OM. Except for turnip leaf, all UT substrates incubated with the control inoculum had a similar (clover-grass silage 2) or higher VFA production compared to the PT substrates. The incubation with roughage inoculum led to a lower VFA production for UT turnip leaf and both types of clover-grass silage than their PT counterparts.

Table 4. Fermentation endpoint characteristics of untreated and pretreated substrates.

Substrate	Treat-ment	Control inoculum			Roughage inoculum		
		OMCV	totVFA	NH_3	OMCV	totVFA	NH_3
		mL g^{-1} OM	mmol g^{-1} OM		mL g^{-1} OM	mmol g^{-1} OM	
Corn silage	UT	260	4.80	4.19	247	5.78	3.76
	PT	158	4.28	4.84	175	4.04	4.66
Rye grass	UT	223	5.09	4.47	225	5.13	4.81
	PT	201	4.73	5.38	226	4.95	5.29
Turnip leaf	UT	301	5.74	7.65	297	5.91	7.32
	PT	271	5.96	8.18	297	6.06	8.03
Turnip tuber	UT	234	6.39	6.06	203	6.51	5.59
	PT	234	6.04	8.18	236	6.19	5.38
Jerusalem art.	UT	373	7.17	4.04	370	7.46	3.98
	PT	315	6.54	5.24	331	6.30	5.11
Clover grass sil.1	UT	241	5.32	5.51	238	5.68	5.18
	PT	226	5.12	5.55	216	6.30	5.26
Clover grass sil.2	UT	302	5.95	5.14	311	5.57	4.92
	PT	265	5.95	5.82	276	5.79	5.84
SEM		6.07	0.29	0.10	13.6	0.23	0.07
Model est. (P-values):							
Pretreatment		0.462	0.109	<0.001	0.020	0.014	<0.001
Substrate		0.005	<0.001	<0.001	<0.001	<0.001	<0.001
P x S (interaction)		0.990	0.788	<0.001	0.039	0.002	<0.001

NH_3, ammonia; OM, organic matter; OMCV, organic matter corrected volume; PT, pre-treated substrate; totVFA, total volatile fatty acids; UT, untreated substrate.

For UT turnip leaf and both types of clover-grass silage, the incubation with the roughage inoculum led to lower VFA production in comparison with their untreated counterparts. For both inocula, except for turnip leaf, all PT substrates had a higher NH_3 production than the UT substrates.

Gas production kinetics

The gas production data were fitted using a biphasic model (Groot *et al.*, 1996). The results are shown in Table 5. For the control inoculum, a highly significant interaction between pre-treatment and substrate was found for the maximum rate of gas production (R_{max}) and the time at which it occurred (T_{Rmax}) in both the first and the second phase ($P \leq 0.002$). The roughage inoculum showed a highly significant interaction between pre-treatment and substrate for all parameters in both phases ($P \leq 0.040$). The values for asymptotic gas production did not differ significantly between the UT and PT substrates, or for the different inocula.

Table 5. Parameters of gas production of UT and PT substrates with two different inocula.

Substrate	Treat-ment	Phase 1				Phase 2			
		A_1	$T1/2_1$	T_{Rmax1}	R_{m1}	A_2	$T1/2_2$	T_{Rmax2}	R_{m2}
		mL g^{-1}OM	h	h	mL h^{-1}	mL g^{-1}OM	h	h	mL h^{-1}
Control inoculum									
CS	UT	54	15.9	6.5	7.6	242	19.4	23.3	3.7
CS	PT	71	3.4	1.6	13.2	97	31.3	14.1	2.4
RG	UT	97	4.6	2.6	13.5	137	34.5	33.0	4.8
RG	PT	80	7.8	1.9	6.7	136	35.7	31.2	5.2
TL	UT	154	8.6	4.3	11.3	85	18.6	12.9	9.1
TL	PT	103	8.9	3.0	9.9	155	14.2	18.0	13.7
TT	UT	203	3.9	1.9	32.7	101	16.1	17.4	10.9
TT	PT	186	9.3	4.9	12.6	110	17.9	15.4	14.4
JA	UT	171	5.4	2.9	27.6	236	5.4	5.3	71.0
JA	PT	206	4.9	3.8	33.6	112	16.4	15.0	8.4
CGS1	UT	123	6.1	2.9	12.7	122	24.5	21.2	5.0
CGS1	PT	148	16.1	2.4	6.5	115	26.0	22.9	4.9
CGS2	UT	176	3.8	2.7	32.9	118	17.4	16.1	9.0
CGS2	PT	168	10.7	1.4	12.5	125	19.2	16.7	6.7
SEM		40	3.9	0.6	1.1	26	1.1	0.9	0.7
Model est. P-values:									
Pretreatment		0.910	0.405	0.055	<0.001	0.074	<0.001	<0.001	<0.001
Substrate		0.052	0.753	0.040	<0.001	0.171	<0.001	<0.001	<0.001
P x S (interaction)		0.936	0.254	0.002	<0.001	0.021	<0.001	<0.001	<0.001

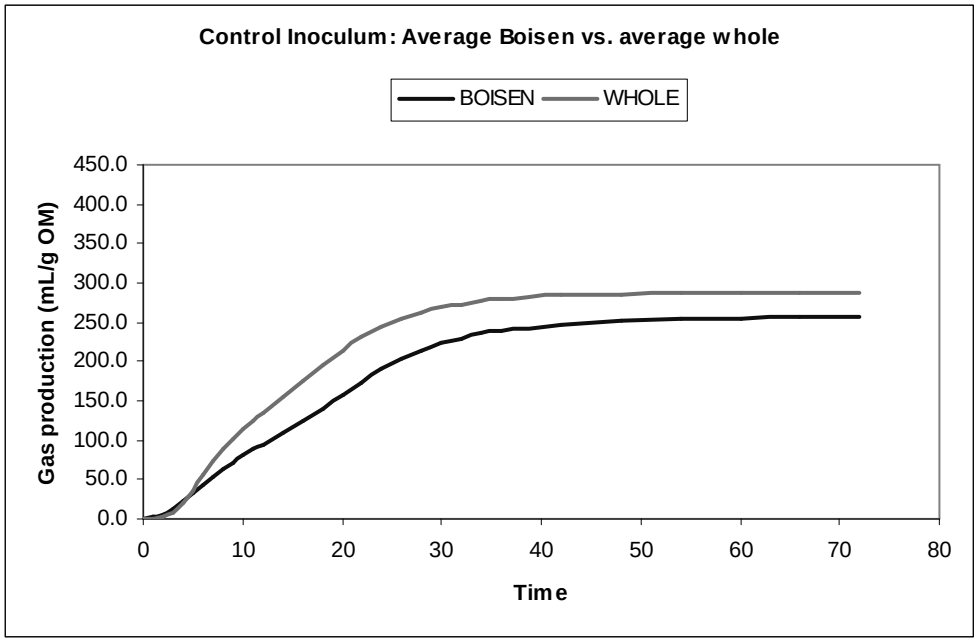
Table 5. Continued.

Substrate	Treat-ment	Phase 1				Phase 2			
		A_1 mL g^{-1}OM	$T1/2_1$ h	T_{Rmax1} h	R_{m1} mL h^{-1}	A_2 mL g^{-1}OM	$T1/2_2$ h	T_{Rmax2} h	R_{m2} mL h^{-1}
Roughage inoculum									
CS	UT	101	5.9	.	10.3	82	30.7	.	2.9
CS	PT	103	5.4	2.2	12.3	124	27.8	26.8	4.8
RG	UT	145	25.7	2.9	5.7	142	38.4	24.6	4.8
RG	PT	125	8.3	1.4	9.7	83	20.9	35.2	7.5
TL	UT	74	4.8	4.7	8.2	175	16.7	20.1	10.3
TL	PT	220	5.4	2.6	27.0	80	19.3	14.1	13.8
TT	UT	86	4.7	3.2	13.3	224	15.6	19.1	15.0
TT	PT	212	6.7	1.5	16.7	181	6.7	13.6	67.0
JA	UT	233	6.1	3.0	31.8	101	17.3	6.5	7.9
JA	PT	126	7.2	4.9	11.0	115	28.1	15.8	4.2
CGS1	UT	198	29.3	3.7	5.6	82	29.6	24.6	3.7
CGS1	PT	184	4.9	1.1	30.6	118	18.4	27.6	10.1
CGS2	UT	148	8.9	3.8	12.5	145	20.4	17.4	7.7
CGS2	PT	86	4.7	1.6	13.3	224	15.6	18.1	15.0
SEM		26	4.4	0.7	1.7	20	0.9	0.9	1.4
Model est. P-values:									
Pretreatment		0.356	0.018	0.004	<0.001	0.029	<0.001	0.002	<0.001
Substrate		0.009	0.042	0.131	<0.001	0.053	<0.001	<0.001	<0.001
P x S (interaction)		0.018	0.039	0.040	<0.001	0.001	<0.001	<0.001	<0.001

A, asymptotic gas production (ml/ g OM); CGS1&2, clover grass silage 1 & 2; CS, corn silage; JA, Jerusalem artichoke, RG, rye grass; R_{max}, maximum rate of gas production (mL/h); TL, turnip leaf; T_{Rmax}, time at which the maximum rate of gas production occurs (h); TT, turnip tuber; $T_{1/2,}$ halftime of A (h).

An exception was the UT corn silage incubated with the control inoculum, which showed very little gas production during phase 1 with 54 mL g^{-1} OM and 350% higher gas production in the second phase with 242 mL g^{-1} OM. In the first phase, the averaged $T_{1/2}$ was reached after ¼ of the total run time (72 h), except for the PT substrates during incubation with the roughage inoculum, where $T_{1/2}$ was obtained after ½ of the total time. When comparing the results of control inoculum to the roughage inoculum, $T_{1/2}$ is numerically higher for UT compared to PT substrates (7 vs. 12 h and 19 vs. 24 h, respectively) and shows a numerical decrease for PT substrates (9 vs. 6 hrs and 23 vs. 20 h) in both phases.

During phase 1, the average R_{max} for PT was 13.6 mL h^{-1} (control inoculum) and 12.5 mL h^{-1} (roughage inoculum), while in phase 2 it only reached as high as 7.9 mL h^{-1} and 7.5 mL h^{-1}, respectively. The variation in R_{max} in the second phase was high and ranged between 2.9 mL h^{-1} for the PT corn silage to 67 mL h^{-1} for the



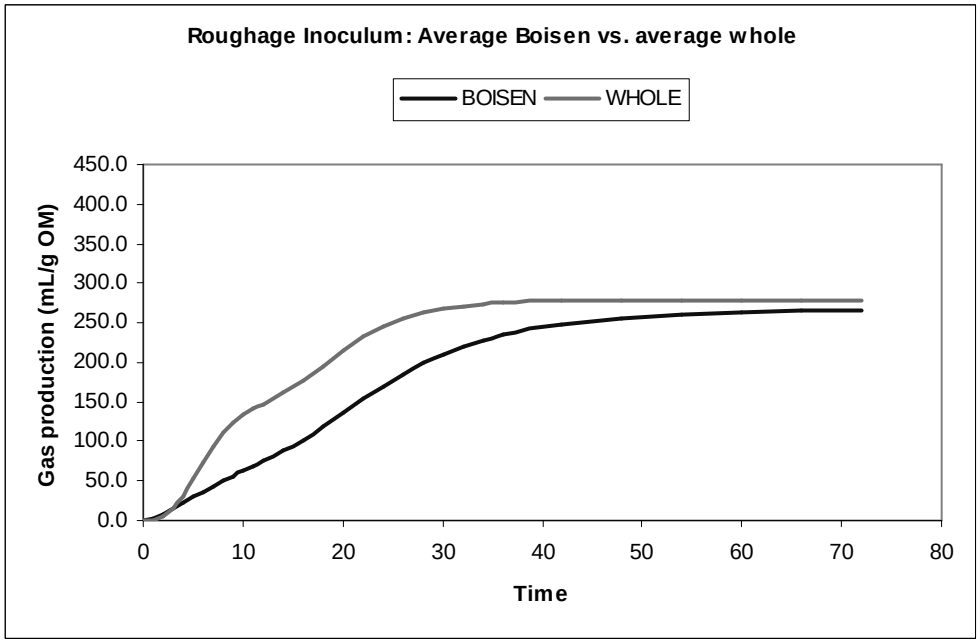


Figure 2. Cumulative gas production over time (h) for pre-treated (BOISEN) and untreated (WHOLE) substrates after incubation with two types of inocula, control and roughage.

PT Jerusalem artichoke. In the first phase, T_{Rmax} was reached at an earlier time for the pre-treated substrates compared to the whole substrates for both the control (P = 0.055) and the roughage inoculum (P = 0.004). The second phase showed that pre-treated substrates reached T_{Rmax} at a later timepoint for both inocula ($P \leq$ 0.002). Figure 2 shows the cumulative gas production curves of pre-treated and whole substrates when incubated with either a control or a roughage inoculum.

Discussion

Proximate, fibre and starch analysis

On average, the PT substrates showed higher fibre contents except for turnip leaf and a higher ash content except for ryegrass in comparison with the UT substrates. The accumulation of CF resulted from the degradation of fractions like starch and readily soluble sugars that are easily accessible to enzymatic degradation. Therefore, the PT substrates showed similar (ryegrass and clover-grass silage 2) or lower (all other substrates) CP, starch and sugar content when compared to their UT counterparts, resulting from the proteolytic and hydrolysing activities of pepsin and pancreatin. The remaining CP fraction in the PT substrates resulted from protein sources enclosed in the cell content and thus not accessible to the enzymes, and protein sources associated with the cell wall fraction (Van Soest *et al.*, 1994). Due to the accumulation of insoluble components, the NDF, ADF and ADL contents increased after pre-treatment because degradation by mammalian enzymes of these components is impossible (Hall, 1994). It is interesting that with UT turnip leaf, the NDF content was smaller (204 g kg^{-1} DM) than the ADF content (352 g kg^{-1} DM), whilst for PT turnip leaf and both the UT and PT turnip tuber NDF and ADF content was almost alike. This might be caused by a high pectin content known to be present in brassica forages. In the acid detergent fibre analysis pectin forms precipitating gels that contaminate the ADF fraction and hence, influences the actual ADF value (Van Soest *et al.*, 1994; Van Soest *et al.*, 1991; Hall, 1994; Cassida *et al.*, 2007).

Organic matter loss

One aim of the study was to compare the loss of organic matter between enzymatic incubation (Boisen & Fernández, 1997), *in vitro* fermentation (Williams *et al.*, 2005a) and a combination of both methods. Our results show considerably higher OML for UT and PT substrates using the *in vitro* fermentation method compared to enzymatic incubation following Boisen & Fernández (1997). Coles *et al*. (2005) concluded that a shortcoming of Boisen & Fernández's conventional enzymatic incubation method is that it would only simulate the enzymes of hindgut

microbes, but not the fermentation process itself. Therefore, and in light of the results of our study, it can be presumed that enzymatic incubation underestimates the real digestibility. Carlson *et al*. (1999) showed a lower OML of 56% for clover-grass silage compared to our values, which ranged from 73% to 85%, whilst Ly *et al*. (1995) found a digestibility for Jerusalem artichoke of about 86% vs. our values which ranged from 93% to 98%. Therefore, further studies need to be conducted which compare the results of different *in vitro* methods with *in vivo* studies.

The rank orders of the substrates for different methods were almost the same. Considering these rankings, the Boisen & Fernández (1997) procedure represents the easiest and cheapest method for assessing total tract digestibility of feedstuffs in practice. However, further research is necessary to determine which method shows the highest correlation to the real digestibility as determined *in vivo.* Comparing the OML from fermentation for UT and PT substrates, a pre-treatment of substrates does not seem to be necessary. The observations from the current trial have to be replicated, and a broader range of roughages differing in quality need to be included to choose those that are most efficient with respect to nutrient utilization. It remains unknown whether the OML obtained after *in vitro* fermentation shows the true degradability of OM, because it is not clear how much OM was truly degraded and how much was used for building up microbial mass (Blümmel *et al*., 1997).

Fermentation end point characteristics

For all PT substrates, lower gas production was observed, showing that pre-treatment leads to a substrate which produces less gas per g OM. It can be assumed that the constituents of the OM are different from those in untreated material and therefore less available to fermentation. Perhaps for the same reason a tendency towards decreased VFA production occurred following pretreatment, in contrast to increased NH_3 production, whilst Bauer *et al*. (2003) attained lower NH_3 production. Even though UT and PT Jerusalem artichoke substrates had the lowest fibre and cell wall contents, they produced the highest amount of gas per g OM. For the UT this can be attributed to the high sugar content, however, in PT the sugar content has reduced to zero, and is the high gas production solely resulting from the non-soluble fibre fraction. Furthermore, when incubated with control inoculum both showed also the highest VFA production whilst during the incubation with roughage inoculum, only the UT substrate showed the highest VFA production. The fermentation of Jerusalem artichoke might be energetically the most beneficial one for pig nutrition. The level of VFA production in the caecum can provide good prediction of the degree of fermentation of the dietary fibre (Freire *et al*., 2000), but it is not known yet how much energy is provided by

VFA. It remains unclear whether pre-treatment of substrates leads to a material comparable to ileal chyme, since non-enzymatic processes, such as some kinds of microbial fermentation in the end of the small intestine, occur in the upper digestive tract *in vivo* which could not be simulated with the pre-digestion step (Bindelle *et al*., 2007b; Jensen & Jørgensen, 1994; Ratcliff, 1991).

Furthermore, significant amounts of endogenous components, such as digestive enzymes and sloughed cells, are also added during the digestive process (Bauer *et al*., 2003), and these also cannot be simulated with *in vitro* methods. The influence of different inocula on OML, NH_3 and VFA is not obvious in this trial, although this was proved by Jørgensen *et al*. (2007). Further investigations are required to develop a method that better simulates the microbiological environment in the gastrointestinal tract (GIT), which is extremely complex and also highly influenced by the concentration and composition of endogenous nutrients (Williams *et al*., 2005a; Williams *et al*., 2001).

Gas production kinetics

Fermentation was generally slower for PT substrates compared to UT substrates for the control inoculum, e.g. as illustrated in Figure 2. This might have been due to 1) microbes which were not adapted to roughage in the control inoculum (where no roughage was fed), and 2) to a possible loss of some rapidly fermentable components during pre-treatment. Thus, it seems obvious that the remaining substrate incubated with control inoculum comprised more slowly fermentable components (Bauer *et al*., 2003). The first phase showed the gas production of soluble and therefore easy fermentable components. The insoluble parts require colonization of microbes and further hydrating before fermentation starts; this would occur in the second phase (Groot *et al*., 1996). Regarding the incubation with roughage inoculum, one can observe that the colonisation of insoluble parts was fostered which is reflected in shorter T1/2 for the majority of substrate over both phases.

It is assumed that rapidly fermentable feed products can be fermented earlier in the GIT whilst more slowly fermentable feeds are fermented later in the tract. The results of measuring cumulative gas production may provide an indication of where the product is likely to be fermented in the GIT (Williams *et al*., 2005). The results of gas production kinetics between the first and second phases were highly variable for all factors, i.e. substrates, pre-treatment and inocula sources. These differences are undoubtedly related to differences in the composition of the substrates. Williams *et al.* (2005a) fermented only untreated substrates in their study. Thus, it has not been confirmed yet whether enzymatic pre-treatment and different inocula have a significant influence on gas production.

Conclusion

Enzymatic incubation appears to be an adequate method for ranking feedstuffs for their fermentability. The influence of different inocula is not obvious in this trial but needs further proof. Further research is also necessary to determine which method shows the highest correlation with *in vivo* values. In this trial it has to be emphasized that only seven different feedstuffs with one replicate each have been examined. More substrates with more replications should be tested to further assess the effects of pre-treatment on fermentation patterns and end-point characteristics.

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Chapter 3

Effect of diet change on *in vitro* fermentation capacity and composition of large intestinal microbiota of sows

Adaptation of faecal microbiota after a diet change in sows

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Submitted

Abstract

Background *In vitro* gas production studies are routinely used in pig nutrition to assess the metabolic capacity of intestinal microbiota obtained from animals adapted to a certain diet. This *in vitro* study was designed to assess whether 19 days of adaptation are sufficient to obtain faecal inocula with a stable microbial composition and activity for *in vitro* gas production. Eighteen multiparous sows were equally assigned to two treatments: one group was changed from a high to a low fibre diet (HL-treatment) while the other group was changed from the diet low in fibre to the diet high in fibre (LH-treatment). Faecal samples were repeatedly obtained (day 1, 4, 7, 10, 13, 16 and 19) after the diet change and the prepared inocula used for incubation of three contrasting substrates: oligofructose, soy pectin and cellulose. In addition, the microbiota was characterised using a recently developed phylogenetic microarray comprehensively targeting pig GI tract microbiota.

Results Day of fermentation run had an effect ($P < 0.05$) on total gas production for the fast fermentable substrates oligofructose and soy pectin. For cellulose, all measured fermentation parameters were higher ($P < 0.05$) for the HL-treatment. The diet change led to significant changes in relative abundance of specific bacteria, especially *Bacteroidetes* which increased and *Bacilli* which decreased for the LH-treatment, while the changes were opposite for the HL-treatment.

Conclusion Changing the diet of sows led to differences in fermentation activity and composition of microbiota over time. Adaptation of the microbiota as assessed by gas production occurred faster for animals changed from a low to a high fibre diet for fast fermentable substrates compared to animals changed from a high to a low fibre diet. Overall, adaptation of the large intestinal microbiota of sows as a result of ingestion of low and high fibre diets seems to take longer than 19 days, especially for the ability to ferment slowly fermentable substrates.

Introduction

Dietary fibre is rich in non-starch polysaccharides (NSP) and plays an important role in the nutrition of pigs. Especially for breeding sows or fattening pigs housed under organic farming conditions, a significant proportion of the diet consists of NSP (fibre) rich ingredients. The increased dietary fibre inclusion (e.g. in the form of grass silage) stipulated for these classes of animals is intended to improve animal health and welfare. Ingestion of additional fibre can reduce health problems like enteric diseases including diarrhoea, and can reduce stereotypic behaviour (Williams *et al.*, 2001; De Leeuw *et al.*, 2008). The dietary fibre component can be fermented by microbes residing along the entire gastrointestinal tract, although the majority of the fermentation occurs in the large intestine of pigs. Inclusion of fibre sources in diets of pigs can however, result in a reduced digestibility of the compound feed, due to e.g. shorter digesta transit time. Furthermore, diets high in fibre generally contain a reduced digestible nutrient and energy content which potentially reduces animal performance and as such profitability (Chabeauti *et al.*, 1991; Owusu-Asidu *et al.*, 2006; Bindelle *et al.*, 2008). The extent of fibre fermentation depends on the amount and type of fibre present in the diet as well as the adaptation of the large-intestinal microbiota in terms of composition and activity (Williams *et al.*, 2001; Bach Knudsen, 2012).

A well-established method to estimate fermentability of different dietary fibre sources is *in vitro* gas production where faeces from donor animals are used as an inoculum source. Substrate/fibre sources are incubated, the cumulative gas production measured over time and fermentation end products quantified at the termination of the incubation. The results of such *in vitro* studies can be used to rank fibre sources and draw conclusions on their fermentability, i.e. kinetics of microbial degradation and products formed (Williams *et al.*, 2005a). In such type of *in vitro* studies, faecal donor animals are fed a well-defined diet in order to obtain a standardised microbial community. For many *in vitro* fermentation studies with pigs, a dietary adaptation time of 7 to 14 days has been used when a new diet was implemented (Bauer *et al.*, 2004; Anguita *et al.*, 2006; Bindelle *et al.*, 2009; Martín-Peláez *et al.*, 2009). Interestingly, there is a general lack of information on the time required for the large intestinal microbiota to adapt to the specific diet used in terms of fermentation capacity. Varel *et al.* (1987b) showed an increase of *Bacteroides succinogenes* and *Ruminococcus flavefaciens* content in chyme when 8 month-old barrows of unknown body weight were changed from a control diet (3.3% cellulose) to a diet containing 40% alfalfa meal (14.0% cellulose). Faecal cellulolytic bacteria numbers and cellulase activity were relatively stable for the control fed gilts compared to the gilts fed a high fibre diet which fluctuated greatly over the 86 day study. No plateau was reached after 86 days in terms of faecal

bacteria numbers or cellulose activity. The latter study was conducted with growing gilts and data could have been affected by potential changes in physiology on microbiota composition.

The aim of this study was to investigate the adaptation of faecal microbes in sows over 19 days in relation to a dietary change from a high to a low fibre diet (HL-treatment) and from a low to a high fibre diet (LH-treatment). Faecal microbial activity was evaluated by *in vitro* gas production kinetics and fermentation end-product profiles and changes in microbial composition were assessed by the Pig Intestinal Tract Chip (PITChip; Pérez Gutiérrez *et al.*, submitted; Pérez Gutiérrez, 2010, Chapter 3).

Material and methods

Animals, housing and feed

Eighteen adult multiparous, non-pregnant sows (mean BW ± standard error: 252 ± 25.3 kg) of a commercial crossbreed (Camborough: (Landrace × Large White) × Duroc) were used. Sows were housed individually in single pens of 3 × 4 m with 50% of the floor area solid concrete and 50% slatted floor. No bedding material was provided. Before allocation of the experimental diet to the sows, each animal received 3 kg of a compound feed (Table 1) at maintenance level (calculated as 1% of live weight, CVB, 2010) for at least three weeks.

Table 1. Composition of the standard compound feed fed to sows.

