COMMUNAUTÉ FRANCAISE DE BELGIQUE
ACADÉMIE UNIVERSITAIRE WALLONIE-EUROPE
FACULTÉ UNIVERSITAIRE DES SCIENCES AGRONOMIQUES DE GEMBLOUX

IN VITRO CHARACTERISATION OF DIETARY FIBRE FERMENTATION IN THE PIG INTESTINES AND ITS INFLUENCE ON NITROGEN EXCRETION

Jérôme BINDELLE

Essai présenté en vue de l'obtention du grade
de docteur en sciences agronomiques et ingénierie biologique

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Bindelle Jérôme (2008). Caractérisation *in vitro* de la fermentation des fibres alimentaires dans les intestins du porc et de son influence sur l'excrétion azotée (thèse de doctorat en anglais). Gembloux, Faculté universitaire des Sciences agronomiques, 146 p., 18 tabl., 9 fig.

Résumé :

Ces dernières années, une attention croissante est portée aux fibres alimentaires (FA) en nutrition porcine. La croissance bactérienne consécutive aux fermentations intestinales des FA provoque un transfert de l'excrétion de N de l'urée dans l'urine vers des protéines bactériennes dans les fèces avec comme conséquence une réduction de l'émission de NH_3 des lisiers. L'objectif de cette thèse était d'étudier la relation entre la fermentescibilité des FA, la croissance bactérienne dans les intestins et l'excrétion de N.

Dans la première partie, une méthode *in vitro* de gaz-test utilisant un inoculum bactérien vivant utilisée chez les ruminants a été adaptée au porc. L'utilisation pour préparer l'inoculum de contenu du colon a été comparée aux fèces et il a été conclu que ces dernières pouvaient remplacer le contenu intestinal, évitant l'usage d'animaux canulés. Deuxièmement, l'importance d'une hydrolyse à la pespine-pancréatine prélable à la fermentation pour simuler la digestion dans l'estomac et l'intestin grêle a été mise en évidence. Finalement, l'influence du poids corporel et du contenu en fibres de la ration des donneurs de fèces sur les cinétiques de production de gaz a été mise en évidence. Lors de l'étude d'un sujet spécifique à une catégorie de porc, il est dès lors recommandé d'utiliser des animaux de cette même catégorie comme donneurs de matières fécales pour préparer l'inoculum.

Dans la seconde partie de la thèse, la synthèse protéique (SP) par les microbes fécaux a été mesurée lors de la fermentation de diverses source d'hydrates de carbone purifiées ou des ingrédients avec des contenus en FA variables, en utilisant dans l'inoculum du NH_4Cl enrichi en ^{15}N. Les résultats ont montré que SP variait entre 9,8 et 22,9 mg N g^{-1} d'hydrate de carbone fermenté en fonction du taux de fermentation et de la teneur en FA solubles de l'hydrate de carbone. Ces observations *in vitro* ont été confirmées au moyen d'expériences *in vivo*: SP *in vitro* passait de 1,51 à 2,35 mg N g^{-1} ration tandis

que le rapport d'excrétion *in vivo* N-urinaire:N-fécal diminuait de 2,171 à 1,177 avec des rations contenant des niveaux croissants de FA solubles.

Bindelle Jérôme (2008). *In vitro* characterisation of dietary fibre fermentation in the pig intestines and its influence on nitrogen excretion (thèse de doctorat). Gembloux, Belgium : Gembloux Agricultural University, 146 p., 18 tabl., 9 fig.

Summary :

Increasing attention has been paid to dietary fibre (DF) fermentation in the large intestine of pigs during the past years in pig nutrition. The bacterial growth supported by DF intestinal fermentation induces a shift of N excretion from urea in urine to bacterial protein in faeces that reduces NH_3 emission from the manure. The objective of this thesis was to investigate the relationship between DF fermentability, intestinal bacteria growth and the N excretion.

In the first part, an *in vitro* gas-test method using a living bacterial inoculum developed for ruminants was adapted to the pig. The use of pig colonic content was compared to faeces for the preparation of the inoculum and it was concluded that faeces could replace intestinal content, avoiding the use of cannulated animals. Secondly, the influence of a pespin-pancreatin hydrolysis prior to the fermentation in order to simulate digestion in the stomach and the small intestine was demonstrated. Finally, the influence of the faeces donnor bodyweight and the dietary fibre content of its diet on the gas production kinetics was shown. When studying a topic related to a specific category of pig, it is recommended to use animals from the same category as faeces donors to prepare the inoculum.

In the second part of the thesis, the amount of protein synthesis (PS) by faecal microbes fermenting different sources of purified carbohydrates, or ingredients differing in DF content, was measured using ^{15}N-labelled NH_4Cl in the inoculum. PS ranged between 9.8 and 22.9 mg N g^{-1} fermented carbohydrate according to the rate of fermentation of the carbohydrate and its soluble fibre content. These *in vitro* observations were confirmed through *in vivo* experiments with diets containing increasing levels of soluble DF: *in vitro* PS passed from 1.51 to 2.35 mg N g^{-1} diet while *in vivo* urinary-N:fecal–N excretion ratio decreased from 2.171 to 1.177.

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First of all, I would like to sincerely thank Mr. André Buldgen, Professor at the Department of Animal Production of the Gembloux Agricultural University (FUSAGx) and Mr. Pascal Leterme, Researcher at the Prairie Swine Centre (Saskatoon, SK, Canada). They both accepted to supervise this thesis, to share their knowledge in animal nutrition and give me all the means required to complete this work. I must also thank them for their contribution in the elaboration of the experimental protocols, in the interpretation of the data and in the correction of the articles.

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Last but not least, I want to thank Stéphanie, my wife, and Émilien, Heitor and Darius, my sons, for the understanding, the encouragement and the funny questioning on the odd experiments with pigs. I dedicate them the following song:

O porquinho

(Vinicius de Moraes and Toquinho, A Arca de Noe 2, Polygram, 1981)

Muito prazer, sou o porquinho. Eu te alimento também.
Meu couro bem tostadinho, quem é que não sabe o sabor que tem.
Se você cresce um pouquinho, o mérito, eu sei, cabe a mim também.
Se quiser, me chame, te darei salame e a mortadela branca, rosa e bela num pãozinho quente.
Continuando o assunto, te darei presunto. E na feijoada, mesmo requentada, agrado a toda gente.
Sendo um porquinho informado, o meu destino bem sei.
Depois de estar bem tostado, fritinho ou assado, eu partirei.
Com a tia vaca do lado, vestido de anjinho, pro céu voarei.
Do rabo ao focinho, sou todo toicinho. Bota malagueta em minha costeleta numa gordurinha.
Que coisa maluca, minha pururuca é uma beleza, minha calabresa no azeite fritinha.

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Abbreviations

ADF, acid detergent fibre

ADL, acid detergent lignin

BNI, bacterial nitrogen incorporation

CFU, colony forming unit

CIUF, Conseil interuniversitaire de la Communauté Française de Belgique (Brussels, Belgium)

CUD, Commission universitaire au développement (Brussels, Belgium)

CP, crude protein

DE, digestible energy

DF, dietary fibre

G_f, maximum gas volume

HF-S, high fibre, soluble diet

HIGH-I, high fibre, low soluble diet

HIGH-S, high fibre, high soluble diet

INS, insoluble fibre diet

INT, intermediate fibre diet

KNU, kilo novo units

L, lag time

LOW-I, low fibre, low soluble diet

LOW-S, low fibre, high soluble diet

ME, metabolizable energy

MF, medium fibre diet

$\mu_{t=T/2}$, fractional rate of degradation

NDF, neutral detergent fibre

NSP, non-starch polysaccharides

OH, oat hulls

OM, organic matter

r, simple correlation coefficient

r^2, simple coefficient of determination

R^2, multiple coefficient of determination

RMSE, root mean square error

RS, resistant starch

SBP, sugar beet pulp

SCFA, short-chain fatty acids

SD, standard diet

T/2, half time to asymptotic gas production

VHF, very high fibre diet.

INTRODUCTION

Introduction

The research presented in this manuscript aimed to investigate the relationships between the fermentability of DF and its influence on nitrogen excretion pathways. It was initiated in the framework of a cooperation project financed by the Belgian Co-operation for Development (CIUF-CUD, CERCRI project) between the Department of Animal Production of the Gembloux Agricultural University (Gembloux, Belgium) and the Department of Animal Science of the National University of Colombia (Palmira, Colombia). The project aimed to improve the local swine production in South-Eastern Colombia. Feeding strategies used by the farmers were evaluated and it appeared that fibrous ingredients such as crop by-products, fruits or tree leaves were available in order to partially replace the prohibitive concentrates used in swine nutrition. The wide range of ingredients that had to be evaluated, pointed out the usefulness of an *in vitro* method for the rapid screening of fibrous ingredients, before investigating the most interesting among them *in vivo*. The scope of the method that was adapted from ruminant studies, was enlarged to study the functionality of dietary fibre in pigs reared in the tropics as well as those reared in temperate environments. For the latter, the influence of intestinal fermentation of dietary fibre on nitrogen excretion in pigs was also investigated.

This manuscript is a compilation of published articles and is structured as follows: after a review of the literature on the nutritional and environmental consequences of dietary fibre in pig nutrition, the research strategy that was developed in this thesis and the results are presented. Finally, a general conclusion and future prospects are drawn.

CHAPTER I

Article 1:

Nutritional and environmental consequences of dietary fibre in pig nutrition : A review

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Running head:
Dietary fibre in pig nutrition

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Keywords: pigs, dietary fibre, fermentation, nitrogen pollution, volatile fatty acids, forages

Abbreviations: CP, crude protein ; DE, digestible energy; DF, dietary fibre; NDF, neutral detergent fibre; NSP, non-starch polysaccharides; OM, organic matter; RS, resistant starch; SCFA, short-chain fatty acids

This article is accepted for publication in *BASE.*

1. Introduction

The second half of the XXth century has seen a sharp increase of the world pork (*Sus scrofa*) production, which reaches nowadays a herd of 964 million pigs (FAO, 2006). Intensification of the rearing techniques, breeding programs and genetic progresses have resulted in lower production costs. However, intensive production systems have caused nitrate leaching and phosphorus accumulation in the soils receiving pig manure. These systems also induced animal welfare concerns such as stereotypies in gestating sows and human health problems such as the development of a gut microflora resistant to antibiotics (Manero *et al.*, 2006). All these issues seriously question the social and environmental sustainability of intensive pig production (Basset-Mens and van der Werf, 2005).

During the last 15 years, different solutions have been proposed to cope with these problems. Efforts have been spent to formulate diets that better meet the pig's requirements or contribute to reduce odour and pollutant excretion. In particular, attention is paid to dietary fibre (DF), for its capacity to reduce ammonia emission (Nahm, 2003; Aarnink and Versetegen, 2007) and to improve gut health (Williams *et al.*, 2001; Montagne *et al.*, 2003) and pig welfare (Meunier-Salaun, 1999; Courboulay *et al.*, 2001).

Increasing fibrous ingredients provided by the food industry are now incorporated in rations for pigs, despite the negative impact of DF on performances due to lower digestibility of dietary energy and protein (Noblet and Le Goff, 2001) and fatter carcasses. The use of forages, rich in DF, is also envisaged in more extensive systems such as herbage in outdoor production systems (Rivera Ferre *et al.,* 2001; Blair, 2007) or in tropical countries where alternative feeding systems are studied, developed and extended (Pérez, 1997, Leterme *et al.*, 2007) because grains are needed for human consumption.

The present review examines the influence of DF fermentation in the pig intestines on the digestive processes. Their consequences on pig protein and energy nutrition, health concerns and environmental issues in intensified and in more extensive tropical production systems are also discussed.

2. Dietary fibre fermentation

1. Dietary fibre definition and chemical structure

DF is commonly defined as all plants polysaccharides and lignin that are resistant to hydrolysis by human digestive secretions (Trowell, 1976). This definition is also commonly used for all non-ruminant animal species, including the pig. DF covers a wide range of carbohydrates known as non-starch polysaccharides (NSP) that include pectins, cellulose, hemicelluloses, β-glucans and fructans. Oligosaccharides and resistant starch are also considered in the DF fraction. As shown in Table 1, the hydrolysis of these carbohydrates invariably produces the same pentoses, hexoses, deoxyhexoses and uronic acids (Chesson, 1995).

Table 1. Classification of common non-digestible carbohydrates (Chesson, 1995; Bach Knudsen, 1997; Montagne *et al.*, 2003; Sajilata *et al.*, 2006)

Type of carbohydrate	Constituent monomers	Solubility, water holding capacity	Common sources in pigs diets
Oligosaccharides, 3 < DP < 10			
Fructo- and galacto-oligosaccharides	Fructose, galactose, glucose	+	Soybean meal, peas, rapeseed meal, cereal, milk products
Polysaccharides, 10 < DP			
Starch			
Physical inaccessible starch (RS1)	Glucose	-	Whole or partly milled grains and seeds, legumes
Crystalline resistant granules (RS2)	Glucose	-	Raw potato, sweet potato, some legumes, plantain, high amylose maize
Retrograded amylose (RS3)	Glucose	-	Cooled heat-treated starchy products
Non starch polysaccharides (NSP)			
Cellulose	Glucose	-	Most cereal, legumes and forages, plant cell wall
Hemicellulose	Glucose, Rhamnose, xylose, galactose, fucose, arabinose	+/-	Cereal, legumes hulls
β–glucans	Glucose	+	Barley, oats, rye
Pectins	Uronic acids	+	Fruits, chicory and sugar beet pulp
Fructans and inulins	Fructose, glucose	+	Yam, rye, Jerusalem artichoke, chicory

DP, degree of polymerisation

The physiological properties of NSP and their fermentability are poorly predictable from the monomeric composition and are more related to their solubility, viscosity, physical structure and water-holding capacity (Asp, 1996).

Starch is susceptible to hydrolysis by salivary and pancreatic enzymes. However, the hydrolysis is not always complete (Sajilata *et al.*, 2006). A part of the starch, termed "resistant starch" (RS), escapes digestion in the small intestine and reaches the large intestine because of physical inaccessibility (RS1 according to Cummings and Englyst, 1995), crystalline structure (RS2) or amylose retrogradation after cooking (RS3). Resistant starch is also considered as a DF (Chesson, 1995).