Ingredient	g kg^{-1}
Barley	300
Wheat middlings	200
Corn	100
Rape seed, extracted	75
Wheat	50
Soy hulls	50
Soy beans, extracted	46
Palm kernel expeller	46
Sunflower seed expeller	38
Vinasse/melasse	20
Oil	14
Linseed	15
Vitamin/mineral premix	46

Sows were paired by BW and randomly assigned to one of two dietary treatments: a diet high or low in fibre. During the experiment the low-fibre diet consisted of the standard compound feed (Table 1), which was fed to meet the maintenance energy requirements of non-pregnant sows while the diet high in fibre consisted of a daily amount of the standard compound feed to meet 70% of the energy maintenance requirement of non-pregnant sows (calculated as 0.7% of BW; CVB, 2010) supplemented with 3 kg of fresh grass silage (estimated 60% NSP/DM; Sappok *et al.*, submitted). The daily feed/silage provided a contrast in the fibre intake by sows with the reduction in the amount of compound feed offered implemented to ensure a high intake of grass silage.

The compound feed was fed in two equal portions at 8:00 and 16:00 h throughout the study, whereas the grass silage was provided in 1 kg-portions at 8:00, 12:00 and 16:00 h. All sows had free access to drinking water and were fed their respective diet for three weeks. At the start of the study (day 1), the group previously fed the high-fibre diet received the low-fibre diet (HL-treatment) and the sows fed the low-fibre diet received the high-fibre diet (LH-treatment). Feed samples (compound feed and silage) were collected at the same day as faecal sampling, pooled at the end of the experiment and used for chemical analyses. Handling of the animals was approved by the ethical committee of Wageningen University and was in accordance with the Dutch law on the use of experimental animals, with amendments made to this law in accordance with Council Directive 86/609/EEC (http://wetten.overheid.nl/ BWBR0003081/).

Faecal collection and inocula preparation

At the start of the main experimental period (day 1), the nine sows in each treatment group were randomly assigned to three subgroups of three animals each so that three inocula per treatment were obtained. Faeces (100 to 200 g) were manually collected directly from the rectum of each sow at seven time points, i.e. day 1, 4, 7, 10, 13, 16 and 19 after the diet change. The faecal samples were immediately stored in CO_2 pre-flushed plastic containers, placed on crushed ice, transported to the laboratory within 1 h after collection and prepared for incubation. Faecal samples were collected and the inocula were prepared at the same time in the same order on each sampling day to reduce variation in fermentation activity due to sampling. In the laboratory, individual faecal samples were homogenized and fresh faeces (±6.5 g) of each of the three sows in a subgroup were pooled into one sample. To each pooled sample, 180 mL of a 0.9% NaCl solution was added and the mixture homogenized with a hand blender for 1 min where after the homogenized mixture was filtered through a nylon cloth (pore size 40 µm, permeability 30%; PA 40/30; Nybolt, Zürich, Switzerland). One mL of

filtrate was collected for the profiling of microbial composition and immediately frozen at -80°C. The filtrate was added to a bicarbonate-phosphate buffered solution as described by Williams *et al.* (2005a) in the ratio 1:16.8 on a weight/volume basis. The preparation of each inoculum was carried out under a constant flow of CO_2 to ensure that anaerobic conditions were maintained.

Substrates

The three substrates used for incubation were chosen based on their contrasting fermentation characteristics (own observations in our laboratory): oligofructose (highly fermentable), soy pectin (highly fermentable) and cellulose (slowly fermentable). Oligofructose (Orafti ® P95) was obtained from Orafti (Tienen, Belgium), soy pectin (Soyafibre-S-DA 100) from Fuji Oil Company Ltd. (Ibaraki, Japan) and cellulose (Vitacel® powdered cellulose for food, LC 200) from J. Rettenmeier & Soehne GmbH + Co (Rosenberg, Germany). All substrates were air-dried and in powder form.

Incubation

During each of the seven fermentation runs (day 1, 4, 7, 10, 13, 16 and 19), three fermentation bottles were incubated simultaneously per inoculum-substrate combination. Per run and inoculum, one blank bottle without substrate was included to monitor background fermentation. Substrates (±0.5 g) were accurately weighed into 300 mL fermentation bottles (Schott, Mainz, Germany) previously filled with CO_2. Subsequently, 89 mL of buffered inoculum solution was dispensed into each bottle, which was then placed in a shaking water (40 rpm) bath at 39°C. Bottles were attached to an 'automated gas production system' to allow the measurement of cumulative gas production (Cone *et al.*, 1996). Incubation was terminated after 72 h of incubation. After the pH of the fermentation fluid was recorded (Hanna Instruments pH meter; Woonsocket, RI, USA) and fermentation fluid was collected for analyses of short chain fatty acids (SCFA) and for ammonia (NH_3) determination. Samples were stored at -20°C.

Chemical analyses

Feed samples and substrates were analysed for dry matter (DM; ISO 6496, 1999), ash (ISO 5984, 2002) and crude protein (CP; ISO 5983, 2005). Feed samples were also analysed for crude fat (CFAT; ISO 6492, 1999) and for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents according to the methods described by Goering and van Soest (1972). The filtrates from diluted pooled faecal samples were analysed for DM and pH. Short

chain fatty acids were measured using a GC and NH_3 was analysed colorimetrically as described by Bosch *et al.* (2008).

Microbial composition profiling

The analysis of faecal microbiota was carried out using an updated version of a recently developed phylogenetic microarray comprehensively targeting pig GI tract microbiota. The design of the microarray, the so-called pig intestinal tract chip (PITChip), was developed and successfully applied by Perez Gutierrez *et al.* (submitted). The updated version of the PITChip (V2.0) used in this study contained over 3300 oligonucleotides targeting 782 bacterial phylotypes reported to occur in the porcine gastrointestinal tract.

DNA extraction from filtered faecal pool samples mixed with 0.9% NaCl solution was performed according to the repeated bead beating (RBB) procedure described by Salonen *et al.* (2010) with minor modifications reported by Yu and Morrison (2004). The DNA was isolated by sequential precipitations and finally purified using the QIAamp DNA Stool Mini Kit columns (Quiagen, Hilden, Germany) according to the manufacturer's recommendations. A fluorometric Quant-iT™ *PicoGreen*® reagent (Molecular Probes, Eugene, OR, USA) was used to measure the DNA content. Additionally, Nanodrop sprectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) and 0.8% agarose gels were used for the quantification and qualification of the DNA extracts.

Hybridization and analysis of the generated data was performed using procedures as described by Rajilić-Stojanović *et al.* (2007, 2009) and modified by Peréz Gutiérrez *et al.* (submitted) for pigs. Two primers (T7prom-Bact-27-for and Uni-1492-rev) were used for amplification of bacterial 16S rRNA gene fragments from 10 ng extracted DNA. PCR products were purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany) and DNA concentration was measured spectro-photometrically (ND-1000, NanoDrop ® Technologies, Wilmington, DE, USA). *In vitro* transcription of the 16S rRNA genes carrying the T7-promoter was performed with the RibosobeSystem (Promega, La Jolla, CA, USA). After 2 h incubation at room temperature, DNA was digested with the Qiagen RNAse-free DNAse kit (Quiagen, Hilden, Germany) and RNA was purified with the RNeasy Mini-Elute Kit, (Quiagen, Hilden, Germany). The RNA concentration was quantified spectro-photometrically. The *in vitro* transcribed RNA was coupled with CyDye using post-labelling reactive dye (Amersham Biosciences, Little Chalfont, UK) dissolved in DMSO for fluorescent labelling. The labelling reactions were incubated during 90 min in the dark at room temperature. The reaction was stopped by addition of hydroxyl-amine and incubation in the dark for 15 min. After termination of the reaction, RNAse-free

water was added and the labelled RNA quantified. Custom microarrays of the 8×15K format were synthesized by Agilent Technologies (Agilent Technologies, Palo Alto CA, USA). Hybridization of the arrays and labelled samples was performed at 62.5°C for 16 h in a rotation oven (Agilent) where after slides were washed in several steps. Data extraction from scan images was done with the help of the Agilent Feature Extraction software, version 9.1 (http://www.agilent.com).

Calculations and statistical analyses

Total gas production was calculated as the amount of gas produced per gram organic matter (OM) of substrate initially incubated (termed OMCV = organic matter corrected volume; in mL g^{-1} OM) after fitting a monophasic model (Groot *et al*., 1996):

$$OMCV = (A/(1+(C/t)^{B}))$$

where OMCV is the total gas produced (mL g^{-1} OM), A is the asymptotic gas production (mL g^{-1} OM), B is a constant determining the sharpness of the switching characteristic of the curve, C is the time at which half of the asymptotic gas production is reached (h) and t represents the time (h). The maximum rate of gas production (R_{max} in mL h^{-1}) and the time (h) at which it occurred (T_{Rmax}) were calculated according to the following equations (Bauer *et al*., 2001):

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

$$T_{Rmax} = C \times (((B - 1)/(B + 1))^{(1/B)})$$

The gas production parameters (e.g. OMCV, C, R_{max}) and end point parameters concentrations of NH_3 and total SCFA (calculated as sum of acetate, propionate, butyrate, iso-butyrate, valeric acid and iso-valeric acid), the molar proportions of the three main SCFA (acetate, propionate and butyrate) and branched chain ratio (*BCR*) are shown as means. Means per substrate were calculated from three inocula per run, which in turn were calculated from the three simultaneously incubated bottles used per inoculum and substrate. *BCR* was calculated as (iso-butyric + iso-valeric acid / total amount of SCFA).
The data were analysed per substrate as a repeated measurement in the MIXED procedure of SAS 9.2 (SAS, 1989), using the following model:

$$Y_{ijk} = \mu + T_i + D_j + (T_i \times D_j) + \varepsilon_{ijk}$$

with the main model effects "treatment" (T_i) and "day (of fermentation run)" (D_j), ($T_i \times D_j$) as the interaction between "treatment" (HL vs. LH) and "day" (day 1,4, …,19) and ε_{ijkl} as the error term. Inoculum was used as subject. Day of fermentation run after diet change was treated as a repeated measure assuming a first order autoregressive covariance structure [AR(1)] because AR(1) fitted the data best based on the Bayesian information criterion (BIC) and Akaike information criterion (AIC) values (Littell *et al*., 1998; Tempelman, 2004). In case of significant effects of T_i, D_j or ($T_i \times D_j$), post hoc analyses were performed for testing differences between treatments, days and between days within treatment, respectively, using the Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS.

To assess the significance of observed differences in relative abundance of individual microbial groups, two-tailed, unpaired t-tests were applied using log10-transformed (log10[x+0.01] relative abundance based on PITChip hybridization data. Resulting p-values were corrected for multiple comparisons, using the "q-value" script as implemented in the Bioconductor package for R (2009; Van den Brink & Braak, 1999).

Simpson's Reciprocal Index (1/D) was used to measure the diversity of microbial profiles obtained by the PITChip analysis and was calculated with the equation $1 = 1/\Sigma Pi^2$, where Pi is the proportion of i^{th} taxon. In order to assess the amplitude of overall changes in microbial community composition between the two treatment groups during the experimental period, a principle response curve (PRC) analysis was carried out using relative abundance of level 2 phylogenetic groups as species data in a redundancy analysis. Results of day 1 for the HL-treatment were used as reference point. Treatment was introduced as an environmental variable and the sampling time points as co-variables (Bauer *et al*., 2001).

Results

The pigs remained in good health and their BW was relatively stable throughout the entire study. Sows in the HL-treatment gained 9 kg (not significant) during the trial (from 249 to 258 kg) and sows of the LH-treatment remained stable (255 vs. 255 kg). All of the daily supplied compound feed was consumed by the sows in both groups. The precise silage intake per pig was not quantified but visual estimation of the approximate percentage left over was ~25%. Sows changed from the low to the high fibre diet required three days to fully accept the silage. The chemical analysis of compound feed, grass silage and substrates is shown in Table 2. The fresh silage contained 553 g kg^{-1} DM and a similar concentration of CP per unit DM compared to the compound feed but more than double the amount of NDF

Table 2. Proximate analyses of the compound feed and grass silage fed to sows and fermentation substrates used in the *in vitro* fermentation assay.

Feed/Substrate	DM	ASH	CP	CFAT	NDF	ADF	ADL
	g kg^{-1}	g kg^{-1} DM					
Feed	886.3	66.9	164.2	41.2	217.4	111.2	24.2
Grass silage	928.5	96.4	169.6	35.2	474.3	280.6	19.9
Oligofructose	957.3	0.6	0.6	-	-	-	-
Soy pectin	883.3	82.7	52.4	-	-	-	-
Cellulose	941.4	3.2	0.0	-	-	-	-

ADF, acid detergent fibre; ADL, acid detergent lignin; CFAT, crude fat; CP, crude protein; DM, dry matter; -, not determined; NDF, neutral detergent fibre.

and ADF on DM basis. The soy pectin contained 52.4 g CP/kg DM and an ash content of 82.7 g/kg DM, while the CP and ash content of the oligofructose and cellulose substrate were below 3.2 g/kg DM.

Fermentation characteristics

The average gas production of the blank bottles was 15.4 ± 7 mL as measured over all 5 fermentation runs. No run-effect could be observed for the blanks. Table 3 shows the mean gas production (OMCV), halftime (*C*) and maximal rate of gas production (R_{max}) obtained during fermentation of the substrates oligofructose, soy pectin and cellulose. A significant treatment×day interaction ($P < 0.05$) was found for OMCV for oligofructose and soy pectin. Day of fermentation run had a significant effect on oligofructose and soy pectin fermentation ($P < 0.05$) with a higher OMCV for the LH-treatment on day 1 compared to day 13 and 19 for oligofructose and with a higher OMCV on day 1 compared to day 10, 16 and 19 for soy pectin. For OMCV from cellulose, the HL-treatment yielded higher values than the LH-treatment (P<0.05 for treatment). The halftime at which asymptotic gas production was reached (*C*) was relatively similar for the readily fermentable substrates oligofructose and soy pectin compared to cellulose, 7.6 and 8.4 compared to 24.5 h. A significant treatment×day interaction ($P < 0.05$) occurred for *C* during the fermentation of oligofructose. Post-hoc analyses did not reveal any significant differences between days within treatments. Maximal rate of gas production was similar for the substrates oligofructose and soy pectin compared to cellulose, about 67.9 and 68.2 vs. 14.7 mL h^{-1}.

A significant treatment×day interaction ($P < 0.01$) was found for R_{max} during fermentation of oligofructose and soy pectin. The interaction for the fermentation of oligofructose was mainly caused by a higher R_{max} for the HL-treatment on day 1

Table 3. *In vitro* fermentation parameters (OMCV, *C* and R_{max}) after 72 h of *in vitro* fermentation for three substrates fermented with faecal inoculum of sows receiving high or low fermentable diets over a 19 day period after a dietary change.

Parameter	Substrate	Treatment	Days after diet change							Pooled SEM	*P*-values		
			1	4	7	10	13	16	19		Treatm.	Day	Treatment x Day
Organic matter corrected gas volume (OMCV in mL g^{-1} organic matter)													
	Oligofructose	High-Low	379	386	399	406	398	385	390	8.5	0.562	**0.028**	**0.002**
		Low-High	415^{a}	399^{ab}	388^{ab}	396^{ab}	367^{b}	388^{ab}	362^{b}				
	Soy pectin	High-Low	355	371	372	360	363	352	358	6.6	0.795	**0.013**	**0.029**
		Low-High	387^{a}	356^{ab}	371^{ab}	353^{b}	356^{ab}	354^{b}	346^{b}				
	Cellulose	High-Low	347	338	380	389	364	374	357	17.7	**0.037**	0.259	0.122
		Low-High	362	322	331	324	310	329	309				
Halftime of asymptotic gas production (*C* in h)													
	Oligofructose	High-Low	7.5	7.9	7.3	7.4	7.6	7.5	7.2	0.2	0.168	0.811	**0.011**
		Low-High	7.3	7.1	8.1	7.8	7.5	8.0	8.1				
	Soy pectin	High-Low	7.8	8.0	8.2	8.8	8.8	8.5	8.1	0.3	0.392	0.223	0.215
		Low-High	9.0	8.3	8.6	8.9	8.3	8.4	8.1				
	Cellulose	High-Low	23.3	23.5	24.5	22.9	22.0	23.9	24.5	1.4	0.155	0.198	0.109
		Low-High	27.5	24.2	23.7	28.0	24.1	26.6	23.8				
Maximal rate of gas production (R_{max} in mL h^{-1})													
	Oligofructose	High-Low	84.1^{a}	74.2^{ab}	67.6^{ab}	69.0^{ab}	60.6^{b}	57.9^{b}	57.4^{b}	4.3	0.726	0.157	**0.004**
		Low-High	61.2	69.3	61.3	70.9	73.5	72.3	71.7				
	Soy pectin	High-Low	74.9	66.4	68.0	66.7	63.7	64.8	64.9	2.6	0.378	0.620	**0.004**
		Low-High	66.3	71.3	66.9	66.0	71.2	69.6	73.9				
	Cellulose	High-Low	17.0	16.7	16.7	17.2	17.3	16.1	13.9	1.4	**0.030**	0.744	0.762
		Low-High	13.5	13.5	13.7	11.5	12.7	13.0	12.6				

[a,b] means with different superscripts within row differ significantly ($P < 0.05$); High-Low, sow group changed from high to low fibre; Low-High, sow group changed from low to high fibre; SEM, standard error mean.

compared to day 13, 16 and 19. Further, a decrease of R_{max} could be seen for the HL-treatment and an increase could be seen for the LH-treatment during fermentation of oligofructose and soy pectin. Treatment had an effect only on cellulose fermentation ($P < 0.05$), with the HL- treatment showing faster fermentation compared to the LH-treatment.

Table 4 shows means of SCFA concentration, acetate:propionate ratio and NH_3 concentration obtained during fermentation of the substrates oligofructose, soy pectin and cellulose. Fermentation of oligofructose and soy pectin yielded higher SCFA concentrations compared to cellulose, both 10.7 vs. 8.5 mmol g^{-1} OM. A significant treatment×day interaction ($P < 0.01$) was found for the concentration of SCFA for oligofructose. Day of fermentation run had a significant effect on oligofructose fermentation ($P < 0.001$) with a higher SCFA concentration for the HL-treatment on day 19 compared to day 1 to 13. No effect was found for soy pectin and a treatment effect for cellulose ($P < 0.05$) with the HL-treatment yielding higher values than the LH-treatment.

The acetate: propionate ratio increased for the HL-treatment during fermentation of oligofructose and soy pectin and decreased for the LH- treatment. A significant treatment×day interaction occurred for the ratio during the fermentation of oligofructose ($P < 0.05$) and soy pectin ($P < 0.01$). Post-hoc analyses did not reveal any significant differences between days within treatments for oligofructose. Day of fermentation run had a significant effect on soy pectin fermentation ($P < 0.05$) with a higher ratio for the HL-treatment on day 16 and 19 compared to day 1 and 13. No effect was observed for cellulose.

Fermentation of soy pectin yielded the highest NH_3 concentration, followed by oligofructose and cellulose (1.76, 1.46, and 1.29 mmol g^{-1} OM). No treatment×day interaction was found for NH_3. A day effect was observed ($P < 0.05$) during fermentation of oligofructose and soy pectin, but no effects for fermentation of cellulose.

Analysis of faecal microbial composition

Large intestinal microbiota dynamics was determined in sows during the first 19 days after a change in diet from high to low fibre and from low to high fibre, respectively. Table 5 shows the abundance and development of microbial composition in the sow's large intestinal microbiota on different days after the diet change (day 1) as measured by two-tailed, unpaired t-tests, corrected for multiple comparisons, for both treatments.

Table 4. Concentrations of short chain fatty acids, acetate: propionate ratio and ammonia after 72 h of *in vitro* fermentation for three substrates fermented with faecal inoculum of sows receiving high or low fermentable diets over a 19 day period after a dietary change.

Parameter	Substrate	Treatment	Days after diet change							Pooled SEM	*P*-values		
			1	4	7	10	13	16	19		Treatment	Day	Treatment x Day
Short chain fatty acids (mmol g^{-1} OM)													
	Oligofructose	High-Low	10.3^{b}	10.7^{b}	10.5^{b}	10.8^{b}	10.3^{b}	11.4^{ab}	12.2^{a}	0.22	0.221	**<0.001**	**0.007**
		Low-High	10.6	11.3	10.5	10.5	9.9	11.1	10.7				
	Soy pectin	High-Low	10.7	11.1	10.9	10.6	10.2	10.9	11.4	0.36	0.204	0.194	0.391
		Low-High	11.0	11.1	10.5	10.2	10.1	10.8	10.1				
	Cellulose	High-Low	9.1	8.8	9.0	9.2	9.0	8.2	9.9	0.47	**0.046**	0.307	0.104
		Low-High	8.2	8.9	7.7	8.0	7.2	8.3	8.2				
Acetate: propionate ratio													
	Oligofructose	High-Low	1.21	1.51	1.64	1.66	1.46	1.63	1.53	0.13	0.171	0.275	**0.022**
		Low-High	1.60	1.63	1.49	1.22	1.18	1.37	1.22				
	Soy pectin	High-Low	1.54^{b}	1.87^{ab}	1.78^{ab}	1.92^{ab}	1.43^{b}	2.12^{a}	2.01^{ab}	0.12	0.170	**0.042**	**0.001**
		Low-High	1.95	1.90	1.68	1.64	1.50	1.61	1.46				
	Cellulose	High-Low	1.39	1.86	1.87	1.85	1.66	1.46	1.79	0.45	0.712	0.837	0.782
		Low-High	2.04	1.69	2.01	1.68	1.73	1.76	1.60				
Ammonia (mmol g^{-1} organic matter)													
	Oligofructose	High-Low	1.40	1.35	1.49	1.45	1.57	1.49	1.54	0.04	0.246	**0.044**	0.201
		Low-High	1.48	1.38	1.46	1.45	1.49	1.42	1.40				
	Soy pectin	High-Low	1.70	1.66	1.85	1.72	1.86	1.78	1.86	0.05	0.362	**0.028**	0.112
		Low-High	1.83	1.77	1.77	1.63	1.73	1.71	1.70				
	Cellulose	High-Low	1.34	1.11	1.25	1.24	1.33	1.28	1.40	0.05	0.552	0.222	0.071
		Low-High	1.31	1.33	1.36	1.22	1.29	1.27	1.28				

[a,b] means with different superscripts within row differ significantly ($P < 0.05$); High-Low, sow group changed from high to low fibre; Low-High, sow group changed from low to high fibre; SEM, standard error mean.

Table 5. Relative abundance (%) of genus-like phylogenetic groups of faecal microbiota (relative contribution > 1%) for two groups of sows; one switched from diets high in fibre to diets low in fibre (HL) and vice versa (LH) over a 19 day period after a dietary change.

Higher taxonomic group	Group	Day 1			Day 4			Day 7			Day 10			Day 13			Day 16			Day 19		
		Treatment			Treatment			Treatment			Treatment			Treatment			Treatment			Treatment		
		HL	LH	q-value	HL	LH	q-value	HL	LH	q-value	HL	LH	q-value	HL	LH	q-value	HL	LH	q-value	HL	LH	q-value
		%	%		%	%		%	%		%	%		%	%		%	%		%	%	
Bacteroidetes	*Alistipes*-like	6.07	3.01	0.237	5.86	3.94	0.474	4.28	5.53	0.190	2.87	4.65	0.131	2.87	5.37	**0.033**	2.94	4.62	**0.023**	3.06	5.01	0.089
	Bacteroides distasonis-like	2.19	1.09	0.069	1.86	1.83	0.983	1.47	2.21	**0.046**	0.97	2.27	**0.031**	1.11	2.61	**0.033**	1.40	2.03	0.091	1.24	2.19	0.061
	Uncultured *Bacteroidetes*	0.86	0.88	0.636	0.97	0.53	0.983	1.00	1.00	0.862	1.12	0.75	0.219	0.57	0.83	0.150	0.87	0.91	0.471	0.77	0.73	0.760
	Uncultured *Porphyromonadaceae*	1.80	1.14	0.293	1.49	1.49	0.983	1.24	1.73	0.409	0.93	1.48	0.115	0.98	1.76	**0.033**	0.97	1.58	**0.021**	1.00	1.49	0.097
	Uncultured *Prevotella*	5.74	3.60	0.134	5.52	4.14	0.983	5.18	5.73	0.670	3.77	4.86	0.206	3.81	5.94	0.064	3.81	4.86	**0.032**	3.67	5.26	0.076
Bacilli	*Enterococcus*-like	2.69	2.62	0.527	2.71	2.78	0.983	2.45	2.59	0.714	2.58	2.69	0.482	2.52	2.54	0.424	2.55	2.65	0.273	2.48	2.77	0.064
	Lactobacillus acidophilus-like	0.47	0.83	0.237	0.48	0.67	0.983	0.75	0.23	0.091	1.05	0.23	**0.031**	1.20	0.30	0.089	0.68	0.34	0.105	0.78	0.24	0.106
	Lactobacillus amylovorus-like	0.51	0.67	0.483	0.49	0.53	0.983	0.85	0.21	0.091	1.06	0.19	**0.031**	1.16	0.29	0.100	0.69	0.32	0.126	0.72	0.20	0.129
	Lactobacillus delbrueckii-like	0.99	1.19	0.483	1.09	0.99	0.983	1.35	0.54	0.198	1.77	0.56	**0.043**	1.81	0.75	0.150	1.21	0.83	0.190	1.21	0.58	0.268
	Lactobacillus plantarum-like	0.56	0.81	0.241	0.56	0.61	0.983	0.62	0.49	**0.029**	0.93	0.53	0.171	1.08	0.49	**0.033**	0.82	0.51	0.100	0.95	0.49	0.071
	Lactobacillus salivarius-like	1.35	1.33	0.525	1.25	1.42	0.349	1.29	1.37	0.668	1.36	1.49	0.219	1.31	1.44	0.089	1.41	1.50	0.091	1.41	1.48	0.141
	Streptococcus bovis-like	0.71	1.38	0.483	0.79	1.20	0.983	0.82	0.87	0.755	0.80	0.86	0.401	0.83	0.85	0.382	1.22	0.79	0.100	1.52	0.77	0.076
	Streptococcus suis-like	0.36	1.62	0.391	0.40	1.14	0.981	0.45	0.59	0.684	0.47	0.52	0.517	0.65	0.47	0.189	1.18	0.42	**0.048**	1.72	0.42	0.055
Clostridium cluster I	*Clostridium perfringens*-like	1.80	2.03	0.494	1.26	1.16	0.983	1.72	1.16	0.190	2.25	1.53	0.162	2.39	1.34	**0.033**	1.93	1.23	**0.021**	2.04	1.28	0.055
Clostridium cluster IV	*Anaerotruncus*-like	0.99	1.14	0.340	1.15	1.17	0.983	1.16	1.09	0.679	1.11	1.02	0.294	1.10	0.93	0.064	1.06	1.03	0.174	1.05	1.04	0.712
	Clostridium cellulosi-like	2.12	2.22	0.485	2.14	2.28	0.983	2.26	2.30	0.728	2.06	1.99	0.514	2.02	1.90	0.208	1.97	1.98	0.470	2.00	2.09	0.441
	Papillibacter cinnamivorans-like	1.18	1.29	0.483	1.31	1.33	0.983	1.26	1.24	0.827	1.28	1.25	0.516	1.34	1.19	0.135	1.35	1.33	0.413	1.33	1.36	0.594
	Ruminococcus bromii-like	0.82	0.87	0.483	0.85	0.92	0.983	0.93	0.95	0.804	0.85	0.94	0.181	0.88	0.97	0.203	1.00	1.01	0.429	0.98	1.02	0.531
	Ruminococcus callidus-like	2.25	2.45	0.483	2.36	2.31	0.983	2.45	2.39	0.804	2.50	2.32	0.276	2.46	2.16	0.132	2.35	2.30	0.359	2.28	2.31	0.507
	Sporobacter termitidis-like	8.24	9.22	0.440	9.02	9.70	0.789	9.22	9.14	0.828	8.57	8.62	0.614	8.57	8.14	0.257	8.90	8.70	0.358	9.06	8.78	0.551
	Subdoligranulum-like	0.98	1.07	0.440	0.95	1.16	0.349	1.05	1.17	0.575	1.02	1.17	**0.045**	1.02	1.17	0.131	1.13	1.27	0.124	1.14	1.23	0.138
	Uncultured *Clostridia* IV	3.36	3.90	0.237	3.54	3.98	0.983	3.41	3.55	0.614	3.65	3.79	0.371	3.89	3.59	0.166	4.00	3.88	0.271	4.24	3.72	0.114
Clostridium cluster IX	*Mitsuokella multacida*-like	1.13	1.27	0.483	1.24	1.31	0.983	1.08	1.21	0.636	1.04	1.20	0.128	1.14	1.26	0.240	1.26	1.24	0.449	1.31	1.31	0.760
Clostridium cluster XI	*Anaerovorax*-like	1.51	1.70	0.483	1.69	1.75	0.983	1.65	1.53	0.409	1.82	1.64	0.219	1.84	1.52	0.150	1.75	1.68	0.240	1.74	1.72	0.760
	Clostridium difficile-like	2.37	2.92	0.483	2.56	2.44	0.983	2.70	2.16	0.198	3.08	2.52	0.266	3.25	2.35	**0.033**	2.89	2.55	0.091	2.86	2.59	0.276
Clostridium cluster XIVa	*Bryantella*-like	1.09	1.06	0.527	1.09	1.07	0.983	1.05	1.04	0.862	1.05	1.04	0.594	1.06	1.03	0.208	1.06	1.10	0.264	1.02	1.13	0.071
	Butyrivibrio crossotus-like	1.42	1.54	0.405	1.78	1.46	0.349	1.64	1.56	0.755	1.61	1.49	0.152	1.53	1.42	0.203	1.51	1.53	0.429	1.43	1.56	0.239
	Eubacterium rectale-like	2.21	1.79	0.241	1.81	1.81	0.983	1.82	1.88	0.728	1.90	1.99	0.401	1.95	2.11	0.244	1.95	2.03	0.280	1.89	2.07	0.220
	Lachnobacillus bovis-like	1.36	1.37	0.630	1.32	1.32	0.983	1.34	1.36	0.862	1.42	1.41	0.614	1.41	1.38	0.262	1.42	1.42	0.493	1.36	1.36	0.760
	Lachnospira pectinoschiza-like	3.55	3.46	0.527	3.44	3.41	0.983	3.38	3.36	0.862	3.51	3.51	0.614	3.44	3.45	0.402	3.55	3.53	0.449	3.45	3.59	0.316
	Ruminococcus obeum-like	2.73	2.61	0.440	2.76	2.83	0.983	2.62	2.68	0.756	2.70	2.64	0.498	2.68	2.70	0.382	2.88	2.91	0.413	2.77	2.99	0.138
	Uncultured *Clostridia* XIVa	2.02	2.32	0.396	2.11	2.07	0.983	2.24	2.23	0.862	2.29	2.11	0.281	2.26	2.01	0.107	2.19	2.20	0.455	2.15	2.19	0.531
Clostridium cluster XIVb	Uncultured *Clostridia* XIVb	1.50	1.86	0.216	1.54	1.69	0.983	1.80	1.61	0.531	1.79	1.63	0.130	1.67	1.56	0.208	1.68	1.60	0.185	1.68	1.62	0.461
Gammaproteobacteria	*Escherichia coli*-like	0.87	0.96	0.440	0.92	0.86	0.983	1.03	0.94	0.680	1.09	1.04	0.472	1.02	0.95	0.290	1.02	0.92	0.174	0.96	0.89	0.295
Spirochaetes	*Treponema*-like	1.43	1.24	0.237	1.61	1.65	0.983	1.19	1.56	0.564	1.00	1.87	0.104	1.05	1.58	0.113	1.21	1.31	0.119	1.12	1.28	0.258

The largest differences in abundance of bacterial species were observed for the higher taxonomic groups *Bacteroidetes* and *Bacilli*. On day 1 and 4, the animals in the HL-group had a higher abundance of *Bacteroidetes* compared to the animals in the LH-group (16.66 and 15.71% vs. 9.72 and 11.93%). In turn, the abundance of *Bacilli* was lower for animals in the HL-group. From day 7 onwards, however, the relationship was reversed and the LH-group showed a higher abundance of *Bacteroidetes* and a lower abundance of *Bacilli* compared to the HL-group. Significant changes could be seen on day 7, 10, 13 and 16 for several other species in both groups. Except for day 1, *Clostridium perfringens*-like species showed a lower abundance in the LH-group compared to the HL-group, which was significant on day 13 and 16. For the LH-group, *Clostridium difficile*-like species were significantly less abundant on day 13, while most other species showed only non-significant differences.

Microbial diversity as measured by the Simpson's Reciprocal Index is shown in Figure 1. On most days after the diet change, Simpson's Reciprocal Index (1/D) fluctuated around 329 (SD ± 5.15) for the HL-group and around 337 (SD ± 3.95) for the LH-group, except for day 7.

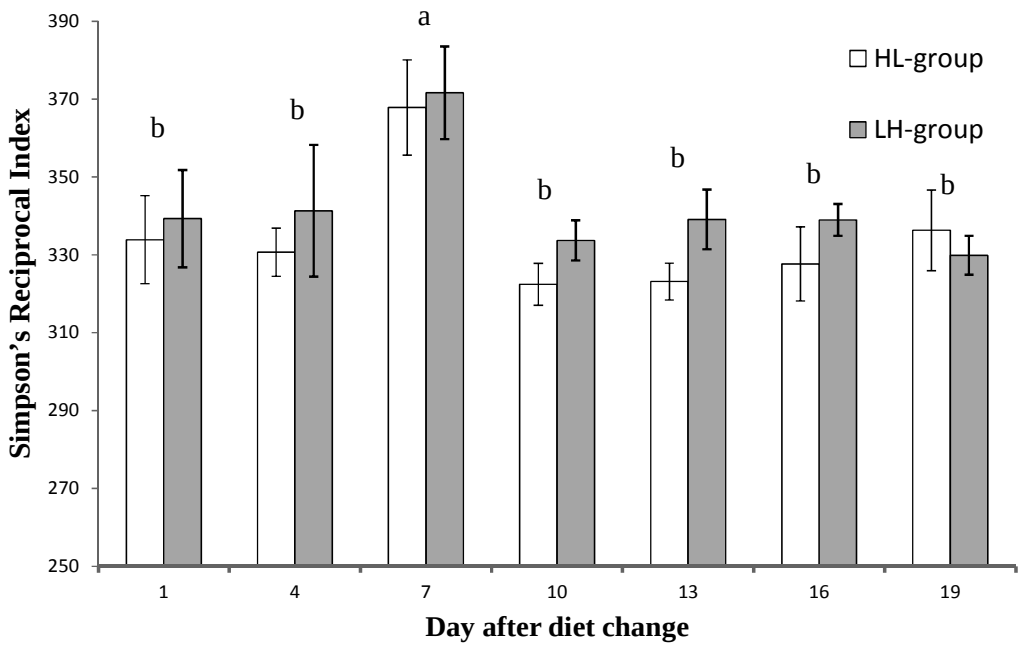

Figure 1. Simpson's Reciprocal Index for both treatment groups at different days after a diet change. A higher index indicates a higher degree of diversity. HL, sow group changed from high to low fibre; LH, sow group changed from low to high fibre. [ab] Days with different superscripts differed significantly from each other ($P < 0.001$), groups did not differ.

During this day, the reciprocal index was significantly higher ($P < 0.001$) for both treatments and reached 368 and 372, respectively. Comparison of the data for the two treatments over time showed an average 2.3% higher reciprocal index for the LH-treatment compared to the HL-treatment although this difference was not significant ($P < 0.13$).

PRC analysis, which allows identification of differences in microbiota composition between treatments in time, showed that the treatment groups were more different towards the end of the experiment compared to the beginning (Figure 2). However, none of the canocical axes were significant. The two treatment groups showed relatively equal microbiota composition on day 4 after the diet change. For the LH-group, the largest change in microbiota composition occurred between day 4 and 7 while for the HL-group, it occurred between day 7 and 10. On day 7, the composition between microbial species changed for both treatments and was reversed compared to day 1 and remained reversed the following days, confirming observations made by univariate analyses of variation in individual microbial groups as described above.

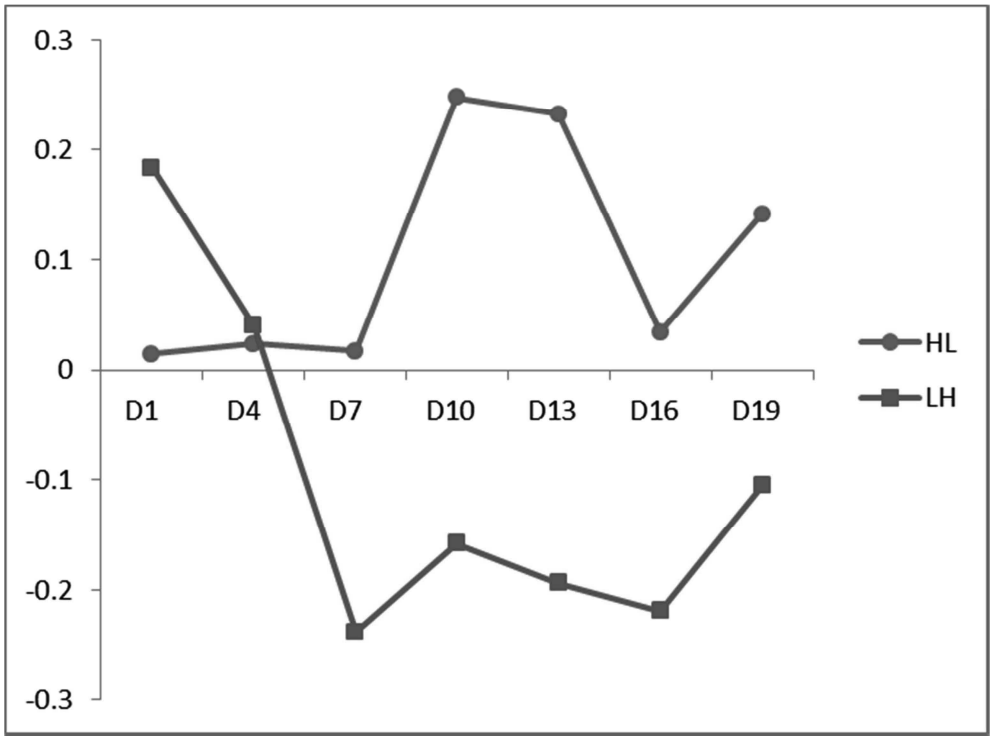

Figure 2. Principal response curves for two dietary treatments after a diet change. HL, dietary treatment changed from high to low fibre; LH, dietary treatment changed from low to high fibre.

The switch of microbial composition between treatments was mainly caused by a change in species of *Bacteroidetes* and *Bacilli* (data not shown) after the diet change; abundance of *Bacteroidetes* was relatively high on day 1 for the HL-group and decreased over time, while abundance of *Bacilli* was relatively low on day 1 for the LH-group and increased.

Discussion

The study reported here was conducted in order to investigate the adaptation of faecal microbes in sows over 19 days when sows were changed from a high to a low fibre diet (HL treatment) and from a low to a high fibre diet (LH-treatment). The high fibre diet (30% NDF) included silage as an NDF source and was expected to lead to a different microbial activity and community compared to the diet low in fibre (22% NDF) which consisted of a standard compound feed for sows.

Differences between fermentation parameters

Fermentation parameters OMCV, *C*, and $R_{max,}$ and end-product profiles (SCFA and NH_3 concentrations and acetate: propionate ratio) did not differ majorly between treatments for the substrates oligofructose and soy pectin, but did differ for cellulose. The faecal microbiota of the animals in both treatments had an overall similar fermentation capacity for highly fermentable substrates but differed for slow fermentable substrates. The OMCV and *C* values presented here are within the range as values found in other studies (Bauer *et al*., 2001; Bindelle *et al*., 2007a; Sappok *et al*., 2012). R_{max} values observed in the latter studies ranged from 55 to 70 mL h^{-1} for oligofructose and soy pectin, which is only slightly lower compared to the R_{max} values obtained using the faeces from the animals on a high fibre diet in the present study (84.1 and 71.7 mL h^{-1}). For the fermentation of cellulose, R_{max} values in these studies (Bauer *et al*., 2001; Bindelle *et al*., 2007a; Sappok *et al*., 2012) ranged between 14 and 19 mL h^{-1} compared to an average value of 12.9 mL h^{-1} found here for the LH-treatment, but were similar to values found for the HL-treatment (16.4 mL h^{-1}). Concentrations of SCFA were relatively similar compared to previous studies (Bauer *et al*., 2001; Sappok *et al*., 2012a) for all substrates, although Jonathan *et al.*(2012) showed a lower concentration of SCFA for oligofructose (6.5 vs. 10.7 mmol g^{-1} OM). In the latter study, donor sows were fed a diet low in fermentable NSP (5%), which could have led to a microbial community in the large intestine which was poorly adapted to the fermentation of oligofructose and therefore did produce low concentrations of SCFA.

The significant difference in gas production found across days of adaptation for oligofructose and soy pectin occurred mainly due to a decreased OMCV over time for the LH-treatment, which was also reflected in a decreased SCFA concentration for soy pectin. The concentration of acetate decreased for both substrates (9% for oligofructose and 19% for soy pectin, data not shown), suggesting a decrease in acetogenic bacteria which results in less gas production. It is known that gas production depends on the composition of SCFA and acetate yields the largest amounts of gas (Beuvink & Spoelstra, 1992). A reason for the decrease in OMCV and shift in SCFA metabolism could be that animals in the LH-treatment were initially adapted to high concentrations of readily fermentable substrates from the low fibre diet which decreased after the diet change. In contrast to the decreased OMCV, R_{max} measured for the LH-treatment group increased for oligofructose and soy pectin (15% and 10%), while it decreased for the HL-treatment (32% for oligofructose and 13% for soy pectin). Gas production (OMCV) remained relatively similar over time for the HL-treatment.

The relatively stable gas production for the animals in the HL-treatment (being initially on a high fibre level) compared to the decrease in gas production of the animals in the LH-treatment indicates that adaptation of the HL-animals was not complete and occurs relatively slow. In turn, the larger difference in R_{max} for the HL-treatment between day 1 and 19 compared to the LH-treatment indicates that adaptation of the large intestinal microbiota was not fully complete for the LH-treatment as well and is likely to further increase. A low R_{max} was found on day 19 for the HL-group compared to the previous days, comparable to the average R_{max} for the LH-treatment, indicating that adaptation was not fully completed. Compared to the other substrates and treatments, the concentration of SCFA increased most during fermentation of oligofructose when animals were changed from a high to a low fibre diet. This might indicate that large intestinal microbiota from sows adapting to a lower fibre diet preferentially ferment oligofructose as apparent from SCFA production compared to microbiota from sows adapting to a high fibre diet. For SCFA concentration, no actual plateau was reached for either treatment after 19 days for any substrate; values for the last 3 days varied 7 to 15%. The animals which were previously on a high fibre diet (HL-treatment) had an overall higher capacity to ferment cellulose compared to the LH-treatment (11% for OMCV and 22% for R_{max}). A reason for this observation could be that animals in the LH-treatment had an overall lower capacity to ferment cellulose or were not fully adapted yet. It is known from previous studies (Sappok *et al*., 2012; Sappok *et al*., submitted) that the capacity of pigs to ferment fibre can vary up to 25% in terms of R_{max} between inocula. Previous studies have shown that adaptation of animals to fibre can take over 86 days in terms of cellulase activity. Varel *et al.* (1984) showed that cellulase activity in faeces of gilts increases rapidly within the

first 5 days after changing to a high fibre diet (35% alfalfa meal), then decreases until 37 days and subsequently increases until 86 days without reaching a plateau. Longland *et al.* (1993) fed growing pigs (25 to 45 kg) diets containing sugar beet pulp or wood-cellulose and concluded that it takes 21 to 35 days before stable values for whole tract digestibility are reached.

It can be concluded that the full fermentation capacity of microbiota from the LH-treatment was not reached yet after 19 days of silage consumption and more time would have been needed for microbes to reach a stable activity. This was an unexpected finding as the majority of *in vitro* fermentation studies adapt pigs to the diets for 7 to 14 days (Bauer *et al*., 2004; Anguita *et al*., 2006; Bindelle *et al*., 2009; Martín-Peláez *et al*., 2009; Awati *et al*., 2006). Thus, animals in the HL-treatment might have also not been fully adapted on day 1 of the actual experiment, after consuming silage for three weeks in the pre-experimental period. Especially for slow fermentable substrates like cellulose, 19 days of adaptation to a high fibre diet is not sufficient to reach constant fermentation capacities. In hind side, a longer period than 19 days would have been advantageous to determine the precise period of total adaptation of pigs to diets contrasting from nearly no or few easy fermentable NSP and with a high level of NSP.

However, it has to be considered that the composition of roughages (grass silage fed to sows) underlies natural variability, due to time and location of harvest, but also due to a varying intake and dietary selection by animals. Roughage intake may have differed in the present study between *in vitro* assessment and this may have contributed to the lack of a stable microbiota composition. Microbiota is continuously adapting to changes in substrate supply and it could be that no steady state can be obtained when roughages are fed. Future studies should examine a more extended time period after a diet change has occurred, especially when the diet changes from low to high fibre.

Dynamics of microbiota composition after diet switch

The largest changes in microbiota species over time were detected for the abundance of *Bacteroidetes*, which was lower for animals on a low fibre diet and relatively higher for animals on a high fibre diet. The results for the first two fermentation runs (day 1 and 4) showed that the abundance of *Bacteroidetes* was lower for the LH-treatment, compared to HL-treatment, suggesting that silage intake directly after the diet change is still minor as compared to later in the experimental period.

On day 7, however, the relative abundance of *Bacteroidetes* was higher for the LH-treatment compared to the HL-treatment, suggesting that the main change in microbial composition after a diet change including grass silage seems to occur in

about 7 days (or earlier) after the diet change. During the following days, abundance and composition of microbiota became relatively stable. This was confirmed with PRC analysis, showing that the difference in microbiota composition between treatments remained relatively constant during the later phase of the experiment (Figure 2).

Only limited information is available concerning the relationship between fibre and microbiota composition in the porcine large intestine. Thirty years ago, Varel *et al.* (1982) reported that especially *Bacteroides succinogenes* and *Ruminococcus flavefaciens* increased when pigs were fed high fibre diets with alfalfa as a fibre source. In line with the previous finding from Varel *et al*. (1982), Lin *et al.* (2011) showed that when fibres were fermented to short chain fatty acids in batch cultures using pig intestinal digesta, the most prominent bands after DGGE of PCR-amplified bacterial 16S rRNA gene fragments were affiliated with *Bacteroidetes* and *Firmicutes*. The observed change in microbial composition for the LH-treatment occurring between 4 to 7 days after the diet change indicates that digesta at this time starts providing noticeably slower fermentable substrates for those members of the bacterial community (likely *Bacteroidetes*) using this as an energy source. In turn, for animals changed to a low fibre diet (HL-treatment) digesta seems to provide less slow fermentable and more readily fermentable substrate after 4 to 7 days after the diet change for those members of the microbial community (likely *Bacilli*) which can utilize more readily fermentable substrates compared to those in grass silage.

The Simpson Reciprocal Index (1/D) showed that the diet change either from low to high or from high to low fibre leads to the highest bacterial diversity on day 7 after the diet change (Figure 1). This might be related to the above mentioned microbial switch for the groups of *Bacteroidetes* and *Bacilli* which had occurred by day 7. The high index indicted that after a diet change more species then usual build up due to the change in fibre-content of digesta, whereas at a later time the diversity seems to decrease again and the level of diversity remains stable, pointing towards establishment of a climax community adapted to the new digesta composition.

Regarding the PCR, the main change in microbial composition occurred at the end of the first week after the diet change and reached a plateau after 16 days. However, it cannot be confirmed yet if this microbial shift remains stable after 19 days.

In future studies, emphasis should be placed on the development of microbial species in relation to fermentation kinetics, using advanced functional microbiomics approaches such as metatranscriptomics and metaproteomics. These approaches, compared to rRNA gene-targeted approaches such as DGGE- and PITChip analysis, can more directly provide information regarding the metabolic

activity of intestinal microorganisms based on the identification of active pathways (Zoetendal *et al*., 2012; Kolmeder *et al*., 2012).

Conclusions

A diet change led to significant changes in microbial composition and fermentation activity regarding gas production and short chain fatty acid concentration within 19 days for readily fermentable substrates oligofructose and soy pectin. Changes in gas production were higher for animals of the LH-treatment compared to the animals of the HL-treatment, indicating a faster adaptation of animals when they are changed from a low to a high fibre diet compared to when they are changed from a high to a low fibre diet for readily fermentable substrates. Overall, adaptation of the large intestinal microbiota of sows as a result of ingestion of low and high fibre diets seems to take longer than 19 days, especially for the ability to ferment slowly fermentable substrates. The main change in microbiota species *Bacteroidetes* and *Bacilli* seems to occur within the first seven days after a diet change.

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Chapter 4

Repeated measurements of *in vitro* fermentation of fibre-rich substrates using large intestinal microbiota of sows

Variation of fermentation kinetics in repeated measures

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Abstract

Background Fibrous ingredients for pig diets can be characterized by *in vitro* fermentation. *In vitro* fermentation methods often use a one-time measurement of gas production during the incubation of test substrates with one faecal inoculum. The representativeness of this approach can be questioned as measuring time and number of animals from which inoculum originates may influence fermentation results. An *in vitro* fermentation trial was conducted incubating three fibrous substrates with three inocula in five replicates (different fermentation runs) to test the influence of run and origin of inocula.

Results Total gas production and maximal rate of gas production differed ($P < 0.05$) between fermentation runs, but less than substrates ($P < 0.01$). The ranking order between substrates remained similar for each run. Fermentation of cellulose led to higher coefficients of variation between inocula compared to the fast fermentable substrates oligofructose and soy pectin. Differences ranged from 2% for total gas production up to 25% for maximal rate of gas production.

Conclusion One fermentation run can provide representative results for substrate ranking. Using multiple inocula mixed from 4 faecal samples each leads to high coefficients of variation for slow fermentable substrates like cellulose. Future studies should examine the optimal number of animals for inocula preparation to decrease variation.

Introduction

In pig nutrition, additional dietary fibre is sometimes added to the daily ration of animals, e.g. sows or organic fattening pigs for animal health and welfare reasons (De Leeuw *et al.*, 2008; Williams *et al.*, 2001). Dietary fibre consists mainly of non-starch polysaccharides (NSP) which are fermented along the gastrointestinal tract. For readily fermentable NSP, the fermentation starts in the distal part of the pig's small intestine, while slow fermentable NSP are mainly fermented by microbes residing in the large intestine. The extent of NSP fermentation depends on the composition, on adaptation and activity of intestinal microbiota, on transit time of digesta, on the amount and type of NSP and on their physicochemical properties (Freire *et al.*, 2000; Bindelle *et al.*, 2008).