2. Gut microbial population and animal health

DF that escapes digestion in the upper part of the gastro-intestinal tract, is potentially available for bacterial fermentation in the large intestine. The anaerobic bacteria concentration in the pig gastro-intestinal tract passes thus from log 7-8 CFU g^{-1} in the stomach and the small intestine to log 10-11 CFU g^{-1} in the large intestine (Jensen and Jørgensen, 1994).

Approximately 90 % of the cultivable bacteria in the pig colon are Gram-positive, strict anaerobes belonging to the *Streptococcus*, *Lactobacillus*, *Eubacterium*, *Clostridium* and *Peptostreptococcus* genus. The Gram-negative represent about 10 % of the total flora and belong to the *Bacteroides* and *Prevotella* groups (Russell, 1979; Robinson *et al.*, 1984; Leser *et al.*, 2002).

The gut microflora of healthy animals is subject to modifications in terms of predominant species according to the diet. The presence of DF seems to play an important role (Moore *et al.*, 1987; Awati *et al.*, 2005). The potential prebiotic influence of DF sources has been investigated in humans and monogastric animals. For example, Mc William *et al.* (2007) observed different bacterial communities in the primary colonizers of 3 insoluble colonic substrates (wheat bran, high amylose starch and mucin). Oligofructose, galacto-oligosaccharides and lactulose were clearly shown to increase Bifidobacteria and *Lactobacilli* in the large intestine of humans (Macfarlane *et al.*, 2006). The addition of guar gum or cellulose to a standard diet was also shown to increase ileal Bifidobacteria and Enterobacteria populations in growing pigs (Owusu-

Asiedu *et al.*, 2006). On the contrary, diets high in fermentable NSP and resistant starch have been associated with increased incidence of clinical swine dysentry in grower pigs and diarrhoea in weaning piglets (Pluske *et al.*, 1998; Pluske *et al.*, 2003).

The stability of the flora depends on numerous bacterial antagonisms between endogenous and exogenous species, including the resistance to colonisation by pathogens (Bourlioux, 1997). The bacteria species and the mechanisms involved are still poorly documented, but they are both of bacterial and animal origin (Williams *et al.*, 2001). The resistance to *Clostridium perfringens*, for example, seems to be linked to the synthesis by *Ruminococcus* species of an antimicrobial substance, which is activated in the presence of trypsin. Conversely, the resistance to *Clostridium difficile* is probably due to an interaction between mucin and the microflora (Bourlioux, 1997).

Furthermore, in acidic environment, SCFA produced by DF fermentation, as presented below, are capable of inhibiting the growth of some intestinal pathogens such as *Escherichia coli*, *Salmonella spp.* and *Clostridium spp.* (Montagne *et al.*, 2003). Butyrate, in particular, seems to play a selective antimicrobial role, since studies in pigs indicate that *Lactobacillus sp.* and *Streptococcus bovis* are less sensitive to *n*-butyrate, compared to *Escherichia coli*, *Salmonella spp.*, *Clostridium acetobutylicum*, *Streptococcus cremoris*, *Lactococcus lactis* and *Lactococcus cremoris* (Williams *et al.*, 2001).

In summary, the presence of DF significantly modifies the microbial equilibrium in the intestines with a positive or detrimental impact on animal health according the DF source and the physiological status of the pig.

3. Fermentation pathways and products

The intestinal bacteria hydrolyse the polysaccharides composing the DF and metabolise their constituent sugars through a series of anaerobic energy-yielding reactions leading to the production of ATP which is used for bacteria basal and growth metabolism (Figure 1) (Macfarlane and Gibson, 1995). Except for Bifidobacteria, the majority of the anaerobes of the large intestine use the Embden-Meyerhof-Parnas pathway, also known as glycolysis, that degrades glucose to pyruvate via glucose-6-phosphate (Prescott *et al.*, 1996), to ferment the carbohydrates (Miller and Wolin, 1996).

Polysaccharides made of pentoses and pectins are first metabolised by the Pentose-phosphate pathway (Macfarlane and Macfarlane, 2003) starting from the pentose to fructose-6-phosphate and glyceraldehyde-3-phosphate via xylulose-5-phosphate (Prescott *et al.*, 1996).

As shown on Figure 1, later steps include methanogenesis from H_2 and CO_2 or formic acid, reductive acetogenesis, butyrogenesis and acetogenesis from acetyl-CoA and propionogenesis via the acrylate pathway or the succinate decarboxylation (Pryde *et al.*, 2002).

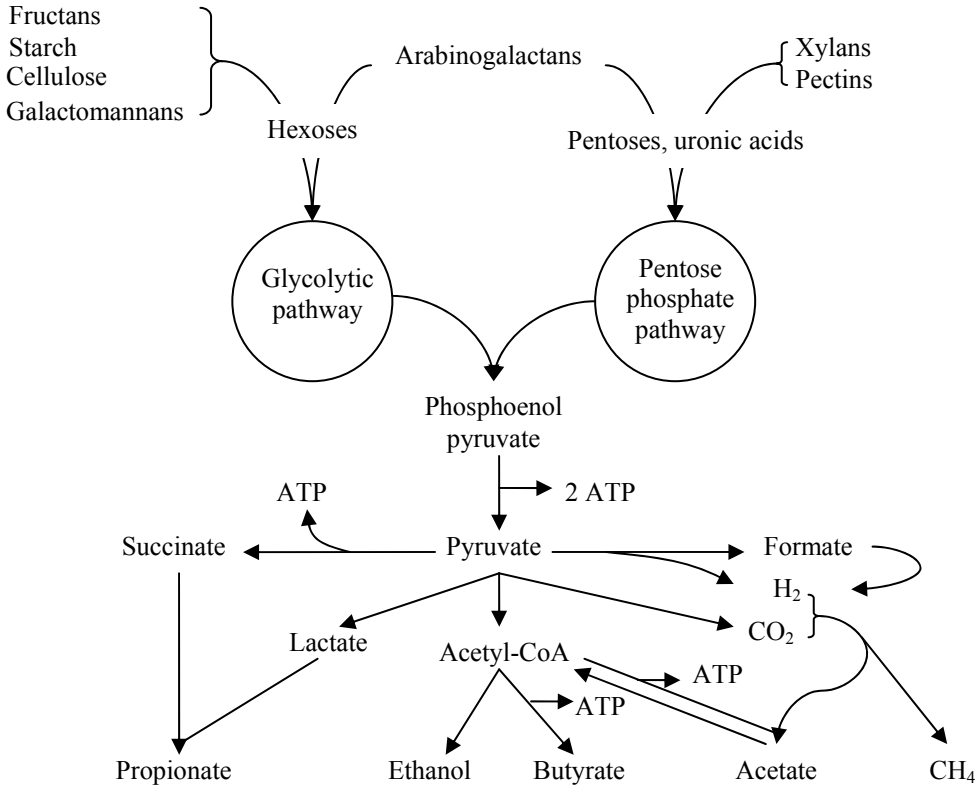

Figure 1. Schematic representation of the pathways for polysaccharides fermentation in the pigs intestines (Macfarlane and Gibson, 1995; Macfarlane and Macfarlane 2003; Pryde *et al.*, 2002).

Short-chain fatty acids (acetate, propionate and *n*-butyrate) and gases (CO_2, H_2 and CH_4) are the main end-products of intestinal fermentation. Other metabolites such as lactate, ethanol and succinate are also formed by different types of bacteria (Drochner

et al., 2004). With the possible exceptions of ethanol, these products do not accumulate in a healthy gut, because they serve as substrate and electron donors for cross-feeding bacteria and are further converted into SCFA (Macfarlane and Gibson, 1995).
The general stoichiometry follows the general equation (1) (Williams *et al.*, 2001):

(1) $$57.5\ C_6H_{12}O_6 + 45\ H_2O \rightarrow 65\ \text{acetate} + 20\ \text{propionate} + 15\ n\text{-butyrate} + 140\ H_2 + 95\ CO_2 + 288\ \text{ATP}$$

Despite this general equation, the amounts and the relative molar proportion of SCFA vary widely. Low SCFA yields recorded with some substrates like wheat bran may equate with incomplete fermentation or may mean that other intermediates are being formed. As presented in the general equation, acetate is the major anion produced during DF fermentation. However, the fermentation of pectin yields 80:12:8 (acetate:propionate:butyrate), other NSP yield 63:22:8 and starch 62:15:23 (Cummings, 1997; Drochner *et al.*, 2004). Resistant starch is indeed known as a butyrogenic substrate of special interest in terms of intestinal health as discussed below (Sajilata *et al.*, 2006). Beside the DF source, the quantity of substrate available also influences the way of its utilisation. Using pure cultures in chemostats, Macfarlane and Gibson (1995) reported that *Bacteriodes ovatus* and *Clostridium perfringens* produced more acetate at the expense of propionate and lactate, respectively when fermenting in a carbon-limited instead of a carbon-excess environment. In the same study, the bacterial growth rate was also shown to influence the SCFA molar ratio.

3. Feeding value of diet enriched in DF

1. Energy loss and metabolic utilisation of SCFA

Increased DF level is associated with a reduced metabolisable energy content of the feed (Noblet and Le Goff, 2001). The overall energy cost in terms of heat production associated with the ingestion and excretion of indigestible fibrous ingredients is minimal and cannot be considered as significant (De Lange *et al.*, 2006). Nevertheless,

beside the unfermented DF, the main loss of energy due to DF is ascribed to the gases of fermentation (CH_4, H_2 and CO_2), the heat of fermentation and the heat due to metabolic utilisation of SCFA. A significant part is also lost as bacterial biomass in the faeces. Even though it has not been clearly quantified yet, this loss was estimated to 0.2 of the neutral detergent fibre (NDF) energy content or 0.5 of the energy content of digestible NDF (Noblet and Le Goff, 2001).

Average energy loss as methane ranges from 0.001 to 0.012 of the gross energy, the highest values being obtained with diets rich in highly digestible DF sources (soybean hulls or sugar beet pulp) (Noblet, 2001). Sows loose a higher proportion of digestible energy (DE) as methane than growing pigs at the same dietary level of fibre (Jørgensen, 2007). This is a consequence of their greater capacity for fermentation due to a higher intestinal transit time (Le Goff *et al.*, 2003).

Energy produced from hindgut fermentation varies from 0.07 to 0.17 of the total available energy, depending on the fermentable carbohydrates content of the diet (Anguita *et al.*, 2006). Fermentation products contribute to the host maintenance energy supply from 0.15 for growing-finishing pigs (Dierick *et al.*, 1989) to 0.3 for gestating sows (Varel and Yen, 1997). This contribution of DF to energy supply is conditioned by the absorption and metabolic utilisation by the host's cells of the SCFA. Propionate is a very effective glucogenic substrate and acetate is less easily taken up by the liver where it stimulates lipogenesis. Unlike propionate and acetate, butyrate does not pass in blood but it is directly metabolised by the colonocytes (Rémésy *et al.*, 1995). Breves and Krumscheid (1997) observed substantial differences between hindgut mucosal uptake of SCFA and serosal release using the Ussing Chamber technique. They also noticed that the proportion of acetate increased at the expense of butyrate, confirming the higher metabolism of butyrate in the colonocytes, as compared to the other SCFA. Butyrate has been shown to regulate epithelial cell growth, to induce differentiation and apoptosis in the small intestine, to increase intestinal cell proliferation in piglets (Kien *et al.*, 2007) and to improve digestive and absorptive capacities of the small intestine in pigs (Claus *et al.*, 2007). Feeding diets rich in pectin to rats, Pirman *et al.* (2007) also noticed that the protein synthesis rates increased significantly in all parts of the intestines, consequently to the fermentation of the DF.

In humans, approximately 90% of the SCFA produced in the large intestine are absorbed (Macfarlane and Gibson, 1995). In growing pigs, Jørgensen *et al.* (1997) observed that less than 1 % of SCFA infused intracaecally was excreted in the faeces. The efficiency of SCFA energy utilisation reached 0.82, 12 % for the building of protein and 88 % for fat production. This is approximately 5 to 10 % lower than starch digested and absorbed in the small intestine (Jørgensen *et al.*, 1997). Combined to the higher loss of energy through methane production, the lower efficiency in utilisation of SCFA energy from DF fermentation compared to glucose explains why the efficiency of DE utilisation for maintenance and growth reaches respectively 0.43 and 0.54 for NDF, as compared to 0.81 and 0.82 for starch (Noblet, 2001).

In brief, among the DF fermentation products, only the SCFA contribute to the energy supply of the animal especially for fat production, but with a lower efficiency compared to glucose. Butyrate however, as major energy source for epithelial cells, improves the health of the intestines.

2. Digestibility

The digestibility of DF varies from 0.40 to 0.60, as compared to the other nutrients (protein, fat, sugars or starch) which are above 0.80 (Noblet and Le Goff, 2001). DF fermentability is also more variable due to high diversity in physical structure and in chemical bounds between monomers. The reduction of energy digestibility will thus vary according to the DF source and the amount of total DF in a diet is an inadequate criterion for predicting energy digestibility (Noblet, 2001). Pastuszewska *et al.* (2000) observed that potato starch and pectins were more extensively fermented than cellulose in the caecum of rats (*Rattus norvegicus*). In growing pigs, NSP digestibility was shown to vary from 0.163 for wheat straw, 0.435 for wheat bran, 0.695 for sugar beet pulp to 0.791 for soybean hulls (Chabeauti *et al.*, 1991). The presence of lignin explains the poor digestibility of wheat straw. Hemicellulose and cellulose composing wheat bran NSP are also less fermentable than the highly-digestible pectic substances of sugar beet pulp and soybean hulls (Karr-Lilienthal *et al.*, 2005). Comparing the influence of soluble and insoluble DF, Owusu-Asiedu *et al.*, 2006 observed that the replacement of 7% maize starch by guar gum (galactomannan) and cellulose in a maize-soybean meal

diet for grower pigs decreased the energy digestibility from 0.878 to 0.866 and 0.849 respectively, while a combination of 7% guar gum and 7% cellulose led to an energy digestibility of 0.758. Conversely, the addition of resistant starch in a pig diet has no influence on the organic matter (OM) digestibility. The faecal digestibility of resistant starch is almost complete as shown by Martinez-Puig *et al.* (2003) who compared the digestibilty of starch in diets containing 250 g of raw potato starch or 250 g of corn starch (0.994 vs. 0.999).

The adaptation of pigs to DF digestion is also a long process that requires 5 weeks (Martinez-Puig *et al.*, 2003). Noblet (2001) calculated that during the 30 to 100 kg period, energy digestibility increases by 0.003 to 0.0045 per 10 kg of live weight for diets containing 4 to 6% of crude fibre. The largest effect is observed between growing pigs and sows. Two different DE values should therefore been provided for fibrous ingredients (Noblet *et al.*, 2003). Energy digestibility is always higher with sows, because of their higher transit time consecutive to their higher gastrointestinal tract volume combined to lower feed intake per live weight (Le Goff *et al.*, 2002).

Thus, beside the lower efficiency in the utilisation of SCFA energy compared to glucose, the low digestibility of some DF sources contributes to their negative impact on the energy content of the diet.

3. Voluntary intake and performances

The bulking capacity of DF reduces the transit time in the entire gastro-intestinal tract and the digestibility of the other nutrients of the diet. An increase in fibre content decreases the mean retention time in the small and the large intestines (Wilfart *et al.*, 2007), reducing the time of exposure of the diet to the host's digestive enzymes (Low, 1982). The amount of digesta flow at the terminal ileum is greater in pigs fed diets with high levels of DF than in pigs fed low-fibre diets (Varel and Yen, 1997). Unlike that in the intestines, the retention time in the stomach can increase in presence of DF, causing earlier satiety due to elongation of the stomach wall (Wenk, 2001). Early satiety is important for the welfare of gestating sows (Meunier-Salaun, 1999), but detrimental in grower pigs where a maximum energy intake is desired. When green forages are fed, intake reduction is even more important, probably due to a poor palatability and a

tilling of the fibrous ingredients increases voluntary intake as shown with tree leaves in sows by Leterme *et al.* (2005).

The slower emptying of the stomach is a consequence of the water-holding capacity of the DF source (Table 1). Soluble DF are therefore efficient to prolong satiety, while insoluble DF have a lower impact (Wenk, 2001). The reduction in digestibility varies also according to the level and type of fibre since the rate of diffusion, towards the mucosal surface, of the host enzymes to the diet and the diffusion of the solubilised components, such as sugars and peptides, are slowed down by the viscosity of the intestinal content, depending on the water-holding capacity of DF (Table 1) (Asp, 1996; Wenk, 2001; Hopwood *et al.*, 2004).

Despite the negative impact on digestibility, farmers in the tropics use extensively fibrous crop by-products and forages as alternative ingredients to prohibitive cereals in pig diets. For example, in Vietnam, the incorporation of 15% spinach or sweet-potato leaves in a diet for Mon Cai × Large White grower pigs increased the crude fibre content of the diets, but also the crude protein (0.172 to 0.182 g CP kg^{-1}DM) and the α-linolenic acid (0.14 g ALA MJ^{-1}ME) contents. This eventually stimulated the growth performances of the pigs (Nguyen *et al.*, 2004). In Nigeria, similar performances were observed in grower pigs fed a diet containing 75% maize and 3 % full fat soybean and a diet containing 27% maize, 38% cassava peels, 9% soybean and 5% palm oil. The inclusion of cassava peels doubled the crude fibre content, but the inclusion of palm oil counterbalanced the decrease in DE content and, finally, the cost saving per kg of weight gain using cassava peels reached 24 % (Balogun and Bawa, 1997). Leterme *et al.* (2006a) also showed that the inclusion of 30% of tropical tree leaves in sow diets did not affect the digestive processes. In their study, tree leaves provided from 8.53 to 12.0 MJ DE per kg DM despite a decrease in energy digestibility. The digestibility of the energy of the leaves alone was calculated to vary from 0.54 to 0.69, while the NDF content ranged from 468 to 310 g kg^{-1}DM.

The use of fibrous ingredients in pig diet as an alternative to cereals may not always be efficient in terms of animal performances but the economical asset of the operation is mostly at the advantage of a substantial substitution (Ogle, 2006). Furthermore, despite their low energy density, tropical unconventional fibrous ingredients were also shown

to provide other valuable nutrients to the animals such as well-balanced protein (Balogun and Bawa, 1997; Leterme *et al.*, 2005 and 2006a) and minerals, especially calcium, iron and manganese (Leterme *et al.*, 2005 and 2006b).

In summary, the lower energy content of high-fibre diets and their influence on satiety reduce growth performances of the animals. This practise may however be interesting for sows or to reduce the feeding costs of growing pigs.

4. Influence of DF on protein nutrition and nitrogen excretion and emission

1. Protein digestibility

As indicated by the following examples, tropical dicotyledons used to feed pigs in the tropics have high CP contents: *Manihot esculenta*, 324 g kg^{-1}DM; *Ipomea batatas*, 244; *Desmodium intortum*, 272; *Amaranthus hybridus*, 261; *Psophocarpus scandens*, 297; *Arachis hypogaea*, 223; *Trichanthera gigantea*, 216; *Morus alba*, 190; *Xanthosoma sagittifolium*, 240 (Leterme *et al.*, 2005; Phuc, 2006; Bindelle *et al.*, 2007). However, an average of 31 % of this nitrogen is bound to the NDF and is not available for the animal (Bindelle *et al.*, 2005). Moreover, green forages are often associated with anti-nutritional factors that interfere with the digestive processes (Phuc, 2006). As discussed above, the reduction in digestibility varies according to the level and type of fibre and its water-holding capacity (Wenk, 2001). High-fibre diets are also known to increase endogenous nitrogen losses (Leterme *et al.*, 1996; Souffrant, 2001) and erosion of the intestinal wall (Varel and Yen, 1997). The source and nature of the DF as well as their physico-chemical properties seem to influence the ileal digestibility of protein. However, controversial results are found in the literature on this topic (Souffrant, 2001).

As a consequence of microbial growth, DF intestinal fermentation reduces protein apparent faecal digestibility and increases faecal N excretion (Mroz *et al.*, 1993) since the bacterial biomass that accumulates in faeces is composed of approximately 625 g kg^{-1}DM of crude protein (Russell *et al.*, 1992). Leek *et al.* (2007), for example, recorded a CP digestibility of 0.82 with barley-based diets, as compared to 0.85 and

0.88 with maize- and wheat-based diets. Simultaneously, barley induced higher N retention than the two other cereals (0.54 vs. 0.47 and 0.41 respectively). Zervas and Zijlstra (2002) also observed that adding fermentable DF sources (soybean hulls or sugar beet pulp) to a low-fibre, high-protein diet reduced the CP digestibility from 0.85 to 0.80 for soybean hulls and 0.74 for sugar beet pulp. In the case of a low protein diet, the reduction was less important but still significant since it passed from 0.82 to 0.76 and 0.74, respectively. This decrease in N digestibility did not affect N retention. In ruminant studies, the nitrogen incorporation per g OM fermented by the flora was shown to vary according to the substrate, with rapid fermentable DF yielding higher bacterial growth than slow fermentable DF (Hall and Herejk, 2001).

The studies by Leek *et al.* (2007) and Zervas and Zijlstra (2002) obviously show that reduced faecal CP digestibility consecutive to intestinal DF fermentation is not necessarily related to lower protein value of the diet. They are consistent with Canh *et al.* (1997) who recorded a N faecal digestibility of 0.85 and a N retention of 0.30 with a grain-based diet, whereas with sugar beet pulp the digestibility was reduced to 0.75 but N retention increased to 0.44.

Bacterial carbohydrate fermentation in pigs mainly takes place in the caecum and the colon, but also in a certain proportion before the end of the ileum (Rowan *et al.*, 1992). As an example, Böhmer *et al.* (2005) found that more than 55% of dietary inulin, a highly fermentable and soluble fructan, was digested in the small intestine. In piglets, the capability of a bacterial inoculum to produce SCFA from fructo-oligosaccharides passes 2 mmol h^{-1} kg^{-1}digesta when bacteria are harvested in the stomach to 16.4 in the distal small intestine, 43.6 in the caecum and 65.1 in the colon (Mikkelsen *et al.*, 2004). This indicates that the population of active bacteria is very low in the stomach, but becomes significant in the distal small intestine. As a consequence, the bacterial biomass accumulation occurring before the intestinal content reaches the large intestine contributes in a small but significant extent to the amino acid requirements of pigs (Torrallardona *et al.*, 2003).

It can be concluded that the presence of DF in the diets lowers the apparent faecal digestibility of the crude protein and possibly the ileal digestibility, but not necessarily the efficiency of nitrogen retention by the animal.

2. *Nitrogen excretion pathways*

As the intestinal content passes through the caecum and the colon, it becomes depleted in fermentable carbohydrates. The energy source for the flora evolves from rapidly fermentable to slowly fermentable DF and, finally, to dietary resistant and endogenous proteins. Bacterial proteolysis induces the production of branched-chain fatty acids (mainly valerate, *i*-valerate, *i*-butyrate), malodorous compounds such as skatole, which contributes to unpleasant smell and taste of boars meat (Jensen and Jensen, 1995), and amines and ammonia, originating from the deamination of amino acids.

However, the combination of different sources of DF affects both the SCFA patterns and the site of fermentation (Henningsson *et al.*, 2002). Substrates with lower rates of fermentation, like wheat bran used in combination with more fermentable NSP or RS, maintain the microbial activity throughout the entire large intestine and decrease proteolysis occurring in the distal colon (Govers *et al.*, 1999). The undigested dietary proteins and the endogenous proteins are in this state of figure used for the building up of bacterial proteins and the intense bacterial growth in the intestine enhances the urea transfer from the blood to the large intestine (Younes *et al.*, 1996; Pastuszewska *et al.*, 2000). As a consequence, urinary N excretion is decreased. Zervas and Zijlstra (2002) showed in their study that the inclusion of fermentable DF from sugar beet pulp and soybean hulls in low protein wheat- and barley-based diets increased faecal N output from 5.1 to 7.7 g d^{-1} and lowered urinary:faecal N excretion ratio, decreasing from 2 for the control diet to 1.3 with soybean hulls and 1 for sugar beet pulp. Total N excretion remained unaffected. Kreuzer and Machmüller (1993) also found that the addition of 100 to 220 g NSP kg^{-1} in pig's diet reduced urinary N excretion from 20 to 28 %. The same observation was made by Canh *et al.* (1997). With a grain-based diet, they recorded a urinary:faecal N ratio of 3.83 vs. 1.21 with a diet containing 250 g kg^{-1} of sugar beet pulp.

3. *Nitrogen emission through manure*

The N-excretion shift from urea in urine to bacterial protein in faeces, as exposed in the previous section, is a potential means for reducing the environmental load of pig facilities (Nahm, 2003). The breakdown of protein in manure is a slow process taking

weeks and even months depending on the temperature. Conversely, the degradation of urea to ammonia and CO_2 covers only several hours (Aarnick and Verstegen, 2007). The ammonia production during 10 d of manure from pigs fed a barley-based diet is limited to 0.066 of the N intake, as compared to 0.113 for maize and 0.121 for wheat. When NSP enzymes are added to the diets, the emission from barley-based diets increases while the emission from maize- and wheat-based diets decreases, confirming the role of the NSP in the reduction of ammonia emission (Leek *et al.*, 2007). Sutton *et al.* (1999) observed that during a 7 d-storage, the manure of pigs fed a grain-based diet lost 0.24 of the initial N as NH_3, as compared to 0.14 in the case of sugar beet pulp. Kreuzer *et al.* (1998) showed that feeds with high contents in pectin and hemicellulose, like citrus pulp and sugar beet pulp, were the most effective DF sources to reduce N loss in manure, as compared to cellulose from rye bran and RS from cassava.

Bach Knudsen *et al.* (1991) observed that SFCA concentration increases from less than 20 mmol/l in the small intestine to 100-140 mmol/l in the caecum. As a consequence the pH drops from 6.6-7.2 to 5.7-6.8 (Jensen and Jørgensen, 1994). The intestinal pH and the content of total SCFA in the digesta are linearly related (Högberg and Lindberg, 2004). The pH was shown to raise again when passing from the caecum to the proximate and latter the distal colon, but this increase was higher with low-fibre diets, as compared to high fibre diets (Bach Knudsen *et al.* 1991; Jensen and Jørgensen, 1994), as a consequence of NH_3 production. The lower pH of faeces and manure of pigs fed diets with high fermentable DF content is also an efficient means for reducing ammonia emission since it is soluble under its protonated form (NH_4^+) (Aarnink and Verstegen, 2007). Canh *et al.* (1998) found that for each increase of 100 g NSP in pig diets, the slurry pH decreased by 0.12 units and ammonia emission was reduced by 5.4 %.

In tropical extensive production systems, decreasing urinary-N emission is also of great interest. Indeed, tropical soils are generally poor in OM and nutrient recycling is critical to maintain productivity since mineral fertilizers are prohibitive to the small farmers. N is the most limiting nutrient in agricultural systems because it can be easily be lost through gaseous losses, leaching or runoff, while large amounts are essential for non-leguminous plant growth (Campbell *et al.*, 1995; Smil, 1999). In mixed livestock-

cropping systems, urinary-N is particularly susceptible to loss despite improved manure collection and storage practises. A reduction in N loss requires to maximise faecal-N excretion (Rufino *et al.*, 2006) as influenced in pig production by the DF content of the diet. However, manure from highly-fermentable fibre diets contains lower proportions of nitrogen directly utilizable by the plants since protein degradation is a slow process (Kreuzer and Machmüller, 1993; Aarnick and Verstegen, 2007).

Finally, the accumulation of bacterial protein in manure consecutive to high-fibre diets is also valorised in integrated fish and pig production systems which are very common in Southeast Asia (Payne and Wilson, 1999). However, these practises require further investigation because they are controversial (Kumar *et al.*, 2004).

5. Conclusion and perspectives

This review of the literature pointed out the influence of the DF source on the microbial flora equilibrium, on energy and protein digestibility and on the N excretion pathways in pigs. The potentialities offered by fibrous ingredients for extensive production systems in the tropics were also illustrated.