Increased inclusion of NSP in pig diets can result in a reduced digestibility of nutrients, because of a lower overall total tract digestibility of especially NSP but also a reduced digestibility of other macronutrients and energy (Owusu-Asiedu *et al.*, 2006; Högberg *et al.*, 2004; Kirchgeßner *et al.*, 1989). This reduced nutrient digestibility associated with inclusion of NSP can result in a decreased animal weight gain. On the other hand, inclusion of NSP in pig diets has several beneficial effects, e.g. reduction in ulcers and constipation (Williams *et al.*, 2001). Microbial fermentation of NSP in the large intestine also yields valuable nutritional substrates, in the form of short chain fatty acids (SCFA), which can contribute between 11 to 30% to the animals' maintenance energy requirements (Yen *et al.*, 1991; Imoto & Namioka, 1978). In addition, SCFA can have antimicrobial effects which prevent establishment of pathogenic bacteria (Cummings, 1983). Inclusion of dietary fibre also can lead to a shift in N-excretion from easy degradable urine-urea to a more stable form of N in faecal microbial protein. This shift in N-excretion can result in reduced ammonia emissions and therefore less environmental pollution (Nykänen *et al.*, 2010; Clark *et al.*, 2005).

In order to optimize the benefits of including NSP in the diets of growing animals, it is important to predict the fermentation capacity of NSP containing feed ingredients which can be rapidly measured *in vitro*. Hereby, NSP substrates are incubated with a faecal inoculum prepared from a faecal pooled sample (Bindelle *et al.*, 2007c; Williams *et al.*, 2005a). *In vitro* fermentation capacity is a poor predictor of *in vivo* digestibility of substrates, but the fermentation results do reflect the *in vivo* situation by showing e.g. a similar ranking-order of substrates (Anguita *et al.*, 2006; Duda, 2004). Many studies use *in vitro* fermentation methods to assess the fermentation capacity of NSP-substrates for pigs, however, only few validation studies have been conducted (Coles *et al.*, 2005). There is still a lack of information on some assumptions commonly used in *in vitro* studies with pigs, like the adaptation time of animals to an experimental diet, repeatability of fermentation

results or the number of pigs used per inoculum. Kass *et al.* (1980) tested the adaptation time of animals to a NSP-rich diet. In most studies, the results of only one fermentation run are taken to be representative for pigs adapted to a particular diet (Bindelle *et al.*, 2007c; Sappok *et al.*, 2009). Also using one pooled faecal inoculum from either three or four donor pigs (to account for between-animal variation) is generally taken to be adequate to obtain valid results for the incubated substrates (Bindelle *et al.*, 2007c; Le Goff *et al.*, 2003; Bauer *et al.*, 2003). Data from human nutritional studies, however, suggest that more than four individuals are required to provide representative faecal inoculum-material (Edwards *et al.*, 1996).

The main objective of the current study was to determine whether inoculum from the same sets of animals (repeated use of inoculum of the same origin) leads to similar fermentation results at successive fermentation runs. Further, the variation in fermentation kinetics and end products between inocula within fermentation run was studied. An *in vitro* gas production trial was conducted with three inocula mixes originating from same sets of 4 pigs fed the same diet for a total of five consecutive fermentation runs/weeks. Three purified indigestible carbohydrate sources (oligofructose, soy pectin and cellulose) varying in fermentability were used as substrate for incubation.

Material and methods

Animals and inoculum preparation

In total, twelve adult (250 to 300 kg) multiparous, non-pregnant sows (Large White × Landrace × Duroc crossbreds) were used as faecal donors for inocula preparation because of their high fermentation capacity (Jørgensen *et al.*, 2007). Sows were housed pairwise in pens (4 × 3 m) with wood shavings and straw as bedding material. All animals were fed a commercially available compound feed (Table 1) and received a daily amount of feed to meet their maintenance requirements (CVB, 2010). The compound feed was based on barley, wheat middlings, maize, extracted rape seed and extracted soy beans (Table 1), and was fed in two equal meals at 8:00 and 16:00 h throughout the five week study. Feed samples were collected each week and combined at the end of the experiment for proximate analysis. All sows had free access to drinking water. Handling of the animals was approved by the ethical committee of Wageningen University and was in accordance with the Dutch law on the use of experimental animals, with amendments made to this law in accordance with Council Directive 86/609/EEC (http://wetten.overheid.nl/ BWBR0003081/).

Table 1. Composition of the standard sow diet.

Ingredient	g kg^{-1}
Barley	250
Wheat middlings	220
Corn	100
Rape seed, extracted	75
Wheat	70
Soy hulls	50
Choco power	50
Sunflower seed expeller	38
Vinasse/melasse	30
Wheat feed flour	25
Soy beans, extracted	20
Oil, fish and palm	15
Linseed	10
Vitamin/mineral mix	47

At the start of the study, twelve animals were allotted into three groups of 4 animals to prepare three inocula. All animals were adapted at least 3 weeks to the compound feed. During each of the following five weeks, faeces of the same four animals were pooled proportionally to ensure inocula of the same origin were obtained for each run (one run per week). Faeces were collected at the same time of the day, at the same day of each week over five consecutive weeks. Faeces collection was done manually with approximately 100 to 200 g per animal collected directly from the rectum. Faeces were immediately transferred into a CO_2 pre-flushed plastic container, which was placed on crushed ice until further processing. Within 4 h of collection, faeces were transported to the laboratory and prepared for incubation. Previous experiments in our laboratory (Bosch *et al.*, submitted) have shown that canine faecal samples, kept under anaerobic conditions can be stored up to 24 h on crushed ice without affecting the extent and kinetics of neither gas production nor fermentation end products. In order to reduce variation due to the sampling time and start of substrate fermentation, the animals were sampled in the same order at the same time for each run and as well as preparation of inocula. In the laboratory, individual faecal samples were homogenized and 5 g faeces of each of the four pigs pooled into one sample. To each pool sample, 180 ml of a 0.9% NaCl solution was added and the mixture homogenized with a hand blender for 1 minute. After homogenising the mixture was filtered through a nylon

cloth (pore size 40 µm, permeability 30%; PA 40/30; Nybolt, Zürich, Switzerland), thus creating the inoculum. The processing of the incubation medium was conducted as described by Williams *et al.* (2005a). The inoculum was added to the medium in the ratio 1:16.8 on a weight /volume basis. All handling while preparing the inocula was carried out under a constant flow of CO_2 to ensure anaerobic conditions. The resulting three inocula were used to test three different substrates (oligofructose, soy pectin and cellulose).

Substrates

Three contrasting fermentation substrates were chosen: oligofructose (highly fermentable), soy pectin (moderately fermentable) and cellulose (slowly fermentable). Oligofructose (Orafti ® P95) was obtained from Orafti (Tienen, Belgium), soy pectin (Soyafibre-S-DA 100) from Fuji Oil Company Ltd. (Ibaraki, Japan) and cellulose (Vitacel® powdered cellulose for food, LC 200) from J. Rettenmeier & Soehne GmbH + Co (Rosenberg, Germany). All substrates were air-dried and in powder form.

Cumulative gas production

During incubation, cumulative gas production over time was measured using an "automated gas production system" (Cone *et al.*, 1996). For each inoculum and each substrate, 3 fermentation bottles were incubated simultaneously as replicates. The experimental set-up was as follows: 3 replicate bottles × 3 substrates × 3 inocula/animal groups × 5 fermentation runs. Per inoculum and run, one bottle without substrate (blank) was included to control background fermentation. Substrate (~0.5 g) was weighed into a 300 mL fermentation bottle (Schott, Mainz, Germany) and after pre-flushing the bottle with CO_2, 89 mL of medium (inoculum + buffer solution) was added, the bottle placed in a shaking water bath and incubated for 72 h at 39°C. After 72 h, incubation was stopped, pH of the fermentation fluid recorded (Hanna Instruments pH meter; Woonsocket, RI, USA) and fermentation fluid collected from each bottle.

Chemical analyses

Feed samples were analysed for dry matter (DM; ISO 6496, 1999), ash (ISO 5984, 2002), crude protein (CP; ISO 5983, 2005), crude fat (CFAT; ISO 6492, 1999) and starch (ISO 15914, 2004), substrates were analysed for dry matter (DM; ISO 6496, 1999), ash (ISO 5984, 2002) and crude protein (CP; ISO 5983, 2005). The results of the proximate, starch and sugar analysis of the diet, calculated NSP as well as the DM, ash, CP and energy contents of substrates are shown in Table 2.

The NSP-level in the sows' diet was 330 g kg^{-1} DM and is representative for diets of non-pregnant sows (Van der Peet-Schweering *et al.*, 2004). The soy pectin substrate contained a relatively high CP content (52 g kg^{-1} DM) compared to the other two substrates ($\geq$ 0.6 g kg^{-1} DM).

Reducing sugars were extracted using 40% ethanol and hydrolysed with HCl according to van Vuuren *et al.* (1993). Gross energy (GE) content was determined by adiabatic bomb calorimeter (model IKA-calorimeter C7000; IKA Werke GmbH & Co. KG, Staufen, Germany) according to ISO 9831 (1998a). The inocula (faecal samples diluted with 0.9% NaCl-solution) were analysed for DM and pH determination while the substrates were analysed for DM, ash, CP and GE. Short chain fatty acid (SCFA) and ammonia (NH_3) concentrations in the fermentation fluid were analysed as described by Sappok *et al.* (2009). In short, SCFA were analysed using a GC (Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with a split/splitless injector operated in split mode (split ratio 1:10) and fitted to a flame ionization detector (FID), using a capillary column (Mega bore EC-1000, length 30 m, i.d. 0.53 mm, film thickness 1.00 μm, Alltech Associates, Inc., Deerfield, IL, USA) with Helium as the carrier gas (50 kPa pressure). Iso-caproic acid was included as an internal standard. NH3 was determined colorimetrically at 623 nm using a spectrophotometer (Cary 50, Varian, Palo Alto, CA, USA) and based on the Berthelot reaction as described by Searle (1984) after deproteinising the supernatant by addition of 10% trichloroacetic acid.

Table 2. Chemical composition of the standard sow diet and fermentation substrates.

Diet/Substrate	DM	ASH	CP	CFAT	Starch	Sugar	NSP	GE	ME
	(g kg^{-1})	(g kg^{-1} DM)						(kJ g^{-1} DM)	(kJ g^{-1} OM)
Sow diet	876	71	165	44	343	49	330	18.1	nd
Oligofructose	957	0	0.6	nd	nd	nd	nd	17.1	8.6
Soy pectin	883	83	52	nd	nd	nd	nd	14.9	8.4
Cellulose	941	2.4	0	nd	nd	nd	nd	16.7	7.2

CP, crude protein; CFAT, crude fat; GE, gross energy; ME, metabolizable energy (for calculation see Material and Methods); nd, not determined; NSP, non-starch poly-saccharides.

Calculations and statistical analyses

The NSP content in concentrates and silages was calculated as DM – Ash – CP – Cfat – starch – (sugar × 0.965); CVB, 2010. Metabolizable energy (ME) of the diet (table 1) was calculated from the SCFA (amount and molar proportions) as: sum of the SCFA (acetate, propionate and butyrate-yield (mmol g^{-1} OM)) multiplied by the molar weight of each SCFA times the energy yield of individual SCFA (kJ g^{-1}) times the energy efficiency (acetate 0.65; propionate 0.71 and butyrate 0.67; Gaedeken *et al*., 1989).

Total gas production was calculated as the amount of gas produced per gram organic matter (OM) of substrate initially incubated (termed OMCV = organic matter corrected volume; in mL g^{-1} OM) after fitting a monophasic model (Groot *et al*., 1996):

$$OMCV = (A/ (1+(C/t)^{B}))$$

where OMCV is the total gas produced (mL g^{-1} OM), A is the asymptotic gas production (mL g^{-1} OM), B is a constant determining the sharpness of the switching characteristic of the curve, C is the time at which half of the asymptotic gas production has been reached (h) and t represents the time (h). The maximum rate of gas production (R_{max} in mL h^{-1}) and the time (h) at which it occurred (T_{Rmax}) were calculated according to the following equations (Bauer *et al*., 2001):

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

$$T_{Rmax} = C \times (((B - 1)/(B + 1))^{(1/B)})$$

The gas production parameters (e.g. OMCV, C, R_{max}) and endpoint parameters like NH_3, SCFA (sum of acetate, propionate, butyrate, iso-butyrate, valeric acid and iso-valeric acid), the molar proportions of the three main SCFA (acetate, propionate and butyrate) and branched chain ratio (*BCR*) are shown as means. Means were calculated from the three inocula per run, which in turn were calculated from the three replicate bottles used per inoculum for each substrate. *BCR* was calculated as (iso-butyric + iso-valeric acid / total amount of SCFA). The data were analysed as a repeated measurement in the the MIXED procedure of SAS 9.2 (1989), using the following model:

$$Y_{ijk} = \mu + R_i + S_j + I(S)_{jk} + (R_i \, x \, S_j) + \varepsilon_{ijk}$$

with fixed model effects R_i and S_j for run and substrate, the random effect $I(S)_{jk}$ of inoculum nested within substrate, $R_i \times S_j$ the interaction between run and substrate and ε_{ijk} the residual error. Run (week) was treated as repeated measure assuming a first order autoregressive covariance structure [AR(1)] because based on the Bayesian information criterion (BIC) and Akaike information criterion (AIC) values, AR(1) fitted the data best (Littell *et al*., 1989; Tempelman *et al*., 2004). An effect of replicate bottles was tested separately but did not contribute significantly to the model for any of the parameters, and was therefore excluded from the model. Differences between main effects R_i and S_j were analysed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS.

The coefficient of variation (CV) of the three inocula values within substrate for each run per fermentation parameter (OMCV, C and R_{max}) was calculated. The resulting five CV values were averaged for each substrate per fermentation parameter and graphically presented. Differences between CV's across substrates within fermentation parameter were analysed by ANOVA using the PROC GLM procedure of SAS.

Results

All animals remained healthy throughout the trial and consumed their feed completely each day. The mean (± SD) DM and ash content and pH of the faecal samples diluted with 0.9% NaCl were 21.3 ± 0.87 g kg^{-1}, 12.7 ± 0.29 g kg^{-1} DM and 7.2 ± 0.33, respectively. The mean CV for gas production of the triplicate bottles per sample incubated was 1.9% and did not exceed 3.0%. The NH_3 and SCFA concentration were 2.01 ± 0.26 and 8.23 ± 1.19 mmol L^{-1}, respectively.

After 72 h, blanks showed an average OMCV of 17.3 ± 8 mL as measured over all 5 fermentation runs. Means (n=3) of the gas production parameters for the three substrates and effects of substrates, run and the interaction between substrate and run are shown in Table 3. The total gas produced (OMCV) was similar for oligofructose (387 mL g^{-1} OM) and cellulose (382 mL g^{-1} OM) and lowest for soy pectin (356 mL g^{-1} OM, $P < 0.05$) for the five repeated measurements (run 1 to 5). During run 4, the incubation of oligofructose yielded a high OMCV (412 mL vs. app. 380 mL g^{-1} OM) compared to the other runs. OMCV results for soy pectin and cellulose were similar over time. There were no differences between runs for the parameters C and R_{max} for individual substrates. The time at which half of the asymptotic gas production was reached did not differ between oligofructose and soy pectin (8.0 h and 8.7 h, respectively) while cellulose had a higher (23.5 h, $P < 0.001$) C value compared to the other two substrates.

Table 3. Means (n=3 inocula) of gas production parameters measured for three substrates in five consecutive runs.

Run	OMCV (mL g^{-1} OM)			*C* (h)			R_{max} (mL h^{-1})		
	OF	SP	Cell	OF	SP	Cell	OF	SP	Cell
1	388	356	380	7.6	8.6	23.3	73.3	65.9	17.9
2	378	352	380	8.0	9.0	23.2	69.9	60.4	19.4
3	374	352	387	8.3	8.5	23.7	63.9	63.8	21.8
4	412	369	389	8.2	8.8	24.9	64.5	60.3	18.3
5	383	351	373	7.9	8.3	25.4	74.8	70.7	16.7
Average	387[a]	356[b]	382[a]	8.0[b]	8.7[b]	24.1[a]	69[a]	64[a]	19[b]
Model SEM		8.25			1.20			3.82	
Mixed-model established p values for main effects and their interaction term									
Run		0.006			0.912			0.020	
Substrate		0.007			<0.001			<0.001	
Substrate × Run		0.583			0.959			0.061	

C, halftime of asymptotic gas production; Cell, cellulose; OF, oligofructose; OMCV, organic matter corrected volume (total gas production); R_{max}, maximal rate of gas production; SP, soy pectin.
[ab] means with different superscripts within row and gas production parameter differ significantly ($P < 0.05$).

Maximal rate of gas production was highest for oligofructose (69 mL h^{-1}), followed by soy pectin (64 mL h^{-1}), and with a lower value observed for cellulose (19 mL h^{-1}, $P < 0.001$).

For all substrates, run had an effect on OMCV ($P = 0.006$) with run 2, 3 and 5 yielding lower values compared to run 4 and also on R_{max} ($P = 0.020$), with run 4 yielding lower values compared to run 5. Substrate had an effect on OMCV ($P = 0.007$) and also on the parameters *C* and R_{max} ($P < 0.001$). No interaction occurred between the main effects of run and substrate.

Ammonia concentration, *BCR* and total SCFA concentration are shown in Table 4. The average NH_3 concentration was higher for soy pectin (3.09 mmol g^{-1} OM, $P < 0.01$) compared to oligofructose (2.64 mmol g^{-1} OM) and cellulose (2.52 mmol g^{-1} OM), while *BCR* was numerically the highest for soy pectin (2.62%) compared to oligofructose and cellulose (2.23 and 2.50%). Incubation of cellulose yielded a

Table 4. Means (n=3 inocula) of fermentation end products for three substrates in five consecutive runs.

Run	SCFA (mg g^{-1} OM)			BCR (%)			NH_3 (mg g^{-1} OM)		
	OF	SP	Cell	OF	SP	Cell	OF	SP	Cell
1	824	828	699	1.93	2.32	2.25	43.7	50.5	42.3
2	706	695	563	2.15	2.55	2.43	44.6	54.5	39.7
3	763	758	688	2.27	2.72	2.44	44.8	56.9	41.9
4	750	729	632	2.39	2.74	2.53	45.5	48.5	51.2
5	711	722	640	2.38	2.79	2.87	45.6	52.2	39.2
Average	751[a]	747[a]	645[b]	2.23	2.62	2.50	44.8[b]	52.2[a]	42.9[b]
Model SEM		27.3			0.27			1.92	
Mixed-model established p values for main effects and their interaction term									
Run		<0.001			0.486			0.237	
Substrate		<0.001			0.114			0.001	
Substrate × Run		0.884			0.873			0.001	

BCR, branched chain ratio; Cell, cellulose; NH_3, ammonia; OF, oligofructose; SCFA, short chain fatty acids; SP, soy pectin.

[ab] means with different superscripts within row and gas production parameter differ significantly ($P < 0.05$).

lower SCFA (9.64 mmol g^{-1} OM, $P < 0.01$) concentration compared to soy pectin and oligofructose (10.95 and 10.86 mmol g^{-1} OM).

For all substrates, there was an effect ($P < 0.001$) of run on SCFA concentration with run 1 yielding higher values compared to run 2, 4 and 5, but not for NH_3 concentration and *BCR*. There was a substrate effect for NH_3 ($P < 0.001$) and SCFA ($P < 0.001$). An interaction was found between run and substrate for NH_3 concentration. Molar compositions for the three main SCFA (acetate, propionate and butyrate) are shown in Table 5. Fermentation of cellulose gave the highest proportion of acetic acid (64.6%) compared to oligofructose and soy pectin (53.7 and 58.4%), but differed only compared to oligofructose ($P < 0.01$). Cellulose yielded numerically the lowest propionate proportion (24.8%) compared to oligofructose (33.8%) and soy pectin (30.7%). Oligofructose-fermentation yielded numerically the highest butyrate proportion (7.3%) compared to soy pectin (5.9%) and cellulose (6.1%). There was an overall run effect (P = 0.041) on acetate, with run 3 yielding lower values compared to run 1.

Table 5. Means (n=3) of the molar proportions of acetate, propionate and butyrate for three substrates in five consecutive runs.

Run	Acetate (%)			Propionate (%)			Butyrate (%)		
	OF	SP	Cell	OF	SP	Cell	OF	SP	Cell
1	56.4	62.2	67.5	32.7	27.9	22.6	6.2	5.4	5.9
2	55.5	58.9	67.2	32.6	30.2	22.9	7.1	5.9	6.0
3	50.8	56.5	63.3	36.9	32.4	25.5	7.9	6.2	6.7
4	52.9	57.1	64.3	34.2	31.6	24.8	7.3	6.0	6.1
5	53.0	57.2	60.9	32.8	31.2	28.2	8.2	6.2	5.8
Average	53.7^{b}	58.4^{ab}	64.6^{a}	33.8^{a}	30.7^{a}	24.8^{b}	7.3	5.9	6.1
Model SEM		2.46			2.12			0.55	
Mixed-model established p values for main effects and their interaction term									
Run		0.041			0.152			0.070	
Substrate		0.009			0.006			0.079	
Substrate × Run		0.952			0.826			0.671	

Cell, cellulose; OF, oligofructose; SP, soy pectin.

[ab] means with different superscripts within row and gas production parameter differ significantly ($P < 0.05$).

For all substrates, run had an effect on the molar proportion of acetate ($P = 0.041$), with run 3 being lower than run 1, but not on the proportion of butyrate and propionate. Substrate had an effect ($P < 0.01$) on the molar proportions of acetate ($P = 0.009$) and propionate ($P = 0.006$). There was no run×substrate interaction. In Figure 1, the average CV between inocula across runs is shown for fermentation parameters OMCV, C and R_{max} and individual substrates. The CV for OMCV was low (between 2 and 5%) compared to the CV of the parameter C (up to 13%) and R_{max} (up to 26%). Cellulose showed a higher CV for the parameter OMCV ($P < 0.05$) and R_{max} ($P < 0.01$) compared to the other substrates and the highest SD for R_{max}.

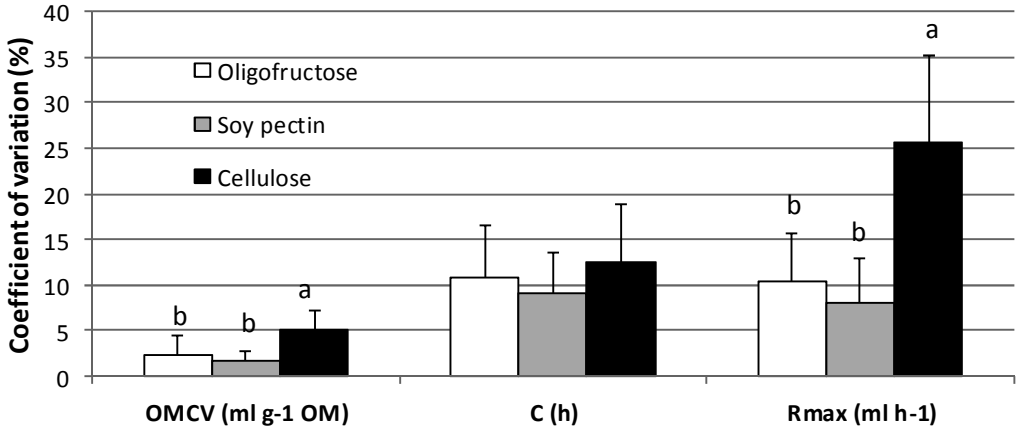

Figure 1. Average coefficient of variation of inocula (n=3) across runs (n=5) for the gas production parameters: organic matter corrected volume (OMCV), halftime (*C)* and maximal rate of gas production (R_{max}) for three substrates.
[ab]Different superscripts within fermentation parameter indicate differences between substrates.

Discussion

Run differences occurred for OMCV, the latter which was mainly due to a very high gas production during oligofructose-fermentation in 4th run. When the OMCV mean of oligofructose for the 4th run was replaced with an average value of other runs, no run effect occurred for the main effects OMCV, *C* and R_{max}. Jonathan *et al.* (2012) also observed high differences in gas produced during oligofructose-fermentation: in a pilot study 385 ml g^{-1} OM were observed and 424 mL g^{-1} OM in the actual experiment. The 10% higher OMCV of oligofructose in run 4 in the current study was not reflected in a higher total SCFA or NH_3 concentration. No increase was observed in OMCV for the other two substrates in run 4. Run differences occurred also for R_{max} as this is calculated from OMCV. There was no effect of run for parameters *C*, NH_3 concentration, *BCR*, propionate and butyrate proportions as opposed to total SCFA concentration and acetate proportion for which a run effect was observed. The high OMCV-value for oligofructose in run 4 may have been caused by a difference in microbial activity in relation to the oligofructose and the composition of microbiota in the inoculum compared to the other runs due to a difference in digesta composition. The latter may have been the result of differences in straw and wood shaving intake, because compound feed

intake was not different across runs as the animals consumed their daily ration completely. Inocula blanks of run 4 showed no differences compared to blanks in other runs.

Many studies reporting *in vitro* fermentation results use inocula from pooled samples of either 3 or 4 animals to account for between-animal variation (Bindelle *et al*., 2007c; Bauer *et al*., 2003). At each of the five runs in the current study, the same animals (3 x 4 animals) were used to obtain faeces which were processed to yield three inocula and used to observe the variation between inocula within runs for fermentation characteristics and individual substrates. Figure 1 shows that the inocula varied the least for OMCV, followed by *C* and R_{max}. The average CV of inocula across runs was higher for cellulose compared to oligofructose and soy pectin for OMCV ($P < 0.05$) and especially for R_{max} ($P < 0.01$). Furthermore, the CV for R_{max} of cellulose showed the highest standard deviation compared to the other substrates. The CV-values show that the gut microbiota of different animals might be of different composition and therefore differently adapted to a substrate, even if pigs are adapted to the same diet. It has been shown that cultivable gut microbiota of individual pigs can differ, even when dietary variations are avoided, suggesting a strong host influence (Pryde *et al*., 1999). Overall the results indicate that microbiota from the three sets of 4 animals show some variation in fermentation activity and that cellulose seems to be the least constant substrate in terms of fermentation. In biological studies, a well-known indication of accuracy of a method is a CV of less than 10%. The high CV for cellulose (up to 25% for R_{max}) indicates that future studies should focus on examining the optimal number of faecal donors per inoculum for slow fermentable substrates in order to reduce the CV.