Further studies devoted to the relationship between DF fermentability and its functionalities are necessary in order to identify appropriate DF sources that reduce ammonia emission, promote intestinal health and still allow fair pig performances. The influence of crude protein bound to NDF on protein availability, bacterial growth and N excretion pathways requires also further investigation. Finally, the loss of energy as bacterial biomass in the faeces should be properly quantified in the future.

In vivo models for describing the fate of DF in the digestive system are difficult and expensive, especially if a wide range of ingredients must be evaluated. There is thus an important potential role for *in vitro* techniques, providing an adequate *in vivo* validation of the method (Coles *et al.*, 2005).

Enzymatic methods based on substrates disappearance during incubation, e.g. the protocol of Boisen and Fernandez (1997), succeed in the prediction of *in vivo* OM and energy digestibility, but limit the understanding of fermentation in the large intestine. The use of a living bacterial inoculum as in the gas production technique, used in ruminant nutrition (Menke and Steingass, 1988), instead of an enzymatic complex such

as viscozyme, allows to record the substrates disappearance during fermentation and also bacterial accumulation, SCFA production and gas release, which are of first importance when the functionalities of DF are considered. As showed by Awati *et al.* (2005), *in vitro* fermentation methods can also highlight the effect of the DF source on the microbial composition. Nevertheless, there is still a lack of proper *in vivo* validation of such studies results.

Finally, numerous studies highlighted the potential of fermentable DF to reduce the environmental load of pig facilities. However, a combined economical and environmental evaluation of this practise is necessary for intensified as well as for extensive production systems.

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CHAPTER II

Research strategy

The main objective of this thesis was to investigate the relationships between the fermentability of DF and its influence on nitrogen excretion pathways.

As expressed previously, *in vivo* models for describing the fate of DF in the digestive system are difficult and expensive, especially if a wide range of ingredients must be evaluated. There is thus a potential role for *in vitro* techniques, provided that such methods are adequately validated *in vivo* (Coles *et al.*, 2005). The first step of the present work was thus to develop an *in vitro* method. Later steps included the use of this *in vitro* method to evaluate of the relationship between DF fermentability, intestinal bacteria growth and N excretion shifts.

Enzymatic *in vitro* methods were developed during the late eighties and early nineties to study digestion in single-stochamed animals. These methods are based on the measurement of substrate disappearance during incubation with several enzymes reproducing digestion in the different parts of the gastro-intestinal tract (Wilfart *et al.*, 2007). Typically, porcine pepsin is used to simulate digestion in the stomach, porcine pancreatin the digestion in the small intestine and, finally, viscozyme, a complex of various polysaccharidases, or cellulase are used to simulate microbial fermentation occurring in the large intestine. If such methods were shown to be able to predict *in vivo* organic matter and energy digestibility (Boisen and Fernandez, 1997), they do not allow the understanding of fermentation in the large intestine. Indeed, no information on fermentation products, gases, SCFA or microbial biomass, is available from enzymatic incubation. In Chapter II, the role of these products on the functionalities of DF was clearly pointed out.

The use of a living bacterial inoculum as in the gas production technique, used in ruminant nutrition (Menke and Steingass, 1988), instead of an enzymatic complex such as viscozyme, would allow the measurement of substrate disappearance during fermentation and also bacterial accumulation, SCFA production and gas release.

When the work of this thesis was initiated in 2001, some attempts had been made to adapt the ruminant gas production technique to pig studies (Christensen *et al.*, 1999; Bauer *et al.*, 2001). For this purpose, rumen liquid was replaced by intestinal content,

diluted in a buffer solution. However, some methodological aspects required further development before the technique could be used in routine. First of all, it was not stated whether an *in vitro* hydrolysis for simulation of the digestion in the stomach and the small intestine prior to the gas test was necessary (Bauer *et al.*, 2001). A first experiment was thus undertaken to measure, on six ingredients, the influence of an hydrolysis prior to the fermentation on the gas production kinetics. Secondly, most of the available protocols required invasive techniques (cannulas) (Christensen *et al.*, 1999; Wang *et al.*, 2004) or slaughtering (Fondevila *et al.*, 2002) for collecting the intestinal content. These cannulas cannot be used in every laboratory, especially in developing countries, where many unconventional, fibrous ingredients are used to feed pigs. A second experiment was thus undertaken in order to investigate whether freshly collected faeces can be used in place of intestinal content for the preparation of the bacterial inoculum. In this experiment, wheat bran and sugar beet pulp were used as substrates.

The results of these two first experiments are grouped in Chapter III of this manuscript and published in *Animal Feed Science and Technology*.

The stage of development of the animals and the composition of the DF in the diet also affect the mean retention time in the digestive tract, modify the composition of the microflora and the activity of the latter in the large intestine and the faeces (Bauer *et al.*, 2001; Williams *et al.*, 2001; Le Goff *et al.*, 2003). The effects of these parameters on the *in vitro* fermentation kinetics were thus studied in two successive experiments. The first one compared the fermentation kinetics of sugar beet pulp when faeces of young pigs, growing pigs and sows were used to prepare the inoculum. The second experiment compared inoculums prepared from pigs fed diets differing in total DF content and variable proportions of soluble and insoluble DF fractions. The results of these experiments investigating the influence of the donor on the fermentation kinetics are presented in Chapter IV and published in *Animal Feed Science and Technology*.

After these methodological clarifications, the *in vitro* fermentation technique was used with slight modifications for investigating the relationship between DF fermentability and N excretion shift since this relationship is still poorly documented, although the source of DF is suspected to influence the growth of the colonic bacterial population

(Kreuzer *et al.*, 1998; Zervas and Zijsltra, 2002). For this purpose, 5 purified DF sources and 3 pepsin-pancreatin hydrolysed ingredients were used in an *in vitro* gas test in which the inoculum was enriched with ^{15}N. The results of this experiment are presented in Chapter V of this manuscript and published in *Animal*.

A last experiment was conducted, using diets differing in DF content and solubility, to investigate the relationship between the N excretion pathways measured *in vivo*, DF fermentability and bacterial protein synthesis measured *in vitro*. This last experiment presented in Chapter VI is submitted for publication to the *Journal of Animal Science*.

Finally, the general discussion reviews the implication of the results, seeks the limits of the *in vitro* method as a first step of validation before it could be used routinely for the prediction of N excretion shifts and draws perspectives for further possible applications of the gas production technique for single-stomached animals.

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CHAPTER III

Article 2:

Effect of inoculum and pepsin-pancreatin hydrolysis on fibre fermentation measured by the gas production technique in pigs

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1. Abstract

Two experiments were undertaken to adapt the *in vitro* gas production technique in syringes, used for ruminants, to fibre fermentation studies in the large intestine of pigs.

In a first experiment, two inocula (faeces and large intestine content) were compared at 4 dilution levels in a buffer solution (0.025, 0.05, 0.1 and 0.2 g ml^{-1}) with 2 substrates: wheat bran and sugar-beet pulp. The accumulated gas produced over 72 h was modelled and the kinetics parameters compared. The time to half asymptote was lower for the intestinal inoculum (5.5 vs. 8.0 h, $P < 0.02$), but the 2 inocula yielded similar fractional rates of degradation (0.16 h^{-1}) and gave equal final gas production (252 ml g^{-1} substrate). No interaction ($P > 0.05$) was observed between inocula and substrates. The dilution of the samples in the buffer solution increased ($P < 0.001$) the lag time (from 0.9 to 2.1 h for dilution rates ranging from 0.2 to 0.025 g ml^{-1}, respectively) and decreased ($P < 0.001$) the rates of substrate degradation (from 0.18 to 0.13 h^{-1}).

A second experiment aimed to study the effect of an *in vitro* pepsin-pancreatin hydrolysis of the sample prior to the gas test. Six substrates were tested: maize, wheat bran, sugar-beet pulp, lupins, peas and soybean meal. The enzymatic hydrolysis affected ($P < 0.001$) the kinetics parameters and the ranking order of the fermented substrates. The lag times also increased for all ingredients. The rate of degradation decreased when peas, lupins, maize and wheat bran were hydrolysed ($P < 0.001$) but it increased with soybean meal ($P = 0.014$) and sugar-beet pulp ($P < 0.001$). Final gas production increased with peas and soybean meal ($P < 0.001$), remained unchanged for lupins and decreased for the other substrates ($P < 0.001$).

In conclusion, the method using faeces as a source of microbial inoculum is reliable to characterise the fermentation kinetics of ingredients in the large intestine of pigs. However, it is important to hydrolyse the substrates with pepsin and pancreatin before the gas tests.

Keywords: pig, fermentation, large intestine, gas production, dietary fibre

2. Introduction

In pigs, the energy provided by the volatile fatty acids produced during the fermentation of dietary fibre in the large intestine can be substantial (Noblet and Le Goff, 2001). Some fibre fractions are also used as prebiotics to favour the development of a beneficial microflora (Williams *et al.*, 2001). However, the rate of fibre fermentation in the pig large intestine has not been thoroughly investigated, possibly due to a lack of reliable methodology.

Recently, attempts were made to adapt the gas production technique, used in ruminant nutrition (Menke and Steingass, 1988), to pig studies (Christensen et al, 1999; Bauer et al, 2001, 2004). Rumen liquid is replaced by faecal material, diluted in a buffer solution.

However, some methodological aspects still need to be investigated before the technique can be used in routine in pigs. For example, it is not clear whether an *in vitro* hydrolysis prior to the gas test, in order to simulate the digestion in the stomach and the small intestine, is necessary. Moreover, the current protocols available require invasive techniques (cannulas) (Christensen et al, 1999; Wang et al, 2004) or slaughtering (Fondevila *et al.*, 2002) for collecting the intestinal content. These cannulas cannot be used in every laboratory, especially in developing countries, where many unconventional, fibrous ingredients are used to feed pigs (Leterme et al, 2005, 2006).

The aim of the present work was to investigate whether freshly collected faeces can be used in place of intestinal digesta and whether an hydrolysis of the ingredients, prior to the fermentation gas test, is required.

3. Materials and methods

1. Experiment 1: Source and dilution of the inocula

The first experiment aimed to replace the rumen fluid used in the Menke and Steingass (1988) method by large intestine content or faecal samples collected in cannulated pigs, testing different dilution levels of inocula in a buffer solution.

Animals and diets

Three growing-finishing female Belgian Landrace pigs (initial bodyweight: 25 kg) were used. The animals were fistulated (Authorisation from the Ethical Committee FUSAGx 02/02) with a T-cannula located in the colon at 20 cm from the caecum-colon junction. During the experimental period, the pigs were individually housed, fed ad libitum a standard commercial diet (Brichart 240, Sombreffe, Belgium) and had free access to water. Intestinal and faecal samples collection started when the pigs had reached a bodyweight of 50 kg and had been adapted to the experimental diet over 5 weeks.

Substrates

Sugar-beet pulp (*Beta vulgaris*) and wheat bran (*Triticum aestivum*), differing in fibre composition, were used as substrates. The samples were ground to pass a 1 mm screen, using a Cyclotec 1093 Sample Mill (FOSS Electric A/S, Hilleroed, Denmark). Their chemical composition is detailed in Table 2.

Table 2. Chemical composition of the substrates (g kg^{-1} DM).

	DM (g kg^{-1})	Ash	Crude protein	Crude fat	NDF	ADF	Hemi-cellulose	ADL	Starch
Lupins	899	37	307	88	226	206	20	18	-
Maize	899	17	91	51	68	22	45	4	779
Peas	865	32	200	13	142	121	21	13	431
Sugar-beet pulp	923	97	78	11	347	224	124	31	-
Wheat bran	882	63	176	48	390	130	260	56	147
Soybean meal	919	68	422	20	125	89	35	10	-

Inocula

In order to reduce variation between animals, the inocula were prepared by mixing the intestinal content or the faeces of the 3 pigs. The samples were collected directly in plastic bags saturated with CO_2 and immediately placed in a water-bath at 39 ºC. A buffer solution (180 ml) composed of salts and minerals (Menke and Steingass, 1988) was added to the bags and samples were subjected for 60 seconds to a mechanical pummelling with a Stomacher Lab Blender 400 (Seward Medical, Norfolk, UK) to suspend fibre-associated bacteria in the liquid (Merry and Mac Allan, 1983). The solution was then filtered through a 250 µm mesh screen and completed with a variable

additional volume of the buffer solution to reach the desired dilution of intestinal or faecal samples in the buffer solution (0.2, 0.1, 0.05 and 0.025 g sample ml^{-1} buffer).

In vitro fermentation

Two hundred mg of substrate were placed in a 100 ml Kolbenprober glass syringe. The gas-tests were performed by transferring 30 ml of an inoculum prepared from intestinal content or faeces of the pigs in the preheated syringes and placing them in an incubator at 39 ± 0.5°C. The volumes of gas released in the syringes were recorded after 2, 5, 8, 12, 16, 20, 24, 48 and 72 hours of incubation. Three syringes per inoculum containing just inoculum (blanks) were systematically included for each run.

The experimental scheme was as follows: (2 inocula x (2 substrates + blank) x 4 dilutions x 3 repetitions) x 2 periods. At the end of the fermentation, the pH of each syringe solution was measured.

Chemical analysis

The substrates were analyzed for their content in dry matter (105 ºC for 24 h), ash (550 ºC for 8 h), nitrogen (Kjeldahl method, crude protein = 6.25 x N content), ether extract (Soxhlet method, using ether), neutral (using Na_2SO_3 and Termamyl : 120 KNU/g, Novo Nordisk, Bagsværd, Denmark) and acid detergent fibres and lignin, using the Fibercap system (Foss Electric, Bagsvaerd, Denmark). Starch was determined, after grinding the samples through a 0.5 mm-mesh screen, according to the method of Faisant *et al.* (1995).

The pH of syringe contents was measured using a 300i/SET pH-meter equipped with a SenTix 20 electrode (WTW, Weilheim, Germany).

Calculations and statistical analysis

The gas volume recorded during the fermentation of each syringe was calculated as follows:

$$(2) \qquad V^{corr}(t) = \frac{\mathrm{V}(t) - V_0 - B(t) * V_0}{W}$$

where $V^{corr}(t)$ (ml g^{-1}) is the gas accumulation to time *t* (h) corrected by the amount of incubated substrate and the production of the blanks, *V(t)* (ml) the volume occupied by

the inoculum and the gas at time t, V_0 (ml) the volume of inoculum transferred in the syringes at the start of the fermentation, $B(t)$ (ml gas per ml of inoculum) the mean gas production at time t of the blanks per ml of inoculum and W (g) the amount of substrate placed in the syringe.

Outlying observations, caused by accidental leakage of gas, were discarded as described by Dagnelie (1975).

Gas accumulation curves were modelled using the mathematical model proposed by France *et al.* (1993):

$$(3) \qquad G = 0, \quad \text{if } 0 < t < L$$
$$= G_f\left(1 - \exp\left\{-\left\langle b(t-L) + c\left(\sqrt{t} - \sqrt{L}\right)\right\rangle\right\}), \quad \text{if } t \geq L$$

where G (ml g^{-1}) denotes the gas accumulation to time, G_f (ml g^{-1}) the maximum gas volume for $t = \infty$ and L (h) the lag time before the fermentation starts. The constants b (h^{-1}) and c ($h^{-1/2}$) determine the fractional rate of degradation of the substrate μ (h^{-1}), which is postulated to vary with time as follows:

$$(4) \qquad \mu = b + \frac{c}{2\sqrt{t}}, \quad \text{if } t \geq L$$

In addition to the kinetics parameters G_f and L, the *T/2* (time to half asymptote when $G = G_f/2$) and $\mu_{t=T/2}$ (fractional rate of degradation at $t = T/2$) were compared in the statistical analysis. At *T/2*, the rate of gas production is in a linear phase, near its maximum.

Statistical analysis of the kinetics parameters were performed by means of an analysis of variance and a classification of means by the Differences of Least Squares Means method using the MIXED procedure of the SAS 8.02 software (SAS Inc., Cary, NC, USA) with the following general linear model :

$$(5) \qquad Y = \alpha + S_i + I_j + D_k + (S \times I)_{ij} + (S \times D)_{ik} + (I \times D)_{jk} + (S \times I \times D)_{ijk} + (P \times I)_{jl} + \varepsilon$$

where Y is the result, α the mean, S_i the fixed effect of the substrate (i = 1, 2), I_j the fixed effect of the source of inoculum (j = 1, 2), D_k the fixed effect of the dilution (k =

1,..., 4), P_l the random effect of the period of inoculum sampling (l = 1, 2) and ε the error term.

2. *Experiment 2: enzymatic hydrolysis*

The second experiment aimed to compare the fermentation kinetics of 6 various substrates submitted or not submitted to a pepsin-pancreatin enzymatic hydrolysis.

Animals and diets

The 3 pigs used in the first experiment and weighing now 70-80 kg, were used as donors of faeces.

Substrates

The following feedsuffs were tested: maize (*Zea mays*), wheat bran (*Triticum aestivum*), peas (*Pisum sativum*), sugar beet pulp (*Beta vulgaris*), lupins (*Lupinus luteus*) and soybean meal (*Glycine maxima*). The samples were ground to pass a 1 mm screen using a Cyclotec 1093 Sample Mill (FOSS Electric A/S, Hilleroed, Denmark). The ingredients were analysed using the methods described in Experiment 1. Their composition is detailed in Table 2.

In vitro enzymatic hydrolysis

The enzymatic hydrolysis of the substrates was performed following the protocol described by Boisen and Fernández (1997).

Substrates samples of 0.5 g were weighed in conical flasks. Phosphate buffer solution (25 ml, 0.1 M, pH 6.0) and HCl solution (10 ml, 0.2 M) were poured into the flasks. The pH was adjusted to 2.0 with 1M HCl or 1M NaOH and 0.5 ml of a chloramphenicol (Sigma C-0378) solution (0.5 g 100 ml^{-1} ethanol) was added. Fresh pepsin solution (1 ml, 25 mg ml^{-1}, porcine pepsin: 2000 FIP-U/g, Merck n° 7190) was finally added. The flasks were closed with a rubber stopper and placed for 2 h under gentle agitation in a water-bath at 39 ± 0.5°C.

After the pepsin hydrolysis, 10 ml of a phosphate buffer solution (0.2 M, pH 6.8) and 5 ml of a NaOH solution (0.6 M) were added to the solution. The pH was adjusted to 6.8 with 1M HCl or 1M NaOH and fresh pancreatin solution (1 ml, 100 mg pancreatin

(Sigma P-1750) ml^{-1}) was added. The flasks were then closed with a rubber stopper and placed for 4 h under gentle agitation in a water-bath at 39 ± 0.5°C.

After hydrolysis, the residues were collected by filtration on a Nylon cloth (42 µm), washed with ethanol (2 x 10 ml 95 % ethanol) and acetone (2 x 10 ml 99.5 % acetone), dried for 24 h at 60 ± 1°C and weighed. Each substrate was tested 24 times (8 replicates x 3 periods).

In vitro fermentation

Hydrolysis residues from the different replicates and periods were accumulated and fermented *in vitro* simultaneously with the corresponding non-hydrolysed substrates, using the procedure described in the first experiment. The inoculum was prepared from faeces at a dilution of 0.05 g ml^{-1} in the buffer solution and incubations were repeated over 2 periods. For each period, 6 syringes per substrate (+ 3 blanks) were incubated at the same time, 3 containing the non-hydrolysed substrate, 3 containing the hydrolysed substrate.

Calculations and statistical analysis

The dry matter disappearance (*dDM*) during the pepsin-pancreatin hydrolysis was calculated as follows:

$$(6) \qquad dDM = \frac{\text{weight of the sample before hydrolysis - weight of the residue}}{\text{weight of the sample before hydrolysis}}$$

The gas accumulation curves were modelled using the model of France *et al.* (1993), as for the first experiment. The four parameters (G_f, L, $\mu_{t=T/2}$ and $T/2$) yielded by the model were used to perform the statistical analysis when comparing the influence of the substrate and the hydrolysis on the fermentation patterns.

The general linear model used in the analysis of variance was:

$$(7) \qquad Y = \alpha + S_i + H_j + (S \times H)_{ij} + P_k + \varepsilon$$

where Y is the result, α the mean, S_i the fixed effect of the substrate (i = 1,..., 6), H_j the fixed effect of the hydrolysis (j = 1, 2), P_k the random effect of the period of inoculum sampling (k = 1, 2) and ε the error term.

The Pearson's correlation calculations, the analysis of variance and the classification of means by the Differences of Least Squares Means method were performed using the CORR and the MIXED procedures of the SAS 8.02 software (SAS Inc., Cary, NC, USA).

4. Results

1. Experiment 1

The gas accumulation curves ($\overset{corr}{V}(t)$) recorded during the fermentation of sugar-beet pulp and wheat bran with inocula produced with large intestine or faecal samples and diluted at 0.05 g ml^{-1}, the dilution used in the second experiment, are illustrated in Figure 2.

The parameters of France model obtained for the substrates fermented with intestinal or faecal inocula at different dilution levels in the buffer are detailed in Table 3. The substrates and dilution levels influenced ($P < 0.05$) these parameters. No interaction ($P > 0.05$) between these factors was observed. The inoculum source influenced ($P = 0.020$) only the time to half asymptote (*T/2*) while an interaction between the substrate and the dilution was observed ($P = 0.029$).

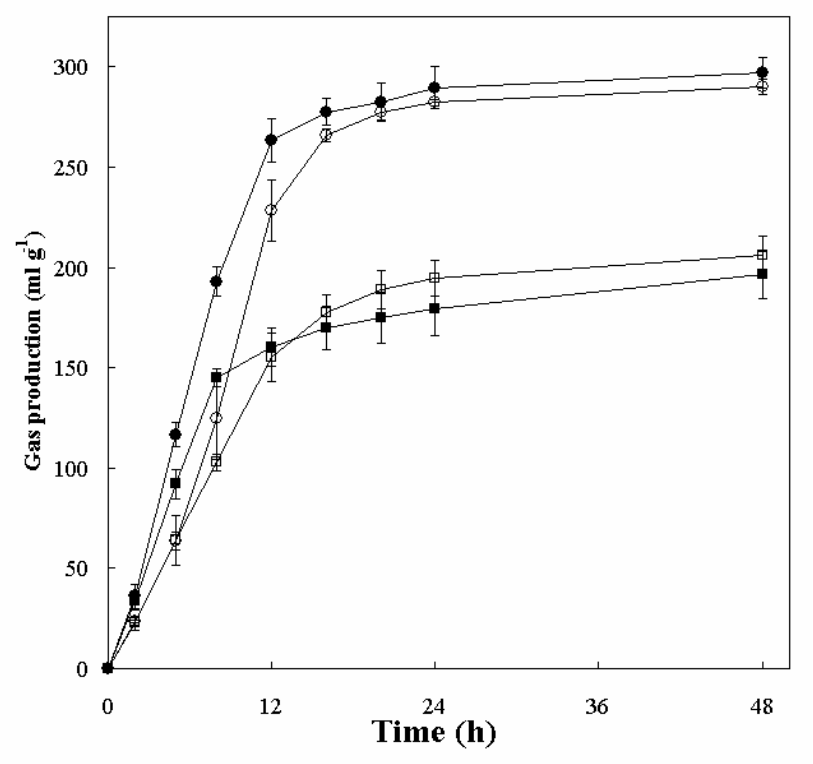

Figure 2. Mean values and standard deviations of the gas accumulation over time (until 48 h) of sugar-beet pulp incubated with large intestine content (●), sugar-beet pulp incubated with faecal inocula (○), wheat bran incubated with large intestine content (■) and wheat bran incubated with faecal inocula (□) (0.05 g ml^{-1} buffer).

The fermentation of wheat bran had a shorter lag time than that of sugar-beet pulp ($P < 0.001$), whatever the inoculum or the dilution. The final gas productions (G_f) and the fractional rates of degradation ($\mu_{t=T/2}$) were higher for sugar-beet pulp ($P < 0.001$). The faecal inoculum yielded a higher *T/2* ($P = 0.020$) compared to intestinal inoculum. The source of inoculum did not affect the lag time (L), $\mu_{t=T/2}$ and G_f ($P > 0.05$).

The dilution of the intestinal and faecal samples in the buffer solution slightly affected ($P = 0.048$) the final gas production, whatever the substrate and the source of inoculum. However, the lag time was longer ($P < 0.001$) when the dilution levels decreased, whereas the fractional rates of degradation decreased significantly ($P < 0.001$) whatever the substrate.

Table 3. Fitted kinetics parameters (means) of the gas accumulation curves modelled according to France *et al.* (1993) for wheat bran or sugar-beet pulp incubated with inocula prepared from large intestine content or from faeces at various dilutions in the buffer (0.025, 0.05, 0.1 and 0.2 g ml^{-1}).

Main effects		N[1]	*L*[2]	$\mu_{t=T/2}$[3]	*T/2*[4]		G_f[5]
Inocula	Large intestine	47	1.5 NS[6]	0.17 NS	5.5 b[7]		250 NS
	Faeces	45	1.3 NS	0.14 NS	8.0 a		253 NS
Dilutions					*Substrates*		
					Sugar-beet pulp	Wheat bran	
	0.2	23	0.9 c	0.18 a	5.0 d	5.2 d	255 a
	0.1	23	0.9 c	0.15 ab	6.2 c	5.9 c	254 a
	0.05	23	1.7 b	0.16 ab	7.3 b	6.4 b	250 ab
	0.025	23	2.1 a	0.13 b	9.0 a	8.5 a	247 b
Source of variation		*d.f.*[8]	*P values*				
Substrate		1	< 0.001	< 0.001	0.002		< 0.001
Inoculum		1	0.760	0.299	0.020		0.466
Dilution		3	< 0.001	< 0.001	< 0.001		0.048
Substrate x inoculum		1	0.081	0.259	0.861		0.052
Substrate x dilution		3	0.291	0.491	0.029		0.198
Inoculum x dilution		3	0.356	0.656	0.081		0.676
Substrate x inoculum x dilution		3	0.991	0.800	0.914		0.383
Variance parameter estimates							
Period x inoculum			0.157	0.0004	0.120		29.8
Residual			0.445	0.0009	0.389		112.2

[1] N, number of observations; [2] *L*, lag time (h); [3] $\mu_{t=T/2}$, fractional rate of degradation at t = *T/2* (h^{-1}); [4] *T/2*, half-time to asymptote (h); [5] G_f, maximum gas volume (ml g^{-1}); [6] NS, non significant; [7] For one parameter, means followed by different letters in the columns differ at significance level of 0.05; [8] *d.f.*, degrees of freedom

2. Experiment 2

The dry matter disappearances (*dDM*) of the substrates after enzymatic hydrolysis and the kinetics parameters (G_f, *L*, $\mu_{t=T/2}$ and *T/2*), calculated for the fermentation of the hydrolysed and non-hydrolysed substrates are presented in Table 4. The analysis of variance revealed differences ($P < 0.001$) in *dDM* between the substrates. Negative correlation coefficients linking *dDM* to the fibrous fractions of the substrates were also found (NDF: r = -0.884 ; ADF: r = -0.832 ; hemicellulose (NDF – ADF): r = -0.906 ; $P < 0.05$). Correlations with the other chemical contents were not significant ($P > 0.05$).

Table 4. Dry matter disappearance during enzymatic hydrolysis (*dDM*) and fitted kinetics parameters of the gas accumulation curves modelled according to France *et al.* (1993) with or without hydrolysis prior to the fermentation with a faecal inoculum at a level of dilution in the buffer of 0.05 g ml^{-1}.