The three substrate sources were chosen (oligofructose, soy pectin and cellulose) to achieve different fermentation patterns to allow testing for effects of time and for source of inoculum. Across all substrates the fermentation patterns showed significant differences for OMCV, *C* and R_{max}. The OMCV was highest for oligofructose and cellulose compared to soy pectin, but the rate of fermentation of oligofructose and soy pectin occurred faster compared to cellulose. The *C* parameter for fermentation of oligofructose and soy pectin were approximately 65% lower compared to cellulose and resulted in an approximately 70% lower R_{max} for cellulose. Compared to Jonathan *et al.* (2012), who used the same substrates for fermentation, the OMCV (Table 2) was in general around 10% higher in our study which was also reflected in a 25% higher SCFA concentration. This can be explained by the fact that the animals in the present study ingested more NSP in the concentrate (33%) and potentially in the form of straw and wood shavings while the sows in the study of Jonathan *et al.* (2012) received an experimental diet high in fat (18.3%) and low in NSP (7%). It was shown by Varel and Pond (1985) that

cellulolytic bacteria increase accounting up to 10% (compared to usually 4 to 6%) of the total culturable flora in the large intestine when diets high in fibre (up to 96% alfalfa) are fed to primiparous gilts. Therefore, large intestinal microbiota of the sows used in the present study can be expected to have been adapted well to the NSP sources and consequently result in a high fermentation capacity.

With respect to the kinetics of fermentation, Bindelle *et al.* (2007a) observed similar values for the parameter *C* for citrus pectin and cellulose (8.0 vs. 23.0 h) as values reported here for soy pectin and cellulose (Table 3). These authors used inoculum from three adult Belgian Landrace sows receiving a commercial diet (16.3% CP, 24.4% starch and 31.2% total dietary fibre). In contrast to the current study, citrus pectin and cellulose yielded more gas (465 vs. 356 mL g^{-1} OM and 397 vs. 382 mL g^{-1} OM) and the gas production of citrus pectin was higher compared to cellulose. Compared to the other pectin sources, the acetate ratio for soy pectin is rather low, about 60% in this study compared to 80% for citrus pectin and sugar beet pectin (Williams *et al*., 2005a), but agrees with 62% reported by Jonathan *et al.* (2012). The differences in pectin fermentation can be due to differences between sources of pectin. Titgemeyer *et al*. (1991) also showed that fermentation of soy fibre leads to higher propionate and butyrate ratios compared to other substrates (sugar beet fibre).

Jonathan *et al*. (2012) showed a much lower halftime for cellulose compared to our study (10.9 vs. 24.1 h, respectively), which is likely due to differences in cellulose composition and a lower activity/population of cellulolytic bacteria caused by lower NSP-ingestion of the pigs. In accordance with results from Williams *et al.* (2005a) and Bauer *et al.* (2001), the fermentation of cellulose gave the lowest total SCFA concentration while fermentation of oligofructose and soy pectin yielded 12 and 13% more SCFA. The relatively low SCFA concentrations for slow fermentable substrates are caused by an energy shortage of available carbohydrates for fermentation which restricts efficient SCFA-production. The overall NH_3 concentration after fermentation was highest for soy pectin with 3.09 mmol g^{-1} OM with the highest *BCR* (2.62%). This shows a relatively high level of protein fermentation compared to the other substrates. In contrast, Jonathan *et al.* (2012) showed the highest NH_3 concentration (2.23 mmol g^{-1} OM) and *BCR* (2.93%) for cellulose. An explanation could be that the increased NSP intake of sows in the current study leads to a high efficiency in formation of microbial protein (Kirchgeßner *et al*., 1989), leading to a self-digestion of microbes after the substrate is fermented (Cone *et al*., 1997). The higher microbial turnover in combination with the higher CP-content of the substrate could be the reason for increased NH_3 concentration and *BCR* for soy pectin. However, one should keep in mind that NH_3 concentration was only measured after 72 h and does not reflect the concentration during or after the substrate was actually fermented.

Despite the relatively high protein fermentation, soy pectin has been shown to supply a considerable amount of metabolizable energy. Oligofructose showed the highest butyrate concentration compared to the other two substrates and can be considered as most beneficial for colonic gut health because it is the preferred energy source by colonocytes (Jha *et al.*, 2011b). The positive correlation between OMCV and total SCFA found by Awati *et al.* (2005) could not be shown in our study. Therefore, OMCV should not be the only indicator for NSP-characterization, but should be used in combination with R_{max} and on SCFA concentration.

Conclusion

Diet-adapted sows fed the same diet over a time period of 5 weeks, when repeatedly sampled for faeces, yield inocula which provide different results in *in vitro* fermentation studies. However, the ranking order between substrates remains similar and is not majorly influenced over time when differences between substrates are sufficiently large. Therefore, the authors regard one fermentation run sufficient to obtain representative results from *in vitro* gas production studies for NSP-characterization. The relatively high coefficient of variation of inocula across runs for cellulose fermentation indicates that the number of animals per inoculum should be revisited, especially for *in vitro* evaluation of slow fermentable substrates in future studies.

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Chapter 5

Large intestinal fermentation capacity of fattening pigs on organic farms as measured *in vitro*

Fermentation capacity of fattening pigs on organic farms

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Submitted

Abstract

Background In accordance to EU regulations organic farms provide pigs with diets high in fibre which may impact the pig's fermentation capacity. The ability of pigs to ferment non-starch polysaccharides (NSP) depends on characteristics of the NSP-source and microbes present in the large intestine of the pigs. To study the fermentation characteristics and it's variation on organic farms, an *in vitro* fermentation study (batch culture method) was conducted using faeces from fattening pigs in the finishing stage from ten organic farms, using three substrates for incubation: oligofructose, soy pectin and cellulose.

Results Pigs from different organic farms showed varying fermentative capacities as assessed by gas production, kinetics and fermentation end products formed ($P<0.001$). Coefficients of variation between inocula within farms varied up to 40% for gas production and kinetics, in particular for incubation with cellulose. No relationship between dietary NSP content and pigs' fermentation capacity of the substrates could be established.

Conclusion Fermentative capacity of pigs reared under organic conditions varies considerable between farms. Finishing pigs reared under organic farming conditions are fast fermenters of oligofructose and soy pectin. More than four donor animals should be used per inoculum to accurately assess *in vitro* fermentation capacity.

Introduction

Fattening pigs produced under organic farming conditions as described in the EU regulations (EEC No 834/2007), receive diets higher in fibre than fattening pigs kept under conventional farming conditions. The higher fibre intake is a result of the consumption of bedding material (straw) and the additional roughage provided with the diet. The stipulated additional fibre/roughage provision is meant to improve animal health and welfare, as ingestion of fibre can reduce enteric diseases, diarrhoea and stereotypic behaviour (Williams *et al*., 2001; De Leeuw *et al*., 2008). Furthermore, reduced N emissions are associated with increased fibre intake (Varel *et al*., 1987a; Bindelle *et al*., 2008). Roughage (e.g. straw, grass silage) consists mainly of non-starch polysaccharides (NSP) which are primarily fermented by microbes residing in the large intestine of the pig. NSP-rich ingredients for concentrate feeds are less costly compared to highly digestible ingredients but an increased NSP content in the diet of pigs can result in a reduced total tract digestibility of nutrients and energy (Raj *et al*., 2005; Freire *et al*., 2000; Jørgensen *et al*., 1996). If this reduced nutrient and energy digestibility cannot be compensated through a higher feed intake, daily weight gain of the animal can decrease.

To obtain the optimal benefit from inclusion of NSP rich ingredients in the concentrate feeds of fattening pigs, it is essential to know their feeding value. A cost-effective and rapid method to obtain an estimate of the fermentative characteristics of NSP-rich ingredients (thereafter called substrates) for pigs is the *in vitro* cumulative gas production technique (thereafter called *in vitro* fermentation). Hereby, substrates are incubated with an inoculum containing intestinal microbes derived from a faecal sample, thus mimicking the fermentation process in the large intestine of the pig (Bindelle *et al*., 2009; Williams *et al*., 2005a). Although *in vitro* fermentation results cannot provide absolute values, it is possible to obtain a relative ranking of NSP-substrates (Coles *et al*., 2005).

Most published *in vitro* fermentation results for substrates are derived from faecal inocula originating from donor animals receiving diets relatively low in NSP. Therefore, results may not be representative for pigs grown under organic farming conditions. The extent and kinetics of *in vitro* fermentation depend on the composition and activity of microbes in the large intestine, which in turn depend primarily on the diet ingested by the donor animal (Van Soest, 1994; Macfarlane *et al*., 1992). In case pigs are fed diets high in NSP as occurs under organic farming conditions, more indigestible carbohydrates become available for the microbial population residing in the large intestine. The additional substrate may increase microbial fermentation activity and further stimulate selective microbial growth (BachKnudsen & Jørgensen, 2001). The aim of this study was to determine the

variation in the fermentative capacity of fattening pigs within and between organic farms using the *in vitro* batch culture method (Williams *et al*., 2005a). Activity of faecal microbes of fattening pigs in the finishing stage from ten organic farms was tested using three substrates; oligofructose (OF), soy pectin (SP) and cellulose (Cell). Additionally, feeding management of each farm was recorded and the nutrient composition of the concentrates and grass/grass-corn silages was analysed.

Materials and methods

Farms and animals

Ten pig farms operating according to the EU regulations for organic farms (EEC No 834/2007) were selected for this study. Farms represented a wide range of feeding regimes and were located either in the Netherlands or Germany such that faecal material could be transported to the laboratory within 4 h after collection. The genetic background of the animals on the Dutch farms was (Dutch Landrace × Great Yorkshire) × Pietrain, while the German farms had two different four-race breeds, (German Edelschwein × German Landrace) × (Hampshire × Duroc) and (German Edelschwein × German Landrace) × (Pietrain × Duroc) as well as a crossbred of German Landrace × Pietrain.

Feeding

All pigs were fed diets high in NSP and had access to an outdoor area and straw as bedding material in line with the EU regulations for organic farms (EEC No 834/2007). Nine farms employed a two- phase feeding strategy (grower phase: 25 to 70 kg BW and finisher phase: >70 to 120 kg BW) and one farm (farm no. 5) employed a three-phase feeding strategy (25 to 60 kg, 60 to 90 kg and 90 to 120 kg BW).

Four farms fed a liquid diet (farm no. 5, 8, 9 and 10), four farms fed diets in meal form (1, 3, 4 and 7) and two farms fed pelleted diets (2 and 6). Five diets were barley-based (2, 3, 6, 7 and 10) while farm no. 8 used 50% bread and >5% brewer's grains (fresh and ensiled) in its diets. Farm no. 1, 4 and 9 fed corn-based diets, with farm 1 and 9 using corn cob mix and farm 4 whole ensiled corn plants. Farm no. 5 fed a wheat-based diet to the pigs. At each farm, the animals had *ad libitum* access to the diet. According to the farmers, average concentrate intake of the pigs used as donors in our study (finishers weighing ca. 90 kg) ranged from 2.6 to 3.0 kg per animal per day. On five farms (6, 7, 8, 9, and 10), additional grass or grass-corn silage was supplied *ad libitum* once daily to the pigs. The daily intake of the different feed components (concentrate, silage, straw) by the animals on each

farm was recorded in a questionnaire with the help of estimates by the farmers and was not quantitatively measured. Water was available *ad libitum* for all animals at all farms. Straw (wheat, barley or rye) was provided on all farms as bedding in the pens at regular intervals, ranging from once daily up to once weekly depending on the farm. All farmers reported that the animals consumed straw, especially after it was freshly provided. Feed samples (concentrate and silage) were collected from each farm on the day of faecal sampling and were assumed to be representative for the daily fed ration during the finishing phase.

Collection and preparation of inocula

Faecal samples were collected from finisher pigs (ca. 90 kg) over a 5-week period. Each week, faeces from pigs on two farms were collected at the same time of the day and the same day of the week. Only two farms were investigated at the same time due to a restricted capacity of the *in vitro* fermentation equipment with farms being randomly allocated to one of the 5 weeks. The pigs were distributed in groups of 10 to 50 pigs in different pens per farm. Depending of pen- and pig number, pigs were randomly chosen from different pens per farm. Faecal samples (100 to 200 g) were manually collected directly from the rectum of the pig, immediately stored in CO_2 pre-flushed plastic containers and placed on crushed ice. After collection, faeces were transported on crushed ice to the laboratory and prepared for incubation. Previous experiments (Bosch *et al*., submitted) showed that canine faecal material kept under anaerobic conditions can be stored on crushed ice up to 24 h without affecting the extent and kinetics of *in vitro* fermentation. Per farm, three inocula were prepared by pooling 5 g of fresh, homogenised faeces of four randomly selected faecal samples. For each farm, three inocula were prepared. To each pool sample, 180 mL of a 0.9% NaCl solution was added and the mixture homogenized with a hand blender for 1 min. The homogenized mixture was filtered through a nylon cloth (pore size 40 µm, permeability 30%; PA 40/30; Nybolt, Zürich, Switzerland), thereby creating the inoculum. The inoculum was then added to a bicarbonate-phosphate buffered solution as described by Williams *et al.* (2005a) in the ratio 1:16.8 on a weight/volume basis. All handlings while preparing the inocula was carried out under a constant flow of CO_2 to ensure that anaerobic conditions were maintained.

Substrates

Substrates were OF (Orafti ® P95, Orafti, Tienen, Belgium), SP (Soyafibre-S-DA 100, Fuji Oil Company Ltd., Ibaraki, Japan) and Cell (Vitacel® powdered cellulose for food, LC 200, J. Rettenmeier & Soehne GmbH + Co, Rosenberg, Germany). These substrates were selected based on their anticipated contrasting

fermentation characteristics (own observations in our laboratory). All substrates were air-dried and provided in a powdered form.

Cumulative gas production

Substrates (±0.5 g) were weighed into 300 mL fermentation bottles (Schott, Mainz, Germany). After pre-flushing the bottles with CO_2, 89 mL of buffered inoculum solution was dispensed into each bottle, which was then placed in a shaking water bath at 39°C shaking at 40 rpm. The bottles were connected to an automated gas production system described by Cone *et al.* (1996) and the cumulative gas production of the incubated substrates was measured for 72h. Three replicate fermentation bottles were incubated per substrate and inoculum. After 72 h, incubation was terminated, pH of the fermentation fluid recorded (Hanna Instruments pH meter, Woonsocket, RI, USA) and fermentation fluid samples collected from each bottle for analyses of short-chain fatty acid (SCFA) and ammonia (NH_3) concentrations and stored at -20°C until chemical analysis.

Analyses

Liquid feed samples and silages were freeze dried before further analyses. All feed samples were analysed for dry matter (DM; ISO 6496, 1999), crude ash (ISO 5984, 2002), crude protein (CP; ISO 5983, 2005), crude fat (CFat; ISO 6492, 1999) and starch (ISO 15914, 2004). Reducing sugars were extracted using 40% ethanol and hydrolysed with HCl according to Van Vuuren *et al.* (1993). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as described by Goering and van Soest (1972). Gross energy (GE) content was determined by an adiabatic bomb calorimeter (model IKA-calorimeter C7000; IKA Werke GmbH & Co. KG, Staufen, Germany) according to ISO 9831 (1998a). Substrates were analysed for DM, ash, CP and GE using similar procedures as for the feed samples. Short chain fatty acids and NH_3 concentrations were analysed as described by Sappok *et al.* (2009).

Calculations and statistics

The NSP-content in concentrates and silages was calculated as DM – crude ash – CP – Cfat – starch – (sugar × 0.965); CVB, 2010. Total gas production was calculated as the amount of gas produced per gram organic matter (OM; DM-crude ash) of substrate initially incubated (termed OMCV = organic matter corrected volume; in mL g^{-1} OM) after fitting a monophasic model (Groot *et al*., 1996):

$$OMCV = (A/(1 + (C/t)^B))$$

where OMCV is the total gas produced (mL g^{-1} OM), A is the asymptotic gas production (mL g^{-1} OM), B is a constant determining the sharpness of the switching characteristic of the curve, C is the time at which half of the asymptotic gas production has been reached (h) and t represents the time (h). The maximum rate of gas production (R_{max} in mL h^{-1}) and the time (h) at which it occurred (T_{Rmax}) were calculated according to the following equations (Bauer *et al*., 2001):

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

$$T_{Rmax} = C \times (((B - 1)/(B + 1))^{(1/B)})$$

The gas production parameters (e.g. OMCV, C, R_{max}) and endpoint parameters like NH_3, SCFA (sum of acetate, propionate, butyrate, iso-butyrate, valeric acid and iso-valeric acid), the molar proportions of the three main SCFA (acetate, propionate and butyrate) and branched chain ratio (*BCR*) are shown as means calculated from three inocula per farm, which in return were calculated from the three replicate bottles used per inoculum and substrate. *BCR* was calculated as ((iso-butyric + iso-valeric acid) / total amount of SCFA)). The data were analysed for differences between substrates and within farms by applying the GLM procedure SAS 9.2 (1989), using the following model:

$$Y = \mu + F_i + S_j + I(F)_{ik} + (F_i \times S_j) + \varepsilon_{ijk}$$

where Y is the dependent variable tested, μ is the mean, F_i is the effect of the farm (confounded with time) i, S_j is the effect of the substrate j, I_k is the effect of the inoculum k within farm i and ε_{ijk} is the residual error. Inocula were nested within farm because farms differed between each other in feeding management. Differences between farms were analysed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS.

Coefficients of variation (CV) for individual farms were calculated from means of inocula (n=3) for the fermentation parameters OMCV, C and R_{max} for each substrate. In addition, CV between inocula within farm were calculated for these parameters.

Results

The DM, ash, CP and GE content of the substrates is presented in Table 1. The chemical composition of concentrates and silages fed to pigs on the ten organic farms are shown in Table 2.

Table 1. Composition of the three substrates used in the *in vitro* fermentation.

Substrate	Dry matter	Ash	Crude protein	Gross energy
	(g kg^{-1})	(g kg^{-1} DM)		(MJ kg^{-1} DM)
Oligofructose	957.3	0.6	0.6	17.1
Soy pectin	883.3	82.7	52.4	14.9
Cellulose	941.4	3.2	0.0	16.7

Table 2. Chemical composition of concentrates and silages fed to pigs on ten organic farms.

Farm no.	DM	Ash	CP	CFat	Starch	Sugar	NSP	NDF	ADF	ADL	GE
	(g kg^{-1})	(g kg^{-1} DM)									(MJ kg^{-1} DM)
*Concentrates**											
1	827	54.0	215	27.2	487	16.8	201	166	75.6	18.9	18.2
2	884	48.3	215	37.8	427	50.5	224	139	69.2	14.2	19.0
3	874	42.4	171	28.4	490	30.6	238	171	73.0	18.1	18.9
4	802	52.1	191	30.5	455	6.2	265	163	78.6	19.8	17.5
5	937	47.3	206	73.0	348	59.5	268	145	71.5	14.7	19.8
6	884	48.7	206	34.1	456	49.4	207	134	59.9	13.9	19.0
7	876	57.4	170	26.4	481	32.0	235	169	90.3	15.5	18.4
8	942	31.1	225	108.0	382	16.8	238	110	75.6	12.7	21.2
9	937	51.1	199	34.9	440	15.6	259	150	76.2	15.6	19.1
10	934	50.7	193	37.4	386	51.5	283	183	87.4	18.7	18.9
*Silages***											
7	955	99.4	91.1	28.4	209	10.8	562	432	250	24.6	18.0
9	925	125	151	38.3	10.6	70.1	608	466	272	22.9	18.4
6	944	133	150	31.3	10.4	60.7	617	492	288	32.0	17.5
10	956	104	104	20.0	20.9	89.7	665	532	322	36.3	17.6
8	935	88.1	105	24.7	11.6	93.7	680	561	332	31.8	18.5

ADF, acid detergent fibre; ADL, acid detergent lignin; CFat, crude fat; CP, crude protein; DM, dry matter; GE, gross energy; MJ, mega joule; NDF, neutral detergent fibre; NSP, non-starch polysaccharides.

* Farms 1 to 5 fed concentrate alone, farms 6 to 10 fed concentrate + silage, farms 1, 3, 4 and 7 fed concentrate meal, farms 2 and 6 fed pellets and farms 5, 8, 9 and 10 liquid; farms 1, 4 and 9 fed corn-based, farm 2, 3, 6, 7 and 10 fed barley-based, farm 5 wheat-based and farm 8 bread-based, farm 5 employed 3-phase feeding strategy, all others 2 phase-feeding.

** Farms 6, 8, 9 and 10 fed grass silage, farm 7 fed corn-grass silage mixture.

The average NSP content of the concentrates ranged from 201 to 283 g kg^{-1} DM. The NSP-content of the silages was higher compared to the concentrates with corn silage (farm no. 7) showing a lower value compared to grass silages (farm no. 6, 8, 9 and 10), 562 g kg^{-1} DM compared to 608 to 680 g kg^{-1} DM. The mean (± sd) pH value, SCFA and NH_3 concentrations of the faecal pool samples diluted with 0.9% NaCl across farms was 6.41 (± 0.22), 15.3 (± 2.0) and 4.3 (± 1.1) mmol g^{-1} OM.

There was a significant farm×substrate interaction ($P < 0.001$) for OMCV, *C* and R_{max} (Table 3). The OMCV and C from Cell showed differences between farms whereas no differences were found when OF or SP were used as a substrate. Inocula incubated with Cell from pigs on farms 3 and 7 resulted in the highest OMCV value while the lowest OMCV value was recorded using inocula from pigs on farms 5 and 9. Values for C when Cell was used as a substrate varied 20% between highest and lowest value with high C values for farms 1 and 7 compared to farms 2, 4, 8 and 9.

Table 3. Mean (n=3 inocula) fermentation parameters according to the effects of farm, substrate and inoculum.

Farm no.	OMCV (mL g^{-1} OM)			*C* (h)			R_{max} (mL h^{-1})		
	OF	SP	Cell	OF	SP	Cell	OF	SP	Cell
1	360	356	342^{cd}	5.49	5.00	33.2^{a}	102^{abcd}	90	11.8
2	354	348	351^{bcd}	4.95	5.26	20.0^{c}	97^{bcde}	89	22.9
3	374	357	401^{a}	4.38	4.64	24.4^{bc}	104^{abcd}	86	14.6
4	356	352	371^{abc}	4.79	4.72	21.1^{c}	88^{de}	59	17.8
5	371	358	322^{d}	5.33	4.91	25.2^{bc}	108^{abc}	84	12.0
6	387	361	352^{bcd}	4.76	4.98	23.8^{bc}	95^{bcde}	77	14.5
7	373	362	384^{ab}	5.16	4.70	31.3^{ab}	110^{ab}	91	11.5
8	381	345	357^{bcd}	4.72	4.86	22.9^{c}	92^{cde}	82	17.9
9	357	353	340^{cd}	5.29	5.39	21.5^{c}	84^{e}	80	17.7
10	364	353	352^{bcd}	5.15	5.06	25.0^{bc}	118^{a}	88	15.9
Average	368	354	356	5.00	4.95	24.8	99.7	82.6	15.7
Model SEM		4.18			0.78			1.72	
Model established P-values for main effects and their interaction term									
Farm		<0.001			0.003			<0.001	
Substrates		<0.001			<0.001			<0.001	
Inoculum(Farm)		0.034			0.450			0.071	
Substrate×Farm		<0.001			<0.001			<0.001	

C, halftime of asymptotic gas production; Cell, cellulose; OF, oligofructose; OM, organic matter; OMCV, total gas production (organic matter corrected volume); R_{max}, maximal rate of gas production; SEM, standard error mean; SP, soy pectin.

^{a,b,c,d,e} means with different superscripts within column differ significantly ($P < 0.05$).

Table 4. Mean (n=3 inocula) fermentation endpoint products according to the effects of farm, substrate and inoculum.

Farm no.	SCFA (mmol g^{-1} OM)			NH_3 (mmol g^{-1} OM)			BCR (%)		
	OF	SP	Cell	OF	SP	Cell	OF	SP	Cell
1	10.47	11.47	9.80	2.35^{bc}	3.40^{a}	2.50^{b}	2.37^{ab}	2.59^{ab}	3.40^{ab}
2	10.74	11.05	10.22	2.42^{bc}	3.24^{ab}	2.82^{ab}	2.24^{ab}	2.68^{ab}	3.82^{a}
3	10.19	10.67	9.67	2.81^{a}	3.41^{a}	2.71^{ab}	2.63^{a}	2.88^{a}	2.96^{b}
4	9.80	10.64	9.46	2.48^{abc}	3.36^{a}	2.78^{ab}	2.29^{ab}	2.61^{ab}	3.74^{a}
5	11.12	12.72	9.96	2.15^{c}	3.22^{ab}	2.73^{ab}	2.08^{ab}	2.34^{ab}	3.35^{ab}
6	11.55	12.07	11.07	2.46^{abc}	3.37^{a}	2.84^{ab}	2.11^{ab}	2.53^{ab}	3.39^{ab}
7	9.91	10.77	10.2	2.65^{ab}	2.44^{c}	2.93^{a}	2.35^{ab}	2.83^{ab}	3.62^{ab}
8	11.39	10.98	10.34	2.26^{bc}	2.95^{b}	2.89^{a}	1.78^{b}	2.16^{b}	3.98^{a}
9	10.75	11.52	9.44	2.24^{c}	2.95^{b}	2.46^{b}	2.39^{ab}	2.72^{ab}	3.97^{a}
10	9.94	10.46	9.03	2.31^{bc}	3.22^{ab}	2.72^{ab}	2.11^{ab}	2.52^{ab}	3.77^{a}
Average	10.59	11.23	9.92	2.41	3.16	2.74	2.23	2.59	3.60
Model SEM		0.21			0.04			0.07	
Model established P-values for main effects and their interaction term									
Farm		<0.001			<0.001			0.001	
Substrates		<0.001			<0.001			<0.001	
Inoculum(Farm)		0.153			0.043			0.526	
Substrate×Farm		0.191			<0.001			<0.001	

[a,b,c] means with different superscripts within column differ significantly ($P < 0.05$).
BCR, branched chain ratio; Cell, cellulose; NH_3, ammonia; OF, oligofructose; OM, organic matter; SCFA, total short chain fatty acids; SEM, standard error mean; SP, soy pectin.