Hydrolysis	*Substrate*	N_1 [1]	*dDM*	N_2 [2]	*L* [3]	$\mu_{t=T/2}$ [4]	*T/2* [5]	G_f [6]
Non hydrolysed	Lupins		-	6	7.3 a [7]	0.11 c	13.7 a	331 a
	Maize		-	5	7.1 a	0.16 b	11.1 b	306 b
	Peas		-	4	6.1 b	0.18 a	9.5 c	295 bc
	Sugar-beet pulp		-	6	5.3 c	0.18 a	8.9 c	291 c
	Soybean meal		-	4	2.5 d	0.09 d	10.7 b	212 d
	Wheat bran		-	6	4.0 e	0.12 c	8.9 c	204 d
Hydrolysed	Lupins	24	0.65 d	5	7.5 a	0.09 c	14.4 a	325 b
	Maize	24	0.86 a	6	7.1 b	0.12 b	12.1 c	279 d
	Peas	24	0.71 c	5	7.0 b	0.13 b	11.8 c	341 a
	Sugar-beet pulp	24	0.34 f	6	7.1 b	0.20 a	10.3 d	268 d
	Soybean meal	24	0.55 e	6	6.9 b	0.11 c	12.7 b	303 c
	Wheat bran	24	0.79 b	5	7.0 b	0.10 c	13.2 b	149 e

Source of variation	*P value*	*d.f.* [8]	*P values*			
Hydrolysis	-	1	< 0.001	< 0.001	< 0.001	0.108
Substrate	< 0.001	5	< 0.001	< 0.001	< 0.001	< 0.001
Substrate x hydrolysis	-	5	< 0.001	< 0.001	< 0.001	< 0.001
Variance parameter estimates						
Period	0.000036		0.011	0.0001	0.052	2.1
Residual	0.000358		0.155	0.0001	0.159	120.9

[1] N_1, number of observations for the hydrolysis; [2] N_2, number of observations for the fermentation; [3] *L*, lag time (h); [4] $\mu_{t=T/2}$, fractional rate of degradation at t = *T/2* (h^{-1}); [5] *T/2*, half-time to asymptote (h); [6] G_f, maximum gas volume (ml g^{-1}) ; [7] For one parameter, averages followed by different letters in the columns differ at significance level of 0.05; [8] *d.f.*, degrees of freedom

The hydrolysis of the substrates before their fermentation affected the kinetics parameters ($P < 0.001$) but an interaction with the substrate was observed ($P < 0.001$). The consequence of the interaction between the hydrolysis and the substrate was that the hierarchy of the means for G_f, *L*, $\mu_{t=T/2}$ and *T/2* was different whether the substrates were hydrolysed or not. For peas and soybean meal, the total gas production (G_f) was increased with the hydrolysis ($P < 0.001$), but the G_f remained unchanged with lupins and decreased with maize, sugar-beet pulp and wheat bran ($P < 0.001$). The hydrolysis of the substrates also induced an increase in lag times (*L*) ($P < 0.01$), except for lupins and maize. The fractional rates of fermentation ($\mu_{t=T/2}$) were lower ($P < 0.001$) when peas, lupins, maize and wheat bran were hydrolysed. For soybean meal ($P = 0.014$) and sugar-beet pulp ($P < 0.001$), the $\mu_{t=T/2}$ parameter increased with the hydrolysis.

No correlation ($P > 0.05$) was observed between any of the four kinetics parameters of the non-hydrolysed substrates and their chemical composition. On the contrary, for hydrolysed substrates, negative correlation coefficients were found, linking the final gas volume to the ADL and hemicellulose contents of the non-hydrolysed substrates (ADL: $r = -0.828$, $P < 0.05$; hemicellulose: $r = -0.960$, $P < 0.01$). Other relationships were also found between ADL or hemicellulose and the lag time (ADL: $r = -0.812$, $P < 0.05$; hemicellulose: $r = -0.899$, $P < 0.05$).

5. Discussion

The buffer solution used by Menke and Steingass (1988) offered an optimal environment to the colic microflora, whether it originated from the large intestine or from faeces. The pH values in the syringes, from 6.7 to 7.0, depending on the source of inoculum and the substrate (data not shown) were consistent with pH values measured in pig large intestines (Bach Knudsen and Hansen, 1991).

The accuracy of the gas volume measurements was also satisfactory. The coefficients of variation were wider during the first 8 h of fermentation (7 to 10 %) and stabilised at around 3 to 4 % after 20 h (Figure 2).

In Experiment 1, the lower $\mu_{t=T/2}$ and higher *T/2* values obtained with faecal inocula could be due to a lower activity of the micro-organisms. According to Jensen and Jørgensen (1994), the activity of the latter is higher in the large intestine than in faeces, even if their concentration is equivalent. The composition in bacteria species may also be different, since it changes with the evolution of the substrate composition (Bach Knudsen, 2001). The absence of differences ($P > 0.05$) for the lag times can be explained by an imprecision of the model since the calculated lag phase covers a short period (< 2 h) during which no experimental data were recorded. However, the activity of the faecal inoculum increases with time, since $\mu_{t=T/2}$ did not differ significantly from that obtained with intestinal content and given that the final gas production (G_f) was similar for both inocula (Table 3). This confirms the fact that the microbial population from the colon and the faeces have similar abilities to ferment a same substrate. Our results are consistent with those of Dung and Udén (2002) and Löwgren *et al.* (1989).

Bauer *et al.* (2004), on the contrary, obtained higher fermentation with faecal inocula compared to colic inocula.

The dilution of the inocula in the buffer solution had no effect on the final gas production (G_f) but influenced the lag times (L) and the fractional rates of degradation ($\mu_{t=T/2}$). This may be related to decreasing concentrations of active bacteria in the inoculum and to the presence in the inoculum of nutrients to which the micro-organisms are adapted. The presence of these nutrients is reflected by the fermentation of the blank samples. In the present experiment, the fermentation of the blanks was significantly lower ($P < 0.05$) after 24 h with the dilution of 0.025 g ml^{-1} compared to the 3 other dilutions (0.2, 0.1 and 0.05 g ml^{-1}) i.e. 1.37 vs. 2.22 ml g^{-1} for faecal samples and 2.39 vs. 3.65 ml g^{-1} for intestinal samples (data not shown). After 72 h, the difference had disappeared, indicating that the inoculum diluted at 0.025 g ml^{-1} had recovered its delay. Therefore, in order to ensure a rapid start of the fermentation, a dilution of the inocula lower than 0.05 g ml^{-1} is not recommended.

As described by Bauer *et al.* (2003), it cannot be stated that the enzymatic hydrolysis prior to fermentation yield a material of similar fermentability to ileal chyme since non-enzymatic processes occurring in the upper digestive tract are not reproduced and since some microbial fermentation are likely to occur in the final part of the small intestine. The hydrolysis concentrates the insoluble dietary fibre in the substrates. For example, with peas, maize and wheat bran, the NDF content of the residues was respectively 248, 168 and 883 g kg^{-1}DM (data not shown) instead of 142, 68 and 390 g kg^{-1}DM before the hydrolysis. As a consequence, the fermentation patterns and the ranking order between the different substrates were affected (Table 4). The hydrolysis also results in the disappearance of part of the soluble fibre. The fermentation of the latter is not taken into account when *in vitro* hydrolysis is performed. Further investigation is required to verify whether their contribution to gas production is significant. Such problem may occur, for example, with sources of soluble fibre such as sugar-beet pulp, lupins or linseed meal (Bach Knudsen, 1997) or with fruits.

The decrease in fermentation intensity ($\mu_{t=T/2}$) observed for various ingredients after *in vitro* hydrolysis (Table 4) can be explained by their lower content in rapidly fermentable components such as free sugars or soluble fibre (Mac Farlane and Mac

Farlane, 1993). For sugar-beet pulp and soybean meal, the hydrolysis prior to the gas test resulted in an increase in fermentation intensity. For soybean meal, it can be explained by its high protein content. According to Blümmel et al (1999), high protein contents (> 400 g kg^{-1}) affect gas production caused by the buffering effect of the NH_3 released during the fermentation. In the case of sugar-beet pulp, the increase in the fractional rate of degradation with the hydrolysis is consistent with that of Hoebler *et al.* (1998).

It can be concluded that the gas production technique is a useful tool to characterise fibre fermentation in the pig large intestine. The microbial inoculum can be prepared from fresh faeces, making the method easier and ethically acceptable. The *in vitro* hydrolysis prior to fermentation significantly affects the fermentation patterns of the substrates but this raises the question of the characterization of ingredients rich in soluble fibre, which hinders the generalisation of the enzymatic treatment and requires further investigation.

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CHAPTER IV

Article 3:

Effect of pig faecal donor and of pig diet composition on in vitro fermentation of sugar beet pulp

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1. Abstract

Two experiments were undertaken to investigate the influence of (1) pig bodyweight and (2) dietary fibre content of the diet on the *in vitro* gas production of sugar beet pulp fibre using faecal inoculum.

In the first experiment, inocula prepared from young pigs (Y; 16-50 kg), growing pigs (G; 62-93 kg) and sows (S; 216-240 kg) were compared. Sugar beet pulp, hydrolysed *in vitro* with pepsin and then pancreatin, was used as the fermentation substrate. The cumulated gas productions over 144 h were modelled and the kinetics parameters compared. Lag times (Y: 4.6 h; G: 6.4 h; S: 9.2 h) and half times to asymptote (Y: 14.7 h; G: 15.9 h; S: 20.8 h) increased with pig bodyweight ($P < 0.001$) and the fractional degradation rates of the substrate differed between the pig categories (Y: 0.110 h^{-1}; G: 0.115 h^{-1}; S: 0.100 h^{-1}; $P < 0.001$). The final gas production was not affected ($P = 0.10$) by the inoculum source.

In the second experiment hydrolysed sugar beet pulp was fermented with 4 inocula prepared from pigs fed diets differing in their total and soluble dietary fibre contents i.e. low fibre diet rich in soluble fibre (LOW-S) or in insoluble fibre (LOW-I) or high fibre diet rich in soluble fibre (HIGH-S) or in insoluble fibre (HIGH-I). The total and the soluble dietary fibre influenced the kinetics of gas production. The presence of soluble fibre decreased the lag times, whatever the total dietary fibre content (2.7 h for LOW-S vs. 3.5 for LOW-I, 4.0 h for HIGH-S vs. 4.4 for HIGH-I; $P < 0.001$). The half times to asymptote were higher with the low-fibre diets ($P < 0.001$) and, for similar total dietary fibre contents, they were lower when the proportion of soluble fibres increased (LOW-S: 9.9 h; LOW-I: 11.4 h; HIGH-S: 8.9 h; HIGH-I: 10.1 h; $P < 0.001$). The fractional degradation rates of the substrate were the highest with the fibre-rich diet containing a high proportion of soluble fibres (0.158 h^{-1}; $P < 0.001$).

In conclusion, the bodyweight of the faeces donors and the dietary fibre composition of the pig diet influence the *in vitro* fermentation kinetics of hydrolysed sugar beet pulp, but not the final gas production.

Keywords: pig, dietary fibre, bodyweight, *in vitro* fermentation, gas-test technique, sugar beet pulp.

2. Introduction

Fibrous ingredients are increasingly incorporated in pig diets in order to reduce the production costs. In the tropics, numerous unconventional ingredients with high fibre content, such as tree leaves, are also used to feed pigs (Leterme *et al.*, 2005, 2006). Dietary fibre bring benefits to the animals in term of well-being (satiety, quietness) and intestinal health (Noblet and Le Goff, 2001; Wenk, 2001).

During the last years, fibre fermentation in the large intestine has received thorough attention since the resulting short-chain fatty acid production both regulates the intestinal micro-organisms and brings energy that can cover from 0.15 of the maintenance energy requirements of growing-finishing pigs (Dierick *et al.*, 1989) to 0.3 for those of gestating sows (Varel and Yen, 1997).

In vivo studies are difficult and heavy to carry out. Therefore, *in vitro* methods were developed for studies in humans (McBurney *et al.*, 1985; Barry *et al.*, 1995) and ruminants (Menke and Steingass, 1988; Theodorou *et al.*, 1994). They are based on the *in vitro* fermentation of a substrate (i.e. the fibre source) by micro-organisms collected in the colon or the rumen. The rate of fermentation is generally evaluated through the measurement of the amount of gas produced during the fermentation.

Recently, the method was adapted to pig studies. *In vivo*, the proportion of slowly fermentable carbohydrates increases as the digesta progresses in the intestine. The microflora, dominated in the caecum and the proximal colon by propionic-producing micro-organisms, becomes more acetic acid-producing and proteolytic in the distal colon (Bach Knudsen, 1991), with variations in pH, redox potential and microbial activity (Jensen and Jørgensen, 1994). Even if the diversity of the environmental conditions in the different sections of the hindgut is not reproduced in *in vitro* methods, it was interestingly stated that faeces can be used as a source of inoculum in place of intestinal contents for the gas-test method (Bauer *et al.*, 2004; Bindelle *et al.*, 2007).

However, other factors should deserve attention. For example, the age of the animals and the composition of the dietary fibre affect the mean retention time in the digestive tract and modify the composition of the microflora and the activity of the latter in the large intestine and the faeces (Bauer *et al.*, 2001; Le Goff *et al.*, 2003; Freire *et al.*,

2000; Williams *et al.*, 2001). To our knowledge, the effect of these parameters on the fermentation kinetics of dietary fibre by the gas-test method has not been studied yet.
The aim of the present work was to investigate the influence of the bodyweight of the inoculum donor and of the dietary fibre composition of the feed given to the animals on the *in vitro* fermentation prepared with faecal samples.

3. Materials and methods

1. *Experiment 1: Bodyweight of the inoculum donors*

Animals and diets

This experiment was carried out using Belgian Landrace pigs : 3 young females, weighing 16 ± 1 kg at the beginning of the collection period and 50 ± 5 kg at the end, 3 growing-finishing females (weighing from 62 ± 6 to 93 ± 15 kg) and 3 sows (weighing from 205 ± 2 to 240 ± 1 kg). The animals received diets formulated to cover their requirements. Special attention was paid to the fibre composition of the diets (Table 5). The young and growing-finishing pigs were kept in groups and fed *ad libitum*. The sows were individually housed and were fed 3.5 kg per day in 2 meals (at 8:00 a.m. and 3:00 p.m.). All the animals had free access to water. The collection of the faeces started when the animals were adapted to the experimental diets, i.e. over 4 weeks.

Substrate and enzymatic hydrolysis

Sugar beet pulp (*Beta vulgaris*), ground to pass a 1 mm-mesh screen with a Cyclotec 1093 Sample Mill (FOSS Electric A/S, Hilleroed, Denmark), was used as the substrate for the gas-test method. Prior to the fermentation, it underwent an in vitro pepsin-pancreatin hydrolysis following the protocol of Boisen and Fernández (1997). Samples of 2 g were weighed in conical flasks. Phosphate buffer solution (100 ml, 0.1 M, pH 6.0) and HCl solution (40 ml, 0.2 M) were poured into the flasks. The pH was adjusted to 2.0 with 1M HCl or 1M NaOH and 2 ml of a chloramphenicol (Sigma C-0378) solution (0.5 g 100 ml-1 ethanol) was added. Fresh pepsin solution (4 ml, 25 g l-1, porcine pepsin : 2000 FIP-U/g, Merck n°7190) was finally added. The flasks were

closed with a rubber stopper and placed for 2 h under gentle agitation in a water-bath at 39 ± 0.5°C.

Table 5. Composition of the experimental diets.

	Experiment 1			Experiment 2			
	Young pigs	Growing pigs	Sows	LOW-I[1]	LOW-S[2]	HIGH-I[3]	HIGH-S[4]
Feedstuffs (g kg^{-1} diet)							
Maize	383	563	622	710	615	229	396
Maize glutenfeed	231	82	0	0	0	409	85
Wheat bran	100	170	253	60	0	170	170
Sugar beet pulp	0	5	0	0	63	55	145
Skimmed milk powder	130	0	0	0	112	77	35
Soyabean meal	120	133	67	182	149	0	115
Soyabean oil	0	0	0	11	3	30	20
L-Lysine HCl	3	0.7	0	0.2	0	1.7	0
L-Tryptophan	0.4	0.2	0	0.2	0.2	3.0	3.0
Salt	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Calcium carbonate	2.1	15.4	20.0	20.0	1.0	0	5.9
Dicalcium phosphate dihydrate	4.9	7.1	15.0	15.0	15.0	7.3	0
Mineral and vitamin premix[5]	22.5	20.0	20.0	20.0	20.0	20.0	20.0
Chemical composition (g kg^{-1} DM)							
DM (g kg^{-1} diet)	898	896	897	893	905	903	908
Crude protein (N X 6.25)	202	177	146	168	161	172	171
Ether extract	59	40	41	57	61	85	66
Ash	71	72	80	58	84	72	64
NDF	165	153	148	83	73	240	171
ADF	54	53	48	33	35	75	73
ADL	8	12	14	6	10	21	42
Starch	319	441	514	525	440	268	329
Total dietary fibre	211	181	176	129	135	308	263
$\frac{\text{Soluble dietary fibre}}{\text{Total dietary fibre}}$ (g g^{-1})	0.02	0.03	0.11	0.06	0.13	0.05	0.10

[1] LOW-I, diet poor in total dietary fibre with high proportion of insoluble fibre; [2] LOW-S, diet poor in total dietary fibre with high proportion of soluble fibre; [3] HIGH-I, diet rich in total dietary fibre with high proportion of insoluble fibre;[4] HIGH-S, diet rich in total dietary fibre with high proportion of soluble fibre; [5] Mineral and vitamin premix, 2507 VAPOR 220 LMT GREEN (Trouw Nutrition, Ghent , Belgium): Vit A 400 IU/g, Vit D3 100 IU/g, Vit E 2.50 mg/g, Vit K3 0.043 mg/g, Vit B1 0.043 mg/g, Betaine 5.0 mg/g, Vit B2 0.14 mg/g, Vit B3 0.35 mg/g, Vit B6 0.088 mg/g, Vit B12 0.00075 mg/g, Vit PP 0.75 mg/g, folic acid 0.0050 mg/g, 6-fytase 26.25 FYT/g, endo-1,4-beta-xylanase 200 U/g, butylhydroxytoluene 0.13 %, potassium iodine 0.0050 %, cobalt carbonate hydroxide 0.0025 %, sodium selenite 0.0021 %, copper(II) sulphate 0.060 %, manganese oxide 0.20 %, zinc oxide 0.50 %, ferrous sulphate monohydrate 0.75 %, L-lysine-HCl 8 %, DL-methionine 2 %, L-théonine 1.625 %, calcium 19.9 %, sodium 7.2 %.

After the pepsin hydrolysis, 40 ml of a phosphate buffer solution (0.2 M, pH 6.8) and 20 ml of a NaOH solution (0.6 M) were added to the solution. The pH was adjusted to 6.8 with 1M HCl or 1M NaOH and fresh pancreatin solution (2 ml, 100 g pancreatin

(Sigma P-1750) l^{-1}) was added. The flasks were then closed with a rubber stopper and placed for 4 h under gentle agitation in a water-bath at 39 ± 0.5°C. After hydrolysis, the residues were collected by filtration on a Nylon cloth (42 µm), washed with ethanol (2 x 25 ml 95 % ethanol) and acetone (2 x 25 ml 99.5 % acetone), dried for 24 h at 60 ± 1°C and weighed. The enzymatic hydrolysis was performed 24 times (8 replicates x 3 periods). Hydrolysis residues from the different replicates and periods were pooled for subsequent *in vitro* fermentation.

The composition of the raw and hydrolysed sugar beet pulp is detailed in Table 6.

Table 6. Dry matter disappearance (dDM) during the pepsin-pancreatin hydrolysis and chemical composition of the raw and hydrolysed sugar beet pulp (g kg^{-1} DM).

	Raw sugar beet pulp	Hydrolysed sugar beet pulp
dDM (g g^{-1})	0.37	-
DM (g kg^{-1} diet)	897	895
Crude protein	79	40
Ether extract	9	ND[1]
Ash	73	82
NDF	360	506
ADF	219	338
ADL	31	84
Total dietary fibre	682	896
$\frac{\text{Soluble dietary fibre}}{\text{Total dietary fibre}}$ (g g^{-1})	0.23	0.21

[1] ND, not determined, as content of this constituent was considered to be negligible.

Inocula

The faeces of each pig were collected directly in 100 ml plastic syringes. The plunger was pushed in order to remove all air and the closed syringes were immediately placed in a water-bath at 39 ºC for transportation to the laboratory. In order to reduce variation between animals, the inocula were prepared by mixing 28.5 g faeces provided by the 3 pigs of the same group in a plastic bag, under permanent flux of CO_2. A preheated (39°C) buffer solution (210 ml) composed of salts and minerals (Menke and Steingass, 1988) was added to the bags and samples were subjected for 60 seconds to mechanical pummelling using a Stomacher Lab Blender 400 (Seward Medical, Norfolk, UK) to suspend fibre-associated bacteria in the liquid (Merry and Mac Allan, 1983). The

solution was then filtered through a 250 µm mesh screen and completed with 1.5 l of the buffer solution in order to reach a dilution of 0.05 g faeces ml^{-1} buffer.

In vitro fermentation

In vitro fermentation was performed by placing 200 mg of hydrolysed sugar beet pulp into a 100 ml Kolbenprober glass syringe. Thereafter, 30 ml of the inoculum prepared from faeces of the 3 groups of donors were added to the preheated syringes, placed in an incubator at 39 ± 0.5°C. The released gas volumes were recorded after 2, 5, 8, 12, 16, 20, 24, 30, 36, 48, 72 and 144 hours of incubation. Three syringes per donor group containing just inoculum (blanks) were systematically included in each run.
The experimental scheme was as follows : (3 inocula x (6 replicates + 3 blanks)) x 3 periods.

Chemical analysis

The raw and hydrolysed sugar beet pulp and the diets, ground to pass a 1 mm-mesh screen, were analyzed for their content in dry matter (105 ºC for 24 h), ash (550 ºC for 8 h), nitrogen (Kjeldahl method, crude protein = 6.25 x N content), ether extract (Soxhlet method, using ether), neutral (using Na_2SO_3 and Termamyl : 120 KNU/g, Novo Nordisk, Bagsværd, Denmark) and acid detergent fibre and lignin, using the Fibercap system (Foss Electric, Bagsvaerd, Denmark). Starch was determined according to the method of Faisant *et al.* (1995) and total and soluble dietary fibre contents were measured following the protocol described by Lee *et al.* (1992), after grinding the samples through a 0.5 mm-mesh screen.

Calculations and statistical analysis

The dry matter disappearance (*dDM*) during the pepsin-pancreatin hydrolysis was calculated as follows:

$$(8) \qquad dDM = \frac{\text{weight of the sample before hydrolysis - weight of the residue}}{\text{weight of the sample before hydrolysis}}$$

The gas volume recorded during the fermentation of each syringe was calculated as follows:

$$(9) \qquad V^{corr}(t) = \frac{\mathrm{V}(t) - V_0 - B(t) * V_0}{W}$$

where $V^{corr}(t)$ (ml g^{-1}DM) is the gas accumulation to time t (h) corrected by the amount of incubated substrate and the production of the blanks, $V(t)$ (ml) the volume occupied by the inoculum and the gas at time t, V_0 (ml) the volume of inoculum transferred in the syringes at the start of the fermentation, $B(t)$ (ml gas per ml of inoculum) the mean gas production at time t of the blanks per ml of inoculum and W (g DM) the amount of substrate placed in the syringe.

Outlying observations, caused by accidental leakage of gas, were discarded as described by Dagnelie (1975).

Gas accumulation curves were modelled using the mathematical model proposed by France *et al.* (1993):

$$(10) \qquad \begin{aligned} G &= 0, && \text{if } 0 < t < L \\ &= G_f\left(1 - \exp\left\{-\left\langle b(t-L) + c\left(\sqrt{t} - \sqrt{L}\right)\right\rangle\right\}\right), && \text{if } t \geq L \end{aligned}$$

where G (ml g^{-1}DM) denotes the gas accumulation to time, G_f (ml g^{-1}DM) the maximum gas volume for $t = \infty$ and L (h) the lag time before the fermentation starts. The constants b (h^{-1}) and c ($h^{-1/2}$) determine the fractional rate of degradation of the substrate μ (h^{-1}), which is postulated to vary with time as follows:

$$(11) \qquad \mu = b + \frac{c}{2\sqrt{t}}, \quad \text{if } t \geq L$$

The kinetics parameters (G_f, L, $\mu_{t=T/2}$, b, c and $T/2$) were compared in the statistical analysis. $T/2$ is the time to half asymptote when $G = G_f/2$. At this time, the rate of gas production is in a linear phase, near its maximum.

Statistical analysis of the kinetics parameters were performed by means of an analysis of variance and a classification of means by the Student-Newman-Keuls method using the GLM procedure of the SAS 8.02 software (SAS inc., Cary, NC, USA), with the following general linear model:

$$(12) \qquad Y = \alpha + I_i + P(I)_{ij} + \varepsilon$$

where Y is the result, α the mean, I_i the effect of the inoculum source (i = 1, 2, 3), $P(I)_{ij}$ the effect of the period (j = 1, 2, 3) and ε the error term.

2. *Experiment 2: dietary fibre composition*

Animals and diets

Twelve Belgian Landrace gilts weighing from 42 ± 4 kg to 88 ± 10 kg were fed *ad libitum* with one of the 4 diets described in Table 5. They were randomly allocated in 4 groups (one group/diet). Two diets were designed to have low total dietary fibre content with low (LOW-I) or high (LOW-S) content in soluble fibre and 2 diets with high total dietary fibre content, with respectively low and high soluble fibre content (HIGH-I and HIGH-S). During the experimental period, the animals were kept in groups and had free access to water. Faeces collection started when the animals were adapted to the diets, i.e. over 4 weeks.

Substrate

Hydrolysed sugar beet pulp was used as a substrate, as described in Experiment 1.

Inocula and fermentation

Inocula preparation and *in vitro* fermentation were performed as described in Experiment 1.

The experimental scheme was as follows: (4 inocula x (6 replicates + 3 blanks)) x 3 periods.

Calculations and statistical analysis

The gas volume recorded during the fermentation of each syringe was calculated as described above.

The gas accumulation curves were modelled and the kinetics parameters compared as described in Experiment 1 using the following general linear model:

$$(13) \qquad Y = \alpha + I_i + P(I)_{ij} + \varepsilon$$

where Y is the result, α the mean, I_i the effect of the inoculum source (i = 1,..., 4), $P(I)_{ij}$ the effect of the period (j = 1, 2, 3) and ε the error term.

4. Results

1. *Experiment 1*

The gas accumulation curves recorded during the fermentation of hydrolysed sugar beet pulp with inocula prepared from young, growing and adult pigs are illustrated in Figure 3. The parameters of the France *et al.* (1993) model developed to describe the fermentation kinetics of sugar beet fibre incubated with the 3 sources of inoculum are detailed in Table 7.

Different lag times (L), half-times to asymptote ($T/2$) and fractional rates of degradation (b, c and $\mu_{t=T/2}$) ($P < 0.001$) were recorded for the 3 inocula. Both L and $T/2$ increased with pig bodyweight ($P < 0.001$). The highest $\mu_{t=T/2}$ were observed with the growing pigs, followed by the young pigs and finally the sows. However, the final gas production (G_f) was not influenced by the source of inoculum ($P = 0.103$).

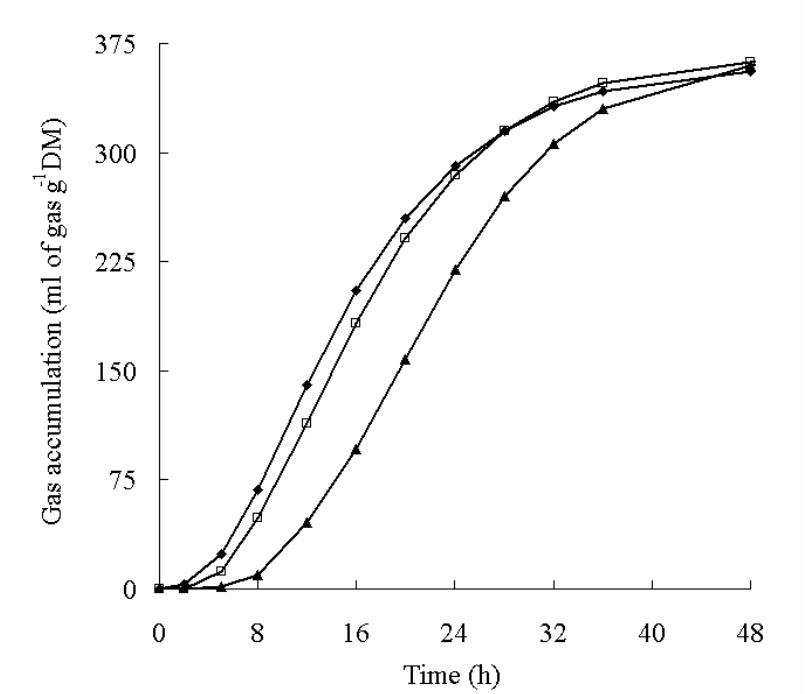

Figure 3. Mean values of the modelled gas accumulation over time (until 48 h) of sugar beet pulp incubated with young pigs (◆), growing pigs (□) and sows (▲) faecal inocula (Exp. 1).