A large variation between farms was also observed for R_{max} for all substrates, but no significant differences between farms were found. High mean values for R_{max} were found when OF was used as a substrate with inocula from farms 5, 7 and 10 compared to farms 4 and 9. Inocula within farm differed significantly from each other for parameter OMCV ($P < 0.05$). A ranking of farms resulted in different orders for each substrate and each parameter.

Results of the NH_3 and SCFA concentration, *BCR* as well as the molar composition for the three main SCFA (acetate, propionate and butyrate) are shown in Tables 4 and 5. There was a significant farm×substrate interaction ($P < 0.001$) for NH_3 concentration and *BCR*, but not for SCFA concentration although there was an overall farm and substrate effect ($P < 0.001$) for the latter (Table 4). Inoculum within farm differed significantly for NH_3 concentration. For the main SCFA (acetate, propionate and butyrate), there was a significant farm×substrate

interaction ($P < 0.001$) (Table 5). The molar proportions of acetate, propionate and butyrate showed a significant farm and substrate effect ($P < 0.001$). However, with respect to the fermentation of SP, the proportion of butyrate showed no farm effect. Inoculum within farm differed significant for the propionate proportion.

The coefficients of variation (CV) for inocula within each farm for individual substrates are shown in Figure 1 for the fermentation parameters OMCV, *C* and R_{max}. The CV-values determined for the OMCV from OF showed that inocula within farms were uniform with CV-values below 5%. In case SP was used as a substrate the CV-values remained below 5% except for farm 8 (CV = 9%, Fig.1). For substrate Cell, the within farm CV for inocula was on average higher than those of OF and SP and more variable for the different farms, ranging between 5 and 12% for farms 1, 4, 5 and 10.

Table 5. Mean (n=3 inocula) molar proportions of the main short chain fatty acids according to the effects of farm, substrate and inoculum.

Farm no.	Acetate (%)			Propionate (%)			Butyrate (%)		
	OF	SP	Cell	OF	SP	Cell	OF	SP	Cell
1	49^{ab}	59^{ab}	56^{ab}	35^{abc}	29^{abcd}	31^{bc}	9.3^{a}	6.9	7.4^{abc}
2	50^{ab}	58^{ab}	48^{cde}	38^{ab}	30^{abc}	40^{a}	5.9^{de}	6.2	6.2^{bc}
3	49^{ab}	59^{ab}	64^{a}	35^{abc}	27^{bcd}	24^{cd}	8.3^{ab}	7.1	6.8^{abc}
4	49^{ab}	58^{ab}	55^{bcd}	35^{abc}	29^{abcd}	32^{b}	8.5^{ab}	6.6	7.1^{abc}
5	55^{ab}	62^{a}	63^{a}	31^{bc}	26^{cd}	23^{d}	6.6^{bcde}	5.6	7.0^{abc}
6	56^{a}	62^{a}	60^{ab}	28^{c}	23^{d}	27^{bcd}	7.4^{abcd}	6.5	6.5^{abc}
7	47^{b}	53^{b}	46^{e}	40^{a}	34^{ab}	39^{a}	6.2^{cde}	6.2	7.9^{abc}
8	49^{ab}	55^{ab}	47^{de}	41^{a}	35^{a}	41^{a}	5.2^{e}	5.5	5.5^{c}
9	47^{b}	56^{ab}	56^{ab}	39^{a}	32^{abc}	29^{bcd}	8.1^{abc}	6.6	8.4^{a}
10	49^{ab}	56^{ab}	54^{bcde}	40^{ab}	34^{ab}	32^{b}	5.6^{de}	5.5	7.5^{abc}
Average	50	58	55	36	30	32	7.1	6.3	7.0
Model SEM		0.85			0.73			0.21	
Mixed-model established P-values for main effects and their interaction term									
Farm		<0.001			<0.001			<0.001	
Substrates		<0.001			<0.001			<0.001	
Inoculum(Farm)		0.218			0.043			0.786	
Substrate×Farm		<0.001			<0.001			<0.001	

Cell, cellulose; OF, oligofructose; SEM, standard error mean; SP, soy pectin.
^{a,b,c,d,e} means with different superscripts within column differ significantly ($P < 0.05$).

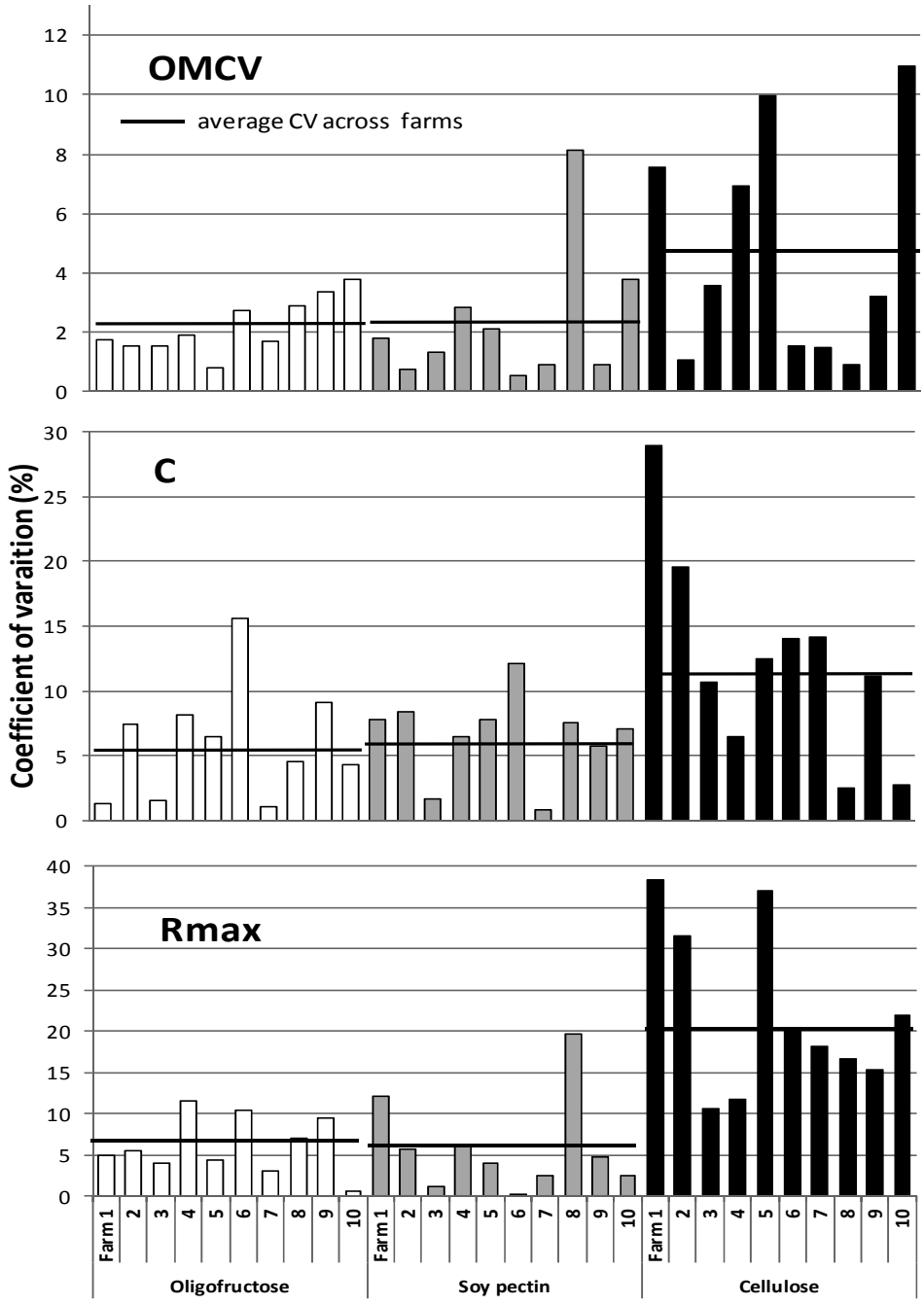

Figure 1. Coefficients of variation (CV) from incocula (n=3) prepared from pig faeces collected on 10 organic farms for: organic matter corrected volume (OMCV), halftime of asymptotic gas production (*C*) and maximal rate of gas production (R_{max}) for three substrates (oligofructose, soy pectin, cellulose).

For the parameter *C* from OF and SP, CV-values for inocula within each farm were below 10% except for farm 6 for both substrates. Incubation of Cell revealed a high variation within farm for farms 1 and 2 (>19%) and below 15% for the other farms. The farm CV's of R_{max} were below 10% for incubation of OF and SP except for farm 4 and 6 (OF) and farm 1 and 8 (SP). Cellulose incubation led to CV-values above 30% for farms 1, 2 and 5, while the other farms showed CV-values ranged between 11 and 22%.

Discussion

The NSP-contents of the concentrates provided to the pigs on-farm was about 33% higher compared to the rations for pigs raised under regular conditions (Högberg & Lindberg, 2004; Carlson *et al*., 1999). In the current study, a lower OMCV-value was observed for OF compared to an earlier study (367 ±11.2 vs. 387 ±14.9 mL g^{-1} OM) where inocula were obtained from non-pregnant sows receiving a 33% NSP diet (Sappok *et al*., 2012). Lower OMCV-values compared to the value reported in the present study were found for OF by Bauer *et al.* (2001, 2004; 340 and 306 mL g^{-1} OM) using pigs weighing 110 kg receiving semi-purified diets low in fermentable carbohydrates. Soy pectin showed similar values for OMCV compared other observations in our laboratory (Sappok *et al*., 2012), 354 vs. 356 mL g^{-1} OM, but lower results compared to Bauer *et al.* (2001; 392 mL g^{-1} OM) who used sugar beet pectin and Bindelle *et al.* (2007a; 465 mL g^{-1} OM) who used citrus pectin. Substrates containing cellulose (brans, hulls and wheat straw) have been reported to yield lower values (Bauer *et al*., 2001; 94 to 342 mL g^{-1} OM) when inoculum of 110 kg pigs receiving semi purified diets low in fermentable carbohydrates was used.

An unexpected result is the low *C*-values (5.00 ±0.34 and 4.95 ±0.24 h) and the resulting high R_{max}-values (99.7 ±10.6 and 82.6 ±9.6 mL h^{-1}) for the fast fermentable substrates OF and SP in the presented study. To the authors' knowledge, no other studies have reported lower values then 7 h for parameter *C* or values exceeding 70 mL h^{-1} for R_{max} for OF and SP fermentation (Bauer *et al*., 2001; Sappok *et al*., 2012; Bindelle *et al*., 2007a; Jonathan *et al*., 2012). This fast occurring fermentation compared to other studies can be an indication of either an exceptional high proportion of bacteria adapted to fast fermentable substrates in the faeces or an overall higher microbial activity, caused through a superior adaptation of the pigs to various fibre sources. All finishing pigs were well adapted for at least 3 weeks to the finishing phase diets high in NSP (Table 2) fed on farm, as well as to the straw provided as bedding. A study by Pellikaan *et al.* (2007) showed that once piglets are adapted to diets, their microbial population seems to be able to

ferment available substrates more readily. *In vitro* Cell fermentation by inoculum obtained from sows (Sappok *et al*., 2012) yields higher values for the fermentation parameters OMCV and R_{max} and a lower *C* value compared to inoculum from finisher pigs. Sows are known to have a larger fermentation capacity compared to growing pigs (ca. 60kg) when the rate of insoluble NSP was increased (Jørgensen *et al*., 2007). The straw consumption of sows (Sappok *et al*., 2012) could have further led to a higher microbial synthesis compared to finishing pigs and might explain the slightly faster and higher gas production during Cell fermentation (Kirchgeßner *et al*., 1989).

In agreement with the varying endpoint results between farms for SCFA, NH_3 and *BCR* presented here, previous authors found that varying levels/composition of dietary fibre in the diet yielded different ratios of SCFA (Wang *et al*., 2004; Bosch *et al*., 2008). The slow fermentable Cell yielded the lowest SCFA concentration and the highest *BCR*, while OF yielded the highest R_{max} but a lower SCFA concentration than SP. Fermentation of fast fermentable SP gave the highest NH_3 concentration. Other studies showed that slow fermentable substrates yield the highest NH_3 concentrations (Bauer *et al*., 2001; Jonathan *et al*., 2008; Bosch *et al*., 2008), due to insufficient fermentable carbohydrate supply for microbial synthesis (Bauer *et al*., 2006). However, fermentation of pectic substances showed relatively high NH_3 concentrations in the above mentioned studies as well compared to other fast fermentable substrates. In the present study the relatively high CP content of SP (Table 1) could be an explanation for the high NH_3 content.

Effects of inocula nested within individual farms were observed for OMCV and NH_3, but not for the other fermentation parameters. Nevertheless, if CV-values for individual farms were calculated (see Figure 1) for the main parameters OMCV, *C* and R_{max}, differences within farm occurred especially for Cell fermentation. Zoetendaal *et al.* (1989) showed a strong genetic link between an individual and its microbial gut community and thus a diverging capacity of an individual to ferment a certain substrate. Therefore it is possible that some pigs within individual farms were better adapted e.g. to Cell fermentation than others.

Although farms varied greatly in management, composition of daily rations fed to pigs and farms were sampled at different time points, the results varied relatively little. The SEM-values of fermentation parameters OMCV, *C* and R_{max} across farms tested at different time points in the current study were half as large (4.18, 0.78 and 1.72) as the SEM within farm tested at different time points during a parallel study (8.25, 1.20 and 3.82; Sappok *et al*., 2012). These lower SEM-values show that fermentation capacity of pigs reared according to organic farming conditions differ less between farms than fermentation capacity of sows within farm when reared under conventional conditions, probably due to their exposure to diverse fibre sources.

Farm 8 showed the highest variation for OMCV during SP fermentation, but the lowest for Cell. Also for R_{max} farm 8 shows the highest CV during SP fermentation, indicating a large variation in fermentative capacity of pigs on this farm. Farm 8 was the only one feeding bread and brewer's grains in their liquid concentrate which might influence fermentation as brewer's grains were fresh ensiled and contained already a population of fermenting microbiota. Interference of this additional microbiota with the one residing in the large intestine could be one reason for an unstable SP fermentation. Farm 1 and 5 showed large CV-values for OMCV and R_{max} for Cell fermentation, but not for fermentation of OF and SP, indicating a stable adaptation to the fast, but not the slow fermentable substrates. Farm 2 showed very low CV-values for OMCV during fermentation of all substrates, while the CV of R_{max} for Cell fermentation was rather high. The high variation for the fast fermentable substrates for some farms shows that fermentation capacity of pigs reared under organic farming conditions is variable. It was expected that a relation could be found between the level of NSP-contents of the diets and the fermentative capacity of the pigs. However, there were no obvious differences between farms that provided silage and farms that did not provide silage. Due to practical on-farm conditions with as little as possible interference in the on-going routine operation of the farm, it was not possible to quantify the daily intake of concentrate and silage on each farm, only estimates were given by the farmers. If it is assumed that pigs on the farms feeding silage ingested 98% of daily DM as concentrate and 2% of the daily DM intake as grass silage (Kelly *et al*., 2007) or amounts of grass silage corresponding to 4% of total energy intake (Danielsen *et al*., 2000), correlations between daily NSP-intake and R_{max}, OMCV or *C* for each substrate were not significant (results not shown). There are many reasons for a lack of a relation between feed and fermentation parameters as animals were not kept under highly controlled laboratory conditions. Feed intake per donor animal was estimated, management between farms differed, different farms used different genotype pigs and housing was not consistent between farms.
However, the results reported here provide a first indication of the fermentative capacity of the microbiota in the large intestine of finisher pigs reared under organic farming conditions. Comparison of the variability in large intestinal fermentation capacity between pigs reared under conventional and organic farming conditions should be undertaken to determine the additional energy yield due to large intestinal fermentation by organically farmed pigs.

Conclusions

Fermentative capacity from pigs reared under organic conditions varies considerably between farms. Finishing pigs reared under organic farming conditions are fast fermenters of oligofructose and soypectin. The high coefficients of variation between inocula within farm especially for cellulose indicate that more than four donor animals should be used per inoculum. *In vitro* fermentation results could not be related to the diets fed on organic farms. More *in vitro* studies should be done with faeces collected on-farm, but under more controlled conditions to find relations between feeding strategy and fermentative capacity, using more animals per inoculum especially for slow fermentable substrates.

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Chapter 6

General discussion

General discussion

In the western world, the main feed ingredients in the diet of commercially farmed pigs contain high concentrations of carbohydrates (e.g. wheat, barley, corn). The research described in this thesis focused in particular on the indigestible, fermentable carbohydrates in the diet of commercially raised pigs kept under organic or conventional conditions. Many feed ingredients contain high levels of these types of carbohydrates, which are not digested by the animal's endogenously synthesised enzymes (e.g. amylase, maltase, lactase, sucrase). However, although not digested, these carbohydrates can be a valuable component in the diet of pigs. Many of these indigestible or poorly digestible feed ingredients are considered unsuitable as food for humans (e.g. straw, grass silage, by-products from the feed industry such as beet-pulp or distiller's grains). The indigestible carbohydrates present in these ingredients consist mainly of non-starch polysaccharides (NSP), which are predominantly fermented in the large intestine of pigs (Bach Knudsen, 2011). Especially breeding sows and pigs kept under organic conditions are fed diets high in NSP (Brouns *et al*., 1995; EC No. 834/2007). Recently, also conventional pig production systems aim to include a higher level of NSP in the diet as this can be beneficial for the animal in terms of health and welfare and are cheaper compared to grains (Verstegen & Williams, 2002; Williams *et al*., 2005b; Le Goff *et al*., 2002; De Leeuw *et al*., 2008). If the diet is properly balanced in terms of amino acid profile and energy, the animal can deposit the maximum amount of protein (Bikker *et al*., 1996). In that case a minimum of absorbed amino acids needs to be deaminated and secreted as urea /ammonia in the urine. In addition, the inclusion of sufficient fermentable NSP in the diet of pigs will result in optimal synthesis of microbial protein in the hindgut (Bikker *et al*., 2007) and less of the nitrogen entering the hindgut will appear as urea /ammonia in the excreta. Thus it will give less ammonia emission compared to grain-based diets (Canh *et al*., 1998; Leek *et al*., 2007; Bindelle *et al*., 2008). However, a high inclusion level of NSP in the diet usually leads to a dilution of the dietary energy content which, if not compensated by higher intake, can result in less energy available to the animal (NRC, 1987; Rijnen, 2003), which in turn may result in a decreased animal performance and profitability (Just *et al*., 1983; Owusu-Asiedu *et al*., 2006). It is therefore, important that potential NSP sources are well characterized in terms of their health benefits and contribution to the energy supply to the animal, so that the most beneficial sources can be included in pig diets to maintain animal performance.

There is a large variety in indigestible NSP sources, ranging from very low fermentable NSP (e.g. cellulose in straw or hay) to the readily fermentable NSP (e.g. pectins in sugar beet pulp) and fructooligosaccharides (Bach Knudsen, 2001).

Not only the digestibility of nutrients in potential NSP sources for inclusion in pig diets is important but also their fermentation potential, as both determine the feeding value. The characterization of NSP sources for pig nutrition in terms of fermentability can be done using *in vitro* methods which mimic microbial fermentation occurring in the intestinal tract of the pig. Several methods have been developed to estimate the fermentation potential of foods/feeds, diets and food/feed ingredients (Coles *et al*., 2005; Boisen & Fernández, 1997; Williams *et al*., 2005a; Cone *et al*., 1996; Bosch *et al*., 2008; Edwards *et al*., 1996). In addition, many *in vitro* studies have investigated various aspects of fermentation in the intestinal tract of pigs (Williams *et al*., 2005; Bauer *et al*., 2003; Bauer *et al*., 2004; Bindelle *et al*., 2007c; Bindelle *et al*., 2009; Anguita *et al*., 2006; Martín-Peláez *et al*., 2009; Becker *et al*., 2003; Rink *et al*., 2011; Jha *et al*., 2011a). However, the majority of these methods have not been thoroughly validated or standardized yet, especially if different classes of pigs (piglets, weaners, growers, sows) are considered. In addition, little emphasis has been placed on the nutrition of the donor human/animal which provide the inoculum in these methods, or the adaptation time of humans/animals to an experimental diet. It should be mentioned that there is some variability in microbiota between animals fed the same diet (Simpson *et al*., 1999; Zoetendal *et al*., 2008).

The above mentioned aspects were the focus of the series of studies conducted and reported in this thesis. The rate of fermentation of NSP depends on the microbiota residing in the intestine of the pig. If an animal is exposed to different types of dietary NSP, these NSP will enter the intestine where they are used as substrates for microbiota. Depending on the NSP source, changes in microbiota occur in terms of composition and activity (Williams *et al*., 2005b). Pigs well-adapted to high dietary concentrations of NSP can be found on organic farms, where the animals receive high levels of roughage in the form of dry or fresh fodder (grazing), silage or straw (EC No. 834/2007). It can therefore be expected that pigs raised on organic farms may have a higher capacity to ferment NSP in the large intestine compared to their counterparts on conventional farms.

Comparison of different *in vitro* methods

The *in vivo* feeding value of different types of NSP has been estimated using *in vitro* enzymatic incubation assays such as that of Boisen and Fernández (1997). These authors developed a three-step enzymatic incubation method where incubation of substrates occurs with pepsin (step 1) and pancreatin (step 2) mimicking pre-caecal digestion by digestive enzymes in the intestine. The third step is the incubation of the substrate with an enzyme complex (Viscozyme) containing a wide range of carbohydrases (arabinose, cellulase, ß-gucanase,

hemicellulase, xylanase and pectinase), mimicking the degradation by enzymes produced by the microbiota in the large intestine. The organic matter loss (OML = indicator for OM-digestibility) of the substrate is measured and the differences between OML between substrates can be used for ranking of substrates. An advantage of the enzymatic incubation is that results correlate well with *in vivo* results and the method has been shown to be highly repeatable (Coles *et al*., 2005). However, by using *in vitro* enzymatic incubation without fermentation, only hydrolysis of those components in substrates will occur for which the enzymes are specialised. If the substrate is an NSP, an underestimation of NSP degradation by microbes will occur and therefore an underestimation of the energy value of the tested substrate. Additionally, the enzymes used in the third step (Viscozyme) in the Boisen and Fernandez (1997) *in vitro* method are considered to be representative for the enzymes excreted *in vivo* by the large intestinal microbiota. A consequence of using Viscozyme is that it does not take into account: 1) the variation occurring *in vivo* between microbiota of donor animals and 2) the possible effect of the microbiota on the duration of the time during which the diet had been ingested by the donor animals. Therefore, it may not be completely representative for the *in vivo* situation.

Another method to estimate the feeding value of NSP is the *in vitro* fermentation method such as that of Williams *et al.* (2005a). In this method, an NSP substrate is incubated with a feacal inoculum mixed from three to four animals, to mimic large intestinal fermentation. Organic matter loss as well as gas production, fermentation kinetics and concentration of end products in the fermentation liquid can be determined. Bauer *et al.* (2003) showed that using enzymatically pre-digested substrates for *in vitro* fermentation lead to significant lower gas production profiles compared to their not pre-digested (untreated) counterparts, due to a loss of rapidly fermentable components during pre-digestion. Furthermore, Bauer *et al.* (2003) showed a profound difference between gas production profiles of pre-digested and undigested substrates on the one hand and original chyme (digesta) obtained *in vivo* on the other hand. These results indicated that using pre-digested substrates is likely to be more representative for the chyme reaching the large intestine *in vivo*.

Both the method of Boisen and Fernandez (1997) and that of Williams *et al.* (2005a) have their shortcomings, but results could be improved when methods are combined. Therefore, the study reported in chapter 2 compared the *in vitro* three-step enzymatic incubation method as developed by Boisen and Fernandez (1997), the *in vitro* fermentation method by Williams *et al.* (2005a) and a combination of both. For the combination, an *in vitro* pre-digestion of the substrates (corn silage, grass silage, Jerusalem artichoke, rye grass and turnip) was performed before *in vitro* fermentation with digestive enzymes pepsin and pancreatin. For fermentation,

two different inocula were used; one inoculum originating from animals receiving a regular compound feed relatively low in NSP (control animals) and one inoculum originating from animals receiving a diet relatively high in NSP (experimental animals), which consisted of a reduced amount of the control diet supplemented with grass and grass silage. Faecal donor animals were adapted for three weeks to the diets. The adaptation time of three weeks was chosen based on the only *in vitro* fermentation studies pro-viding information on adaptation time. In these fermentation studies 7 to 14 days of adaptation to a new diet was regarded as sufficient (Bauer *et al*., 2004; Bindelle *et al*., 2009; Anguita *et al*., 2006; Martín-Peláez *et al*., 2009; Awati *et al*., 2006).

Similar to the results of Bauer *et al.* (2003) the gas production profiles were at a lower level for pre-digested substrates compared to their untreated counterparts. This was expected and can be explained by the loss of nutrients during enzymatic pre-digestion. Therefore, omitting pre-digestion could lead to an over-estimation of fermentability (and therefore the energy value) of NSP in substrates. A ranking of substrates for both treatments in terms of absolute OML showed an identical pattern (Table 1). At present, the pre-digestion of substrates with enzymes (if the substrate is not a purified NSP/indigestible carbohydrate) is now a standard element of the *in vitro* fermentation procedure carried out in our research facilities. Although this procedure is expected to predict the nutrient availability of fibrous feedstuffs more accurately, the relationship to actual *in vivo* values is unknown. Even if results of *in vitro* fermentation with pre-digested substrates do not accurately represent *in vivo* conditions, they may be used to rank the tested NSP substrates. A high ranked NSP substrate would indicate a relatively higher energy yield for the animal compared to a lower ranked substrate. This ranking can provide useful information to select the most beneficial NSP substrates in terms of nutritional value for the animal.

Table 1. Total organic matter loss (ranked) from untreated and pre-digested substrates.

Substrate	Organic matter loss (%)	
	Untreated	Pre-digested
Jerusalem artichoke	98.1	98.3
Turnip tuber	88.5	87.1
Clover-ryegrass silage 1	84.6	85.3
Turnip leaf	81.1	83.0
Clover-ryegrass silage 2	77.9	79.5
Corn silage	67.0	72.3
Rye grass	67.7	68.5

Table 2. Halftimes of asymptotic gas production (*C*) in hours measured *in vitro* for sows and for finishing pigs.

Chapter	Animals	*C* (h)		
		Oligofructose	Soy pectin	Cellulose
3	Sows	7.6	8.4	24.5
4	Sows	8.0	8.7	24.1
5	Finishing pigs	5.0	5.0	24.8

The study in Chapter 2 showed differences between substrates in the rate of fermentation and thus in halftimes (*C*) at which asymptotic gas production was reached. Other studies in this thesis (Chapter 3, 4 and 5) used the purified substrates oligofructose and soy pectin and showed halftimes occurring within the first 10 h of fermentation (Table 2). Data in table 2 indicates that the substrate available for fermentation was depleted long before the fermentation process was stopped (after 72 h). It has to be taken into account that no absorption of fermentation end products occurs *in vitro*. The short chain fatty acids (SCFA) and ammonia (NH_3) formed during fermentation remain in the fermentation bottle and can inhibit production of additional fermentation products during the fermentation process. Awati *et al.* (2006) showed that fast fermentable substrates produce different fermentation end products after 24 and 48 h. It is known that lactic acid can be converted into acetic, propionic and butyric acids by some bacterial species (Bernalier *et al*., 1999). However, concentrations of SCFA from Chapter 2 showed relative higher concentrations for untreated substrates for both inocula and a tendency for higher concentrations of SCFA for the incubation with inoculum from pigs receiving roughage. This indicates that microbiota have more readily fermentable substrate available which results in production of SCFA compared to when substrates are untreated. The microbiota of animals fed roughage in their diet might be better adapted to ferment NSP from roughages and might thus be more efficient in SCFA production compared to microbiota from standard fed pigs (no dietary roughage). In future studies, SCFA can be used for calculation of energy delivery from fermentation of NSP.

Another aspect of fermentation is the fate of nitrogen (N) in the substrate. The studies reported in Chapter 3 and 4 showed considerable amounts of ammonia in the fermentation fluid after 72 h for soy pectin. This is undoubtedly related to the fast fermentable soy pectin in combination with the N content in the fermentation bottle. *In vivo* the aim is to have as much as possible N in the large intestine in the form of microbial protein (Canh *et al*., 1998; Leek *et al*., 2007; Bindelle *et al*., 2008). A high protein fermentation, which is the source of ammonia, is regarded as

less beneficial for the host (Williams *et al*., 2001). In addition, if there is a very low fermentation rate (as with cellulose) this does not enable considerable microbial synthesis. Low microbial synthesis can lead to increased NH_3 production. Cone *et al.* (1997) showed that during incubation with rumen fluid, the fermentable substrate glucose was exhausted very quickly and therefore microbial turnover occurs and NH_3 increases.

Thus, fermentation depends on both the substrate and the inocula used. In that respect, in Chapter 2 two different sources of inocula were used to ferment the substrates: one from the control group (which had been fed solely compound feed) and one from the roughage fed group. Previous *in vitro* fermentation studies showed increased fermentation rates of substrates when the donor animals were fed increased amounts of dietary fibre (sugar beet pulp; Bindelle *et al*., 2007c) and increased SCFA concentrations in the colon when animals were fed high amounts of NSP (ground corn + sugar beet pulp or + wheat bran; Anguita *et al*., 2006). It was expected that the intestinal microbiota of animals which were fed roughage were better adapted to the substrates (Table 1), at least for ryegrass and clover grass silage since those were also part of their diet, than the microbiota from animals who had received solely compound feed. Therefore, an increased fermentation of substrate with the inoculum of donor animals fed the roughage was expected. However, this did not occur and there may be three possible explanations: 1) only one inoculum was used per animal group without replicate and results therefore may not be representative for inocula originating from pigs fed those two different diets, 2) the contrast in the diet of the donor animals was not sufficiently large in NSP or 3) the adaptation time of three weeks for the animals which received roughage was not sufficient to fully adapt the animals.

Microbial activity observed after dietary change

The study reported in Chapter 3 was designed to investigate if three weeks of adaptation to a diet high in NSP (fibre) is sufficient to allow adaption of microbiota in the large intestine of sows. As in the previous study (Chapter 2), the low fibre diet consisted of a control diet (standard compound feed for non-pregnant sows) fed at maintenance level (CVB, 2010) while the high fibre diet consisted of a reduced amount of compound feed (70% of maintenance level) which was supplemented with grass silage (30 % on a dry matter basis), assuming that sows consumed about 1 kg of grass silage (DM). The results showed a decreased gas production (OMCV) especially for the readily fermentable substrates oligofructose and soy pectin after the diet was changed from a low to a high fibre content. After the initiation of ingestion of the grass silage with a high NDF content (47% in the dry matter), the composition of microbiota has probably changed towards one

which is more capable of fermenting slow fermentable substrates and less for fermenting fast fermentable substrates.

Conversely, the microbiota from animals which were adapted to a high fibre diet (HL animals) did not seem to undergo a rapid change with regard to gas production after the diet was changed to a low fibre diet. Animals in this HL-treatment were adapted to a broader NSP spectrum compared to animals in the LH-treatment and maintained their capacity to ferment this broader spectrum of NSP longer after the diet change. On the other hand, the microbiota of the HL-treatment was less stable in terms of fermentation speed (R_{max}) compared to the LH-treatment (14 to 32% decrease compared to an 11 to 15% increase) during fermentation of fast fermentable substrates. When the diet was changed, the capacity of the microbiota to ferment indigestible carbohydrates, and therefore the fermentation process, underwent large changes for the parameters gas production, rate of fermentation and SCFA concentration. Gas production changed more for the LH-treatment than for the HL-treatment while rate of fermentation changed more for the HL-treatment than for the LH-treatment. Faecal microbes from animals in the LH-treatment were adapted to fermentation of readily fermentable substrates which were present in the compound feed and maintained this capacity for about 7 days, even with a low concentration of readily fermentable substrates which were replaced by poorly fermentable NSP. Furthermore, it appears that no plateau had been reached after 19 days for R_{max} during cellulose fermentation and for SCFA concentration during oligofructose fermentation for the HL-treatment (see Chapter 3, Table 3 and 4). It is likely that adaptation of intestinal microbiota of animals to a diet containing grass silage was not complete after a 19 days period.

The microbial profile changed over time after the diet was changed for both treatments and provided additional information on the adaptation of the microbiota in relation to the dietary change. The diet change lead to significant changes in relative abundance for two groups of microbes; *Bacteroidetes* increased and *Bacilli* decreased for the LH-treatment, while the changes were opposite for the HL-treatment. Correlation of the two most affected microbial taxonomic groups showed a negative correlation between *Bacteroidetes* and *Bacilli* when the diet was changed (Table 3).

Table 3. Correlations between two bacterial species and R_{max} measured *in vitro* for two dietary treatments of sows and two substrates.

			HL-treatment				**LH-treatment**	
Bacilli	1				1			
Bacteroidetes	-0.97 **	1			-0.93 **	1		
Rmax OF	-0.83 *	0.880 **	1		-0.46 ns	0.40 ns	1	
Rmax SP	-0.713 *	0.781 *	0.883 **	1	-0.22 ns	0.28 ns	0.624 ns	1
	Bacilli	Bacteroidetes	Rmax OF	Rmax SP	Bacilli	Bacteroidetes	Rmax OF	Rmax SP

HL-treatment, animals changed from high to low fibre; LH-treatment, animals switched from low to high fibre; ns, non-significant; OF, oligofructose; SP, R_{max}, maximal rate of gas production; soy pectin; * $P < 0.05$; ** $P < 0.01$.

When animals on a high fibre diet were subjected to a low fibre diet, the number of *Bacteroidetes* decreased significantly ($P < 0.01$) within 19 days in favour of *Bacilli*. In turn, the animals in the LH-treatment showed a significant decrease ($P < 0.01$) within 19 days in the number of *Bacilli* in favour of *Bacteroidetes*. Furthermore, when R_{max} from fermentation of oligofructose and soy pectin was correlated to abundance of *Bacteroidetes* and *Bacilli* for the HL-group, significant positive correlations between *Bacteroidetes* and R_{max} for the substrates oligofructose ($P < 0.01$) and soy pectin ($P < 0.05$) were observed. As expected, negative correlations ($P < 0.05$) were found between the abundance of *Bacilli* and R_{max} for both substrates. For the LH-treatment, these correlations could be found as well, though not significant within the 19 day period. These results show that a high fibre (NSP) flux in the digesta is related to a high number of *Bacteroidetes*, which in turn leads to a higher fermentation speed. However, this influence seems to be only significant for animals changed from a high fibre to a low fibre diet (HL-treatment) within the observed time period of 19 days, and not for animals in the LH-treatment.

The amount of SCFA produced by the microbes during fermentation of oligofructose and soy pectin increased after the host animals were switched to a diet low in NSP. Table 4 shows the SCFA concentration and the ratios of the main SCFA measured after 72 h for the substrates oligofructose and soy pectin on day 1 and 19 (last day of the experiment) after the diet change.

Table 4. Total short chain fatty acids (SCFA), main SCFA concentrations and ratios of main SCFA measured *in vitro* for two dietary treatments and substrates on day 1 and day 19 after the diet change for sows.

Sub	Treat	Day	Total SCFA	Acetate	Propionate	Butyrate	Ac:Prop:But
			mmol g^{-1} OM		mmol g^{-1} OM		%
Oligofructose							
	HL	1	10.26	5.00	4.13	0.69	49 : 40 : 6.7
		19	12.14	6.59	4.30	0.72	54 : 35 : 6.0
	LH	1	10.57	5.70	3.58	0.84	54 : 34 : 8.1
		19	10.74	5.20	4.26	0.63	49 : 40 : 5.9
Soy pectin							
	HL	1	10.69	5.84	3.77	0.54	55 : 35 : 4.9
		19	11.43	6.74	3.36	0.75	59 : 29 : 6.5
	LH	1	11.02	6.48	3.32	0.69	59 : 30 : 6.2
		19	10.09	5.26	3.71	0.52	52 : 36 : 5.3
Cellulose							
	HL	1	9.07	4.66	3.37	0.57	51 : 37 : 6.3
		19	9.86	5.57	3.09	0.68	56 : 31 : 6.9
	LH	1	8.23	4.80	2.46	0.50	59 : 29 : 6.2
		19	8.22	4.39	2.83	0.52	54 : 34 : 6.3

HL, group of animals switched from high to low fibre; LH, group of animals switched from low to high fibre; SCFA, short chain fatty acids; Sub; substrate; Treat, treatment.

The data for oligofructose and soy pectin showed that microbiota from animals on a low fibre diet produce 7 to 15% more SCFA, except for the fermentation of oligofructose by animals of the LH-treatment. Feeding animals diets containing silage (high in fibre) leads to a lower acetate ratio in favour of propionate compared to diets low in NSP for oligofructose and soy pectin. This could be an indicator that microbes of animals that are used to diets low in NSP (which contain more readily fermentable NSP compared to silage) may also produce more acetate from readily fermentable substrates. Therefore, if one wants to estimate the SCFA composition from a fermentable substrate, it has to be considered that the outcome depends on the diet composition of the donor animal as well. For cellulose, microbes from the HL-treatment were more efficient in terms of total SCFA production, even 19 days after the diet change compared to the LH-treatment, concentrations of SCFA remained at a lower level. However, as for readily fermentable substrates, the ratio of SCFA from cellulose showed a similar shift towards a decreased acetate ratio when fibre increases in the diet. Regarding the

SCFA profiles, the low SCFA concentration observed for the LH-treatment during cellulose fermentation could be an indicator that animals adapted for 19 days to a diet is insufficient for fermentation especially of slow fermentable substrates.
The results of the study reported in Chapter 3 showed that 19 days of adaptation might not be sufficient for microbiota to fully adapt to dietary changes, especially when they are changed from a high to a low fibre diet. This insufficient adaptation in combination with an insufficient contrast between low and high fibre diet could explain why animals in the study reported in Chapter 2 did not show any obvious differences between results achieved with two inocula originating from pigs fed either a control diet (low in fibre) or a silage-enriched diet (high in fibre). There are a number of studies supporting this conclusion. Varel *et al.* (1984) reported that cellulase (enzyme produced from cellulolytic microbes) activity in microbiota from pigs changed to a diet rich in cellulose (NSP) decreased in the first few days but then continuously increased up to 17 weeks after the diet change had occurred. After this period the cellulase activity reached a level which was approximately 13% higher compared to the initially measured activity, indicating increasing numbers/activity of cellulolytic bacteria. Longland *et al.* (1993) and Martinez-Puig *et al.* (2003) concluded that in terms of whole tract digestibility and faecal SCFA excretion, adaptation to several NSP sources such as raw potato starch, sugar beet pulp or wood cellulose can take up to 5 weeks. However, young animals (18.5 to 25 kg) were used for both studies, thus the results might be not representative for adult animals (finishing pigs, ca. 90 kg and sows, ca. 250 to 300 kg). For *in vitro* fermentation studies no data on adaptation to experimental diets high in NSP were available and therefore requires investigation. Other authors investigating aspects of fermentation in the large intestine of pigs have generally used an adaptation period of 7 to 14 days (Bauer *et al*., 2004; Bindelle *et al*., 2009; Anguita *et al*., 2006; Martín-Peláez *et al*., 2009; Awati *et al*., 2006). In hind side, a longer period than 19 days would have been advantageous to determine the precise period of total adaptation of pigs to diets contrasting from low NSP levels to diets with a high level of NSP. In the study reported in Chapter 3, the main changes in bacterial composition and activity occurred within 19 days using diets which are likely to be the widest range in terms of NSP content which are used in practical pig diets. Therefore, the adaptation time of donor animals to diets may be a period of 19 days if animals are already used to some NSP.

Another point of interest is the number of animals required for a representative pooled inoculum. It is likely that the microbiota composition and activity differs between animals and that the microbiota are continuously adapting to changes in diet, climate, stress, medication, injury and other factors. Of these factors, diet can be expected to be one of the major factors especially when roughages are provided *ad libitum*, as these roughages vary in composition due to time and location of

harvest, as well as a varying intake and dietary selection by animals. The study reported in Chapter 4 was designed to examine the influence of time as well as variation between multiple inocula. Multiple inocula originating from the same animals were repeatedly prepared and used for *in vitro* fermentation in order to determine repeatability of the *in vitro* method. Additionally, variation between inocula was investigated to determine if using three or four animals per inocula yields satisfactory results.

Influence of time and multiple inocula on in vitro fermentation results

It is generally accepted that for *in vitro* gas production studies with pigs, one fermentation run and one inoculum mixed from three or four animals are sufficient to provide representative results (Bauer *et al*., 2003 & 2004). Interestingly, until the study in Chapter 4 this assumption had not been validated.

A ring test has been carried out by van Gelder *et al.* (2005) using the same gas production equipment as used in the studies reported in this thesis (described by Cone *et al.*, 1996) and rumen fluid as inoculum to measure variation of gas production profiles during incubation of different substrates within laboratory and between laboratories. Results showed that fermentation profiles were influenced by variation in microbial activity and by changes in air pressure at the end of the run. However, there was a high correlation between fermentation characteristics of different laboratories. It was concluded that the gas production equipment is capable of producing repeatable fermentation characteristics with rumen fluid as inoculum. It should be noted that this conclusion is based on rumen microbiota and not large intestinal microbiota.

In the study reported in Chapter 4, three inocula were repeatedly prepared from faecal pool samples of three different sets of four sows, using always the same sets at each of five consecutive time points (weeks). Faecal pool samples were prepared each run from the same sets of four animals in order to obtain inocula of the same origin over time. All animals were fed the same diet at about maintenance (CVB, 2010) for more than three weeks before the experiment and also during the experiment. The results were shown as the mean of 3 (inocula) per time point and showed that fermentation characteristics differed between runs.

In terms of relative variation, an approximate 2 to 4% difference for the measured parameters OMCV, SCFA and NH_3 between values from the same animals was observed. However, ranking of substrates in terms of fermentability remained similar in each run. It was concluded that despite the observed run differences, which were mainly caused by one substrate outlier/week, one run using inocula obtained from four or more animals is required for ranking of substrates.

However, it remains unknown how many animals are required for the preparation of one inoculum even after the study in Chapter 4. When variation between inocula was considered, a relatively high coefficient of variation between inocula was found for the slow fermentable cellulose. Thus, future studies should determine the number of donor animals per inoculum in order to further reduce variation so that smaller differences between substrates can be tested. The significance of actual variation between inocula could not be statistically proven with the experimental set-up used in this study, as only one inoculum per animal-group was prepared per fermentation run. Therefore, no real replicates per inoculum and fermentation run were available (Udén *et al*., 2012). When variation between inocula was examined using average CV-values in Chapter 4, the variation included a time effect. This means that differences between inocula calculated across runs for the same animals are overestimated.

However, if runs are regarded as repeated observations of 'identical' treatments, information can be provided to report observations on variation between single inocula, each obtained from a different set of four animals. In Table 5, the means of each of the three inocula (A, B, C) across runs (n=5) and CV for each inoculum is

Table 5. Mean (n=5) gas production parameters of three inocula (A, B, C), and coefficient of variation across runs for three different substrates.

Parameter	Inoculum	Substrate					
		Oligofructose		Soy pectin		Cellulose	
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
OMCV (mL g^{-1} OM)							
	A	388[a]	4.9	355	3.0	385[a]	3.3
	B	380[b]	5.3	354	2.4	392[a]	5.4
	C	393[a]	2.9	360	2.4	368[b]	4.6
C (h)							
	A	7.2[b]	12.7	7.88[c]	4.7	22.7[b]	11.7
	B	8.5[a]	4.5	8.89[b]	7.4	23.2[b]	12.0
	C	8.3[a]	4.5	9.18[a]	6.8	26.5[a]	9.1
R_{max} (mL h^{-1})							
	A	74.4[a]	12.8	68.6[a]	5.2	18.5[b]	14.7
	B	61.9[c]	8.1	63.7[b]	9.7	23.0[a]	23.7
	C	71.6[b]	5.7	60.4[c]	11.1	14.9[c]	11.1

A, B, C; inocula, each obtained from a different set of four animals, *C*, halftime of asymptotic gas production; CV, coefficient of variation; OMCV, gas production (organic matter corrected volume); R_{max}, maximal rate of gas production.

[abc] means with different superscripts within column and fermentation parameter differ significantly ($P < 0.05$).

shown in order to show information on single inocula instead of means as in Chapter 4. A contrast analysis was carried out to show differences between inocula. The parameter R_{max} seemed to be more sensitive, as shown by the variation between inocula, compared to OMCV and *C*. The CV values calculated for the different inocula were lowest for the parameter OMCV, just exceeding 5%, while for *C* and R_{max} there is a larger variation, i.e. 12.7% for *C* of inoculum A for oligofructose fermentation and ranging between 9.1 and 23.7% for the parameters *C* and R_{max} for cellulose fermentation. As already shown in Chapter 4, the slow fermentable cellulose shows the highest variation. It should be pointed out that cellulose also has a lower fermentation rate compared to the other substrates. Future studies should be conducted which include replicates of inocula prepared from the same three or four animals, especially for slow fermentable substrates.

Variation in fermentation capacity between organic farms

To the author's knowledge, there are no data available on fermentation capacity of organic fattening pigs on-farm. Organic fattening pigs receive diets with a higher NSP content compared to conventional raised fattening pigs (Cone *et al*., 1997). They are also exposed to a much larger variation in NSP sources, such as straw for bedding and in some cases grass silage. Högberg *et al.* (2004) have stated that an increased ingestion of NSP leads to adaptation of microbes fermenting NSP therefore it was expected that organic raised pigs may have an increased fermentation capacity. In order to test this hypothesis, a trial was conducted where the faeces of organic fattening pigs in the finishing phase (~90 kg) from 10 organic farms were sampled to prepare inocula for an *in vitro* gas production study (Chapter 5).

Per farm three inocula were prepared from four different animals each. Inocula were used for incubation of three substrates, oligofructose, soy pectin and cellulose. A total of five fermentation runs were conducted to determine the fermentation capacities of all 10 participating farms. The results indicated that farms showed differences in fermentation capacity, although far less than expected. The reason was probably that despite the diversity of diets fed on each farm, all diets had in common that they contained a large variation and high amounts of NSP. Additionally, pigs of all farms were exposed to straw and sometimes grass silage throughout their complete fattening period (approximately 13 weeks) which together with the diets high and diverse in NSP resulted in well adapted animals.
Animals from all farms (except for one farm) were fed a compound feed with high CP contents (20% of kg DM, according to requirements of fattening pigs up to 60 kg body weight (BW); CVB, 2010), and the amount of roughage offered did not change (See Chapter 5). When they reached a BW of 60 kg they were changed to

diets containing less protein (ca. 18% of kg DM) in the compound feed. With a daily weight gain of approximately 750 g from 60 to 90 kg BW this means that when reaching a BW of 90 kg they had received the last diet for 5 weeks. One farm used a three-phase feeding strategy, the third phase starting at 70 kg. Thus, these animals at 90 kg had received the last diet for at least 3 weeks. The standard error (SEM) of fermentation characteristics (R_{max} and OMCV) between animals on different farms was smaller compared to the SEM for these fermentation characteristics from the same animals on the same farm (Chapter 4, see paragraph below). Therefore it was concluded that if animals are adapted to a broad range of NSP, they are automatically well adapted to the NSP as tested here. Future studies should investigate fermentation characteristics of pigs adapted to diets high in NSP (e.g. organic) and of pigs adapted to diets relatively low in NSP (e.g. conventional) parallel to each other under controlled conditions, in order to directly compare them. It is further of interest to study the actual amount of energy that will be supplied by fermentation to the animal and how much difference there is between organic (well adapted NSP fermentation) and conventional animals (less well adapted NSP fermentation).

Comparison of sows (Chapter 4) and fattening pigs (Chapter 5)

As mentioned above, the SEM observed for fermentation parameters measured during repeated fermentation runs within farm (where sows were repeatedly used for inocula preparation, Chapter 4) was twice as high compared to the SEM between farms (where fattening pigs from different farms were used for inocula preparation, Chapter 5), as shown in Table 6. It was expected that fermentation capacity from sows within farm would vary less and therefore show lower SEM values between runs compared to fermentation capacity of fattening pigs from different farms, but the opposite was the case. This showed that animals from different farms receiving diets with high varieties and amounts of NSP (Chapter 5,

Table 6. Standard error of the mean (SEM) of fermentation parameters across sows (n=3) and across farms with fatteners (n=10).

Animals	Fermentation parameter		
	OMCV	*C*	R_{max}
	(mL g^{-1} OM)	(h)	(mL h^{-1})
Sows	8.25	1.2	3.82
Fatteners	4.18	0.78	1.72

C, halftime of asymptotic gas production; OMCV, total gas production (organic matter corrected volume); R_{max}, maximal rate of gas production.

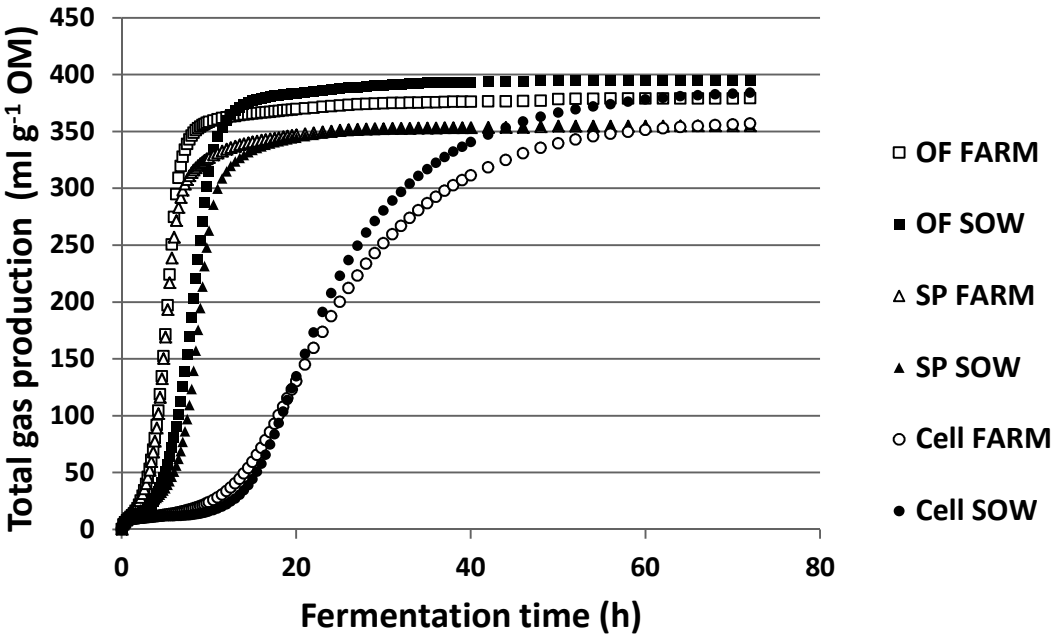

Figure 1. Gas production curves of sows (Chapter 4) and growing-finishing pigs raised on organic farms (Chapter 5) for three substrates oligofructose (OF), soy pectin (SP) and cellulose (Cell). Farm = fattening pigs (90 kg) adapted to high varieties and high amounts of NSP, Sow = Adult non-gestating sows (250-300 kg) adapted to high NSP amounts, but less variety of NSP.

Table 2) for a long period of time (throughout their production cyclus) are overall better adapted compared to animals from one farm receiving the same diet containing a considerable level of NSP but with a relatively low variety of NSP (Chapter 4, Table 1). Furthermore, when comparing studies from Chapter 4 and 5, fattening pigs of 90 kg adapted to high amounts and varieties of dietary NSP showed faster fermentation capacities in terms of C and R_{max} for the readily fermentable substrates oligofructose and soy pectin compared to adult sows of 250 to 300 kg exposed to less variety of dietary NSP. This can be seen in Figure 1 where the slope of gas production for fattening pigs adapted to high amounts and high varieties in NSP is steeper compared to adult sows adapted to considerable amounts and less variety in NSP).

The fattening pigs raised on organic farms were exposed for more than 2 months to varying NSP sources (straw and silage, but also varying components in their compound feeds) and can be considered well adapted compared to sows receiving diets with less variety in NSP. The advantage of the pigs raised on organic farms is mainly in the fermentation speed, regarding C and R_{max} (5 h and 85

mL h^{-1} vs. 7 h 70 mL h^{-1}) for oligofructose and soy pectin. Many authors concluded from previous studies, that sows, possessing the larger fermentation compartment due to their body size, have an increased capacity to utilize fibre compared to young pigs, e.g. in terms of NDF digestibility digestibility (Le Goff, *et al*., 2001) or in terms of fermentative capacity when diets high in insoluble fibre were fed (Jørgensen *et al*., 2007). However, in terms of readily fermentable NSP such as sugar beet pulp, differences in digestibility coefficients were negligible (Bindelle *et al*., 2007c; Jørgensen *et al*., 2007; Noblet & Bach Knudsen, 1997). In the present study it was shown for the first time that in terms of fermentation speed, young pigs (90 kg) fermented the readily fermentable substrates oligofructose and soy pectin almost 40% faster compared to sows (250 to 300 kg), as pigs were exposed to a high variety of NSP sources for long periods of time. This indicates that animals well adapted to a high variety of NSP and to high amounts of NSP throughout their rearing period, they have a very high potential to ferment NSP compared to animals which were raised under conditions where low amounts and a low variety of dietary NSP is provided. Future *in vitro* studies need to be performed where 2 groups of animals of the same age (fattening pigs and sows) are directly compared when exposed for different periods of time to different varieties of NSP to confirm these observations.

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Summary

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Samenvatting

Summary

One of the aspects of commercial pig farming which is becoming increasingly important is the replacement of grains (starch) by indigestible dietary carbohydrates (mainly non-starch polysaccharides, NSP) in pig diets. Grains can be considered high quality feed ingredients, as starch is highly digestible and the main energy source for maintenance and growth of commercially farmed pigs. As grains are however also suitable foods for human consumption, pigs are more and more regarded as competitors to humans for high-quality feed ingredients. Compared to humans pigs are more adept to digest indigestible dietary carbohydrates. Thus, NSP-rich substrates can potentially form an alternative feed source for pig diets. Therefore, it is necessary to identify potential NSP sources and to characterize these NSP sources for their feeding value in diets for pigs. *In vitro* methods are useful tools for characterization of NSP sources, by using enzymes or faecal inocula to incubate the substrate to be tested. The results can be used to predict feeding values of NSP sources in diets for pigs. However, the available *in vitro* methods remain to be validated and standardized in order to improve predictability of nutrient/energy availability from NSP. Recently, it has been shown that using a combination of *in vitro* enzymatic pre-digestion of a substrate with subsequent *in vitro* fermentation provides a good combination to simulate pre-caecal digestion and large intestinal fermentation in the pig. With this procedure, NSP sources can be tested regarding their fermentation kinetics (e.g. fermentation rate) as well as fermentation products such as total gas produced, short chain fatty acid (SCFA) concentrations and ammonia concentration. Especially a high concentration of SCFA would be a selection criterion for potential NSP sources to be included in the diet, since SCFA provide a relative high amount of energy. The results of *in vitro* fermentation depend on a sensitive interaction of the diet fed to the faecal donor pigs, on microbiota present in the faecal inoculum and on the substrate to be tested. Especially the influence of the dietary treatment of the faecal donors has not been given much attention yet. It is known that donor animals fed high amounts of NSP have more potential to ferment a substrate compared to donor pigs which are fed low amounts of NSP. Therefore, the results of *in vitro* fermentation can over- or underestimate the feeding value of NSP sources depending on the diet of donor animals. Additionally, there is still a lack of information on some general aspects of *in vitro* fermentation studies with pigs, such as the adaptation time of donor animals to an experimental diet, repeatability of fermentation results or the variability between multiple inocula. Therefore this thesis describes four studies which were designed to test the above mentioned aspects which are summarized in the following paragraphs.

Since there are enzymatic and fermentative *in vitro* methods to assess degradation of dietary NSP, the first study (**Chapter 2**) was carried out to compare the results of NSP degradation of seven NSP substrates by applying both methods. In addition, a combination of both methods was applied, mimicking pre-digestion and fermentation *in vivo*. Furthermore, two different inocula were used for fermentation: one from faecal donor animals fed a control diet low in NSP and one from faecal donor animals fed a roughage diet high in NSP. The *in vitro* enzymatic incubation method showed 3% to 20% lower values for degradation compared to the *in vitro* fermentation method. The pre-digestion of substrates with enzymes before fermentation led to similar results compared to fermentation of untreated substrates; however gas production kinetics were different. Using two different inocula generated no significant differences. Ranking of feedstuffs in terms of fermentability and organic matter loss remained similar between the different methods. All methods seem to be appropriate tools to rank NSP sources regarding their fermentability/degradation. However, it is concluded that the pre-digestion of substrates in combination with *in vitro* fermentation has the highest potential to predict nutrient availability in the gastrointestinal tract. Unexpectedly, no differences in fermentation kinetics were found between inocula originating either from animals fed diets low in NSP or diets high in NSP, which triggered the question for the following study.

The second study (**Chapter 3**) was designed to investigate if three weeks of adaptation to a diet high in NSP is sufficient to adapt microbiota in the large intestine of sows in terms of *in vitro* fermentability. For this study, two groups of sows were changed from either a high to a low (HL) or from a low to a high (LH) NSP diet. After the diet change, sows were repeatedly used as faecal donors for *in vitro* fermentation of three purified substrates (oligofructose, soy pectin and cellulose) belonging to the group of indigestible carbohydrates. Faeces samples were taken for profiling of microbial composition. As in the previous trial, the low NSP diet consisted of a control diet low in NSP, while the diet high in NSP consisted of the control diet supplemented with grass silage. Day of fermentation run (time) had an effect ($P < 0.05$) on total gas production for the fast fermentable substrates oligofructose and soy pectin. Maximal rate of gas production changed over time for both treatments (LH and HL) and fast fermentable substrates, differing in total 10 to 32% (though not significant). For the slow fermentable cellulose, all measured fermentation parameters were higher ($P < 0.05$) for the HL-treatment. The diet change lead to significant changes in relative abundance especially for two groups of microbes: Abundance of *Bacteroidetes* increased and abundance of *Bacilli* decreased for the LH-treatment, while the changes were opposite for the HL-treatment.

When the diet was changed, the capacity of the microbiota to ferment indigestible carbohydrates and therefore the fermentation process underwent large changes for the parameters gas production, rate of fermentation and short chain fatty acid concentration. Gas production changed more for the LH-treatment than for the HL-treatment while rate of fermentation changed more for the HL-treatment than for the LH-treatment. These results showed that adaptation in terms of microbial activity seems to be incomplete after 19 days for both treatments, especially regarding the fermentation of slowly fermentable substrates. In turn, the microbial composition appears to have reached a stable equilibrium within 19 days after the diet was changed.

The third study (**Chapter 4**) was designed to examine the influence of time on *in vitro* fermentation parameters as well as variation between multiple inocula when same sources (donor animals) of inocula were repeatedly used for *in vitro* fermentation. Therefore, diet-adapted sows fed the same diet over a period of five weeks were sampled for preparation of three inocula, each mixed from four different animals. For each of five consecutive fermentation runs, the same 3 sets of animals were used for inocula preparation to ensure repeated use of inoculum of the same origin. The results showed that fermentation characteristics differed between fermentation runs ($P < 0.05$), but less than substrates ($P < 0.01$). Differences between inocula ranged from 2% for total gas production up to 25% for maximal rate of gas production and were especially high for the slow fermentable cellulose. It is concluded that despite the observed run/time effect, which was mainly caused by one outlier/week, one run is sufficient for testing substrates if differences in fermentability between substrates are sufficiently large. The relatively high coefficient of variation between inocula for cellulose fermentation indicated that the number of animals per inoculum should be revisited, especially for *in vitro* evaluation of slow fermentable substrates. Variation between inocula indicated that pooling of more than 4 animals per inoculum is necessary to reduce variation.

The aim of the fourth and last study (**Chapter 5)** was to determine the variation of the *in vitro* fermentative capacity of fattening pigs within and between organic farms using the *in vitro* batch culture method. Feeding management was recorded to see if fermentation characteristics of farms could be related to NSP-contents in the diets fed on those farms. Pigs from 10 organic farms were sampled to prepare three inocula per farm, each inoculum mixed from four different animals. The results showed that pigs from different farms showed varying fermentative capacities as assessed by gas production, kinetics and fermentation end products formed ($P < 0.001$). Coefficients of variation between inocula within farms varied up to 40% for gas production and kinetics, in particular for incubation with cellulose. No relationship could be established between on-farm feeding practice

(e.g. NSP content in diet) and fermentation capacity of pigs. Fermentative capacity from pigs reared under organic conditions varies considerably between farms. Finishing pigs reared under organic farming conditions are fast fermenters of oligofructose and soy pectin. The high coefficients of variation between inocula within farm especially for cellulose suggest that more than four donor animals should be used per inoculum. *In vitro* fermentation results could not be related to the composition of the diets fed on organic farms.

Overall, it can be concluded that the various *in vitro* procedures lead to different degradation rates. Although the ranking order for substrates in terms of degradation remained similar for different methods, nutrient availability to the animal seems to be best predicted with enzymatic pre-digestion of substrates with subsequent fermentation. To obtain representative fermentation results for faecal donor animals, one single fermentation run seems adequate. However, when donor animals are changed to a new diet for an *in vitro* fermentation study (e.g. a diet differing in NSP) an adequate adaptation time needs to be applied. An adaptation time of 19 days was not enough to adapt animals to a diet with a different content of grass silage. Variation between fermentation results appears to be, at least partly, influenced by age of animals used for inoculum preparation and number of faecal samples mixed per inoculum. Fermentation results obtained from animals adapted to a broad variety and high amounts of dietary NSP throughout their life have probably a higher potential to ferment NSP compared to animals fed only a limited variety and low amounts of NSP.

Based on the conclusions deduced from this thesis, it can be stated that the *in vitro* fermentation method can be improved by using more donor animals and feeding a representative diet to increase predictability of feeding values from NSP feed ingredients. Therefore, important issues to be further revisited are the nutrition of donor animals preferably under controlled conditions, but also comparable to on-farm conditions. Also, groups of animals of different age and weight (growing pigs, finishing pigs, gestating and non-gestating sows) exposed for differing periods of time (rather months than weeks) should be directly compared to see the relative differences in fermentation capacity. Further, the optimal number of animals to be used per inoculum should be investigated. Pig diets based on an improved *in vitro* methodology have the potential to increase economic profit for feed industry and farmers, and also to increase animal health and welfare.

Samenvatting

Een van de aspecten van de commerciële varkenshouderij die steeds belangrijker wordt is de vervanging van granen (zetmeel) door onverteerbare koolhydraten (voornamelijk niet-zetmeel-polysachariden, NZP) in varkensvoeders. Granen zijn hoog kwalitatieve voedermiddelen aangezien het zetmeel goed verteerbaar is en zodoende de voornaamste energiebron is voor onderhoud en groei van commercieel gehouden varkens. Granen zijn echter ook geschikt voor de voeding van de mens en daardoor wordt het varken steeds meer gezien als concurrent voor de mens wat betreft hoog kwalitatief voedsel zoals granen. Daardoor is het noodzakelijk om potentiële NZP bronnen vast te stellen en deze te karakteriseren wat betreft de voederwaarde in varkensrantsoenen. Het is reeds gebleken dat *in vitro* methoden geschikte alternatieven kunnen zijn voor *in vivo* proeven voor karakterisering van voederwaarde van NZP bronnen, door gebruik te maken van enzymen of fecale inocula. De resultaten van deze *in vitro* methoden kunnen gebruikt worden om de voederwaarde van NZP bronnen in rantsoenen voor varkens te bepalen. Deze *in vitro* methoden zijn echter nog niet goed gestandaardiseerd en gevalideerd om een goede bepaling van de energie- en nutriëntenbeschikbaarheid van NZP te verkrijgen. Recent is aangetoond dat door het gebruik van een combinatie van een *in vitro* enzym voorvertering van een substraat met daarna een *in vitro* fermentatie met microbiota, een goede simulatie van zowel dunne als dikke darm vertering bij het varken bereikt wordt. Met deze procedure worden de fermentatie eigenschappen (bv fermentatie snelheid) van NZP alsook de opbrengst aan fermentatie producten zoals totaal geproduceerd gas, kort keten vetzuren (KKVZ) en ammoniak gemeten. Vooral de hoeveelheid KKVZ kan een belangrijk criteria zijn voor potentiële NZP bronnen, aangezien KKVZ een relatief hoge energieopbrengst leveren. De resultaten van *in vitro* fermentatie hangen af van de interactie van het rantsoen dat gevoerd is aan het donor varken, van de microbiota aanwezig in het fecale inoculum en van het te testen substraat. Vooral de invloed van het rantsoen van de fecale donoren heeft tot nu toe nauwelijks aandacht gekregen. Het is waarschijnlijk dat microbiota van varkens die veel en verschillende NZP gevoerd hebben gekregen, ook een beter potentieel hebben om NZP uit substraat te fermenteren dan microbiota van varkens die dit niet hebben gehad. Daarom kunnen de resultaten van *in vitro* fermentatie een onder- of overschatting geven van voederwaarde van NZP, afhankelijk van het rantsoen van de donor varkens. Verder is ook nog niet goed bekend of *in vitro* fermentatie uitkomst ook afhangt van de lengte van de periode waarin het donor varken het voer heeft gehad, de herhaalbaarheid van *in vitro* fermentatie, en de variatie tussen inocula van verschillende varkens. Daarom beschrijft deze

dissertatie vier studies die ontworpen zijn om bovenstaande aspecten te onderzoeken zoals samengevat in de volgende paragraaf.

Aangezien er enzymatische en fermentatie methoden bestaan om de afbraak van NZP *in vitro* te onderzoeken, is in de eerste proef een combinatie van enzymatische voorbehandeling en *in vitro* fermentatie gebruikt om de afbraak van NZP te bepalen (**Hoofdstuk 2**) en vergeleken met alleen fermentatie of alleen enzymen bij zeven NZP substraten. Ook werden twee verschillende inocula gebruikt voor de fermentatie; een van de feces van donor dieren die een controle rantsoen met laag NZP gehalte ontvingen en een van feces van dieren die veel ruwvoer (veel NZP) ontvingen. Door *in vitro* incubatie met enzymen was er een 3% tot 20% lagere afbraak in vergelijking met de *in vitro* fermentatie methode. De voorvertering van de substraten met enzymen voor fermentatie gaf gelijkwaardige resultaten in vergelijking met fermentatie van niet voorverteerde substraten, alhoewel er wel wat verschillen waren in gas productie kenmerken. Tussen de twee inocula waren geen duidelijke verschillen in fermentatie kenmerken. De rangschikking van voedermiddelen op niveau van fermentatie en op organische stof verlies bleef gelijk bij alle methoden. Dus de geteste methoden kunnen gebruikt worden voor de rangschikken van NZP bronnen met betrekking tot de fermentatie/afbraak karakteristieken. Er werd geconcludeerd dat de voorvertering van substraten in combinatie met *in vitro* fermentatie de beschikbaarheid van nutriënten en energie in de darm het beste weergeeft. Het feit dat de inocula van dieren met weinig of veel NZP in het rantsoen geen duidelijk verschil gaf was de aanleiding voor de volgende studie.

De tweede studie (**Hoofdstuk 3**) was ontworpen om te onderzoeken of 19 dagen adaptatie aan een hoog NZP rantsoen lang genoeg was voor microbiota in de dikke darm van zeugen om zich aan te passen met betrekking tot *in vitro* fermentatie studies. Voor deze studie werd het rantsoen van twee groepen zeugen aangepast van ofwel een hoog NZP rantsoen naar een laag NZP rantsoen (HL groep) ofwel van een laag NZP rantsoen naar een hoog NZP rantsoen (LH groep). Na de rantsoen verandering werden de dieren op verschillende dagen als fecale donoren gebruikt voor *in vitro* fermentatie van drie zuivere bronnen (oligofructose, soja pectine en cellulose). Fecale monsters werden ook onderzocht op microbiële samenstelling. Evenals in de proef van hoofdstuk 2 bestond het laag NZP rantsoen uit standaard krachtvoer met laag NZP terwijl dieren op het hoge NZP rantsoen extra kuilgras kregen. Dag van fermentatie (tijd) had effect ($P < 0.05$) op de gas productie (dag effect) in het bijzonder wanneer oligofructose en soja pectine als substraat werd gebruikt. Maximale snelheid van gas productie (R_{max}) veranderde in de tijd voor beide behandelingen (LH en HL) voor de goed fermenteerbare substraten oligofructose en soja pectine, met een verschil van in totaal 10 tot 32% (niet significant). Voor het langzaam fermenteerbare cellulose waren deze

kenmerken van fermentatie alle hoger ($P < 0.05$) met de inocula van de HL dieren. De rantsoenverandering leidde tot significante veranderingen in het relatieve aandeel van microben, in het bijzonder voor twee groepen microben: het aandeel van *Bacteroidetes* nam toe en dat van *Bacilli* nam af voor de LH-dieren terwijl de veranderingen voor de HL dieren tegenovergesteld was. Met verandering in rantsoen veranderde ook de capaciteit van de microbiota om NZP te fermenteren en daardoor ook de gas productie parameters en de KKVZ concentratie. Gas productie veranderde meer voor de LH dieren dan voor de HL dieren terwijl de snelheid van fermentatie (R_{max}) het meest veranderde bij de HL dieren. Adaptatie van de microbiota lijkt nog niet volledig op 19 dagen na rantsoen verandering voor beide behandelingen, vooral voor fermentatie van cellulose. De microbiële samenstelling echter lijkt na 19 dagen een stabiel evenwicht te hebben bereikt .

De derde studie (**Hoofdstuk 4**) was ontworpen om te onderzoeken of tijd invloed heeft op *in vitro* fermentatie parameters en op variatie tussen activiteit van microbiota tijdens fermentatie door microbiota van steeds dezelfde donor dieren. Daarom werden van zeugen die aangepast waren aan een standaard rantsoen gedurende een periode van 5 weken fecale monsters genomen om drie inocula te creëren van vier dieren elk. Bij elke van de in totaal vijf monsternames werd steeds inocula gemengd van dezelfde vier dieren en gebruikt voor *in vitro* fermentatie van soja pectine oligofructose en cellulose. De resultaten lieten zien dat fermentatie karakteristieken verschillend waren tussen de runs ($P < 0.05$) maar de verschillen waren veel minder dan tussen de substraten ($P < 0.01$). Verschillen tussen inocula varieerden van 2% voor totale gas productie tot 25% voor snelheid van gasproductie (R_{max}) en waren vooral hoog voor het langzaam fermenteerbare cellulose. Er werd geconcludeerd dat ondanks het tijdseffect (dat vooral veroorzaakt werd door één uitschieter) een run voldoende is om substraten te testen voor verschillen in fermentatie. De hoge variatie coëfficiënt tussen inocula voor cellulose fermentatie toont aan dat mogelijk voor langzaam fermenteerbare substraten zoals cellulose meer dan vier dieren per inocula nodig zijn .

Het doel van de vierde en laatste studie was het onderzoeken (**Hoofdstuk 5**) van de variatie in fermentatie capaciteit van de microbiota van mestvarkens op biologische bedrijven met de *in vitro* batch methode. Voerschema's werden geregistreerd om na te gaan of de fermentatie karakteristieken voor NZP hiermee samenhangen. Varkens van 10 biologische bedrijven werden bemonsterd voor het verkrijgen van inocula (op een tijdstip drie keer vier dieren om drie inocula te verkrijgen per bedrijf). Resultaten lieten zien dat inocula van varkens op de verschillende bedrijven variëren met betrekking tot fermentatie kenmerken zoals gas productie, kinetiek en fermentatie eind-producten ($P < 0.001$). De variatie coëfficiënten tussen de inocula binnen bedrijf varieerden tot 40% voor gas productie en kinetiek, in het bijzonder voor cellulose. Er werd geen relatie

gevonden tussen de variatie in voerstrategie (aandeel NZP) en de fermentatie capaciteit van inocula van vakens. Deze capaciteit van varkens varieerde behoorlijk tussen bedrijven. Wel kan gezegd worden dat zware mestvarkens op deze bedrijven de oligofructose en soja pectine snel fermenteren. De hoge variatie coëfficiënt tussen inocula voor fermentatie van cellulose binnen bedrijven suggereert dat vier dieren wellicht niet voldoende is voor het inoculum van dat bedrijf. *In vitro* fermentatie had geen relatie met voersamenstelling op deze biologische bedrijven.

De conclusie van de proeven is dat de verschillende *in vitro* technieken ook verschillende resultaten geven in afbraaksnelheid van substraten. De rangschikking tussen substraten blijft gelijk. De nutriëntbeschikbaarheid lijkt het best voorspeld te worden met enzymatische voorvertering gevolgd door fermentatie. Om representatieve fermentatie resultaten te behalen is één run met inocula van de donor dieren voldoende. Echter na een verandering in rantsoen (in NZP gehalte) van donor dieren moet voldoende tijd worden genomen alvorens een inoculum te nemen. Voor volledige adaptatie op bv gras silage toevoeging was de 19 dagen zoals in studie 2 niet voldoende. Variatie in fermentatie resultaten door inocula wordt beïnvloed door het aantal dieren dat gebruikt wordt voor een inoculum. Fermentatie resultaten middels inoculum van dieren die aan een breed spectrum van NZP in het voer zijn gewend vertoond slechts een beperkte variatie op hoog en laag NZP.

De conclusie van het onderzoek in deze dissertatie is dat *in vitro* fermentatie kan worden verbeterd door van voldoende donor dieren een inoculum te nemen en ook door de donor dieren een representatief rantsoen te verstekken om de voederwaarde van NZP ingrediënten beter te voorspellen. Aspecten die nader onderzoek behoeven zijn 1) voeding van de donor dieren, onder gecontroleerde omstandigheden die lijken op die op varkensbedrijven; 2) het type varken qua leeftijd en kenmerken (bv groeiende varkens, zware mestvarkens, drachtig en niet drachtige zeugen); en 3) adaptatieperiode (langer dan 3 weken). Verder dient het optimale aantal dieren per inoculum bepaald te worden. Rantsoenen voor varkens die gebaseerd zijn op een verbeterde *in vitro* methode, hebben het potentieel om de winst te vergroten voor de industrie en de varkenshouders, alsook het dierwelzijn en gezondheid te verhogen.

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Curriculum vitae

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Maria Antonia (Mascha) Sappok was born on April 11th, 1979 in Tübingen, Germany. She graduated in 1998 from the secondary school "Hildegardisschule" in Bochum. After spending one year doing an internship on the therapy-farm "Marienhof" in Hattingen for mentally handicapped people she started her studies of agricultural sciences with emphasis on animals sciences at the "Rheinische Friedrich-Wilhelms-University" in Bonn, Germany. The study was successfully completed in 2005 with animal nutrition as specialisation. During and after her studies she spent one year in Switzerland, USA and Canada to work on different farms. In April 2006 she started a PhD position at the Faculty of Organic Agricultural Sciences, Department of Animal Health and Nutrition at the University of Kassel, Germany, supervising a project with fattening pigs raised in an outdoor system. While working on her thesis in Kassel she also started to collaborate with the Animal Nutrition Group at the Wageningen University, Netherlands, spending 2 months there as a visiting researcher on in vitro hindgut digestion of fibre in pigs. This collaboration resulted in a continuation of her PhD at the Animal Nutrition Group of the Wageningen University from 2009 onwards. The results of the PhD project are described in this thesis. She can be reached by e-mail at: maria.sappok@yahoo.de

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