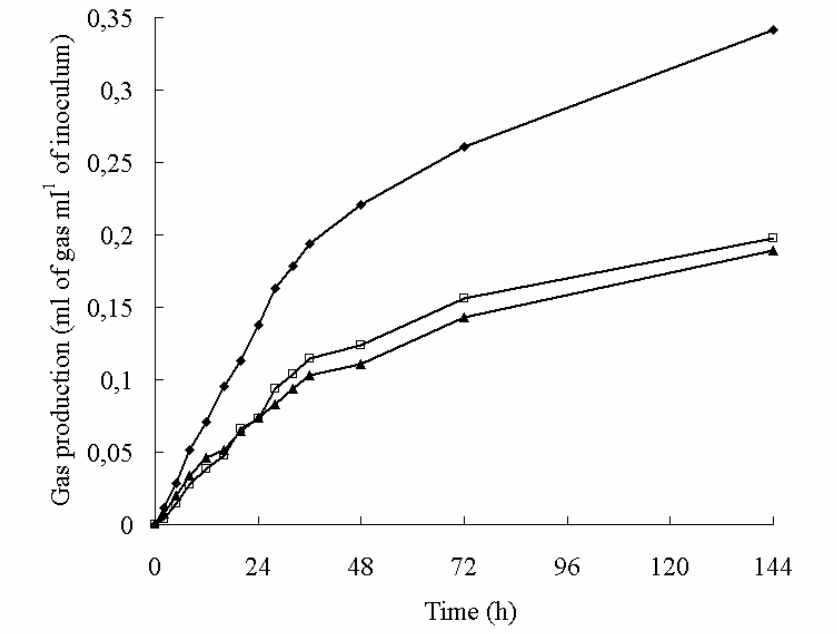

Figure 4. Mean values of the gas production over time (until 144 h) of the blanks (faecal inocula without substrate) from young pigs (◆), growing pigs (□) and sows (▲) (Exp. 1).

Significant differences ($P < 0.05$) were also observed between the gas productions of the blanks (Figure 4 and Table 7). After 8 h of incubation, the inocula provided by the young pigs yielded higher gas productions (0.05 ml gas ml^{-1} inoculum) compared to the growing pigs and the sows (0.03 ml gas ml^{-1} inoculum) ($P = 0.025$). This difference remained until the end of the fermentation process: the gas production of the blanks after 144 h reached 0.34 ml gas ml^{-1} inoculum for the young pigs and only 0.20 and 0.19 for the growing pigs and the sows respectively ($P < 0.001$).

Table 7. Fitted kinetics parameters (means) on the gas accumulation recorded for hydrolysed sugar beet pulp incubated with faecal inocula provided by young pigs, growing pigs and sows, and gas production of the blanks (Exp. 1).

Animals	N[1]	L[2]	$T/2$[3]	b[4]	c[5]	$\mu_{t=T/2}$[6]	G_f[7]
Young pigs	18	4.6[c8]	14.7[c]	0.244[b]	-1.006[a]	0.110[b]	367
Growing pigs	18	6.4[b]	15.9[b]	0.266[b]	-1.214[b]	0.115[a]	366
Sows	18	9.2[a]	20.8[a]	0.312[a]	-2.004[c]	0.100[c]	361
SEM[9]		0.7	0.4	0.040	0.274	0.006	8
Source of variation	*df*[10]			*P-values*			
Inoculum	2	<0.001	<0.001	<0.001	<0.001	<0.001	0.103
Period (inoculum)	6	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	N	V_{5h}[11]	V_{12h}	V_{24h}	V_{72h}	V_{144h}	
Young pigs	9	0.03	0.07[a]	0.14[a]	0.26[a]	0.34[a]	
Growing pigs	9	0.01	0.04[b]	0.07[b]	0.16[b]	0.20[b]	
Sows	9	0.02	0.05[b]	0.07b	0.14[b]	0.19[b]	
SEM		0.01	0.02	0.02	0.03	0.05	
Source of variation	*df*			P-values			
Inoculum	2	0.069	<0.001	<0.001	<0.001	<0.001	
Period	6	<0.001	<0.001	<0.001	<0.001	<0.001	

[1] *N*, number of observations; [2] *L*, lag time (h); [3] *T/2*, half-time to asymptote (h); [4] *b*, parameter of the fractional rate of degradation (h^{-1}); [5] *c*, parameter of the fractional rate of degradation ($h^{-1/2}$); [6] $\mu_{t=T/2}$, fractional rate of degradation at t = *T/2* (h^{-1}); [7] G_f, maximum gas volume (ml g^{-1} DM); [8] For one parameter, means followed by different letters in the columns differ at a significance level of 0.05; [9] *SEM*, standard error of means; [10] *d.f.*, degrees of freedom; [11] V_{5h}, volume produced by the fermentation of the blanks after 5 h of incubation (ml gas ml^{-1} inoculum)

2. *Experiment 2*

The gas accumulation curves recorded with inocula prepared from the growing pigs fed the 4 diets differing in their fibre content are shown on Figure 5. The parameters of the France model calculated for the 4 inocula are detailed in Table 8. The source of inoculum influenced these parameters. The lag times (*L*) and the half times to asymptote (*T/2*) increased with the levels of fibre content and the fraction of insoluble fibre ($P < 0.001$). The dietary fibre content and fibre solubility of the donors also influenced the *b* and *c* parameters ($P < 0.001$).

The highest fractional rate of degradation ($\mu_{t=T/2}$) was recorded with the inoculum from pigs fed the HIGH-S diet followed by the HIGH-I diet, the LOW-S diet and, finally, the LOW-I diet. The final gas productions (G_f) were similar for all the diets ($P > 0.05$), excepted for the LOW-I diet ($P < 0.001$).

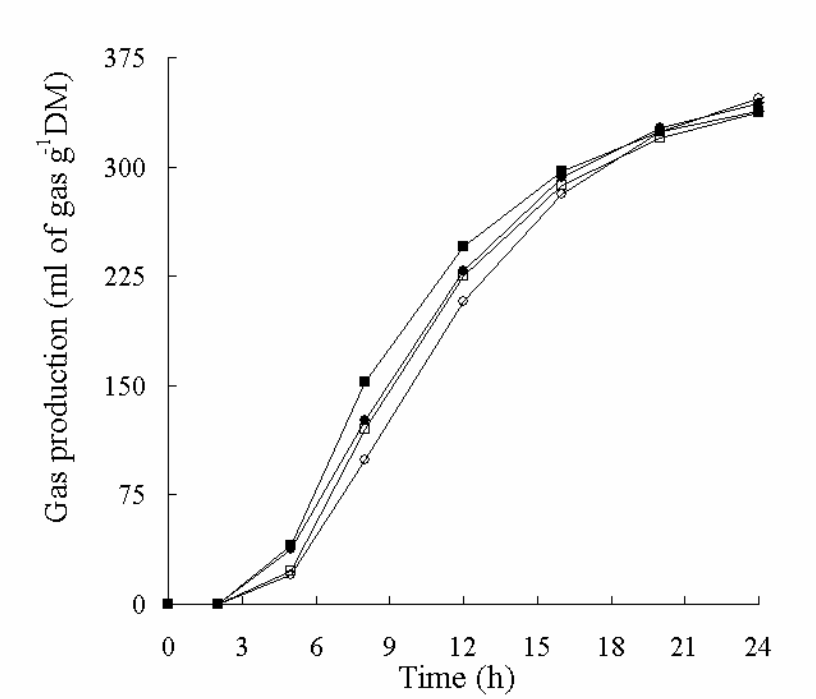

Figure 5. Mean values of the modelled gas accumulation over time (until 24 h) of sugar beet pulp incubated with faecal inocula of pigs fed the HIGH-I (□), HIGH-S (■), LOW-I (○) and LOW-S (●) diets (Exp. 2).

Differences were also observed between the gas productions of the blanks (Figure 6 and Table 8). In the middle of the incubation period, the blanks prepared from the pigs fed the LOW-S and LOW-I diets yielded higher gas productions compared to the HIGH-S and HIGH-I diets ($P < 0.001$).

Table 8. Fitted kinetics parameters (means) on the gas accumulation recorded for hydrolysed sugar beet pulp incubated with faecal inocula of growing pigs fed diets with 4 different fibre contents, and gas production of the blanks (Exp 2).

Diet	N[1]	L[2]	$T/2$[3]	b[4]	c[5]	$\mu_{t=T/2}$[6]	G_f[7]
Low fibre, insoluble	18	3.5[c8]	11.4[a]	0.314[a]	-1.175[c]	0.139[d]	368[a]
Low fibre, soluble	18	2.7[d]	9.9[c]	0.313[a]	-1.038[bc]	0.147[c]	359[b]
High fibre, insoluble	18	4.4[a]	10.1[b]	0.279[a]	-0.812[b]	0.154[b]	356[b]
High fibre, soluble	17	4.0[b]	8.9[d]	0.176[b]	-0.156[a]	0.158[a]	359[b]
SEM[9]		0.4	0.3	0.066	0.428	0.005	6
Source of variation	*df*[10]	*P-values*					
Inoculum	3	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Period (inoculum)	8	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

	N	V_{5h}[11]	V_{12h}	V_{24h}	V_{72h}	V_{144h}
Low fibre, insoluble	9	0.04[ab]	0.09[b]	0.15[b]	0.24[b]	0.27[b]
Low fibre, soluble	8	0.06[a]	0.12[a]	0.19[a]	0.33[a]	0.37[a]
High fibre, insoluble	9	0.03[bc]	0.06[c]	0.09[c]	0.19[c]	0.25[b]
High fibre, soluble	9	0.01[c]	0.04[c]	0.08[c]	0.18[c]	0.24[b]
SEM		0.02	0.02	0.02	0.02	0.03
Source of variation	*df*	*P-values*				
Inoculum	3	<0.001	<0.001	<0.001	<0.001	<0.001
Period (inoculum)	8	0.145	<0.001	<0.001	<0.001	<0.001

[1] *N*, number of observations; [2] *L*, lag time (h); [3] *T/2*, half-time to asymptote (h); [4] *b*, parameter of the fractional rate of degradation (h^{-1}); [5] *c*, parameter of the fractional rate of degradation ($h^{-1/2}$); [6] $\mu_{t=T/2}$, fractional rate of degradation at t = *T/2* (h^{-1}); [7] G_f, maximum gas volume (ml g^{-1} DM); [8] For one parameter, means followed by different letters in the columns differ at a significance level of 0.05; [9] *SEM.*, standard error of means; [10] *d.f.*, degrees of freedom; [11] V_{5h}, volume produced by the fermentation of the blanks after 5 h of incubation (ml gas ml^{-1} inoculum)

5. Discussion

The composition of the fermented substrate (Table 6) shows that the pepsin-pancreatin hydrolysis of the sugar beet pulp induced an important enrichment in dietary fibre, while the proportion of soluble fibre barely changed after hydrolysis (0.21 after

hydrolysis vs. 0.23 before). Modifications in CP, NDF, ADF and ADL content during enzymatic hydrolysis were very similar to the values reported by Bauer *et al.* (2003).

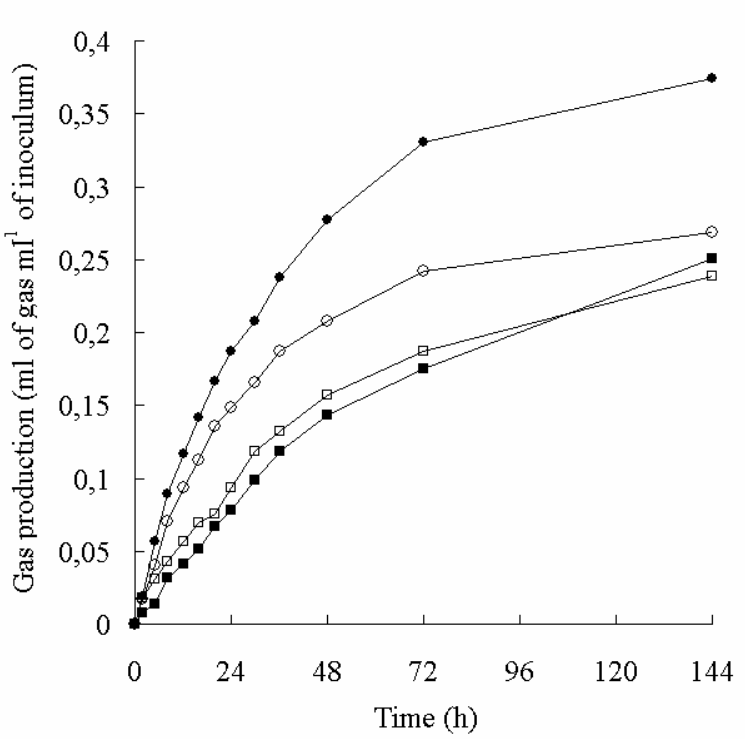

Figure 6. Mean values of the gas production over time (until 144 h) of the blanks (faecal inocula without substrate) from pigs fed the HIGH-I (□), HIGH-S (■), LOW-I (○) and LOW-S (●) diets (Exp. 2).

Even if similar levels of fibre were pursued in the design of the first experiment, the 3 diets moderately differed in terms of NDF, ADF and total dietary fibre contents (see Table 5) because we had to adapt crude protein contents to the specific requirements of the donors and therefore different ingredients were used in the diets (maize, maize gluten feed and wheat bran). The more important differences were observed in starch contents and in soluble dietary fibre proportions. Both components were higher in the sow diet (514 g kg^{-1}DM for starch; 0.109 of soluble dietary fibre) compared to those for young and growing pigs (319 and 441 g kg^{-1}DM for starch; 0.022 and 0.027 of soluble dietary fibre, respectively). These differences might have biased the bodyweight effect through their influence on digestion and microbial activities. In Experiment 2, NDF, ADF and dietary fibre contents of the 4 diets given in Table 5 differed according to the

experimental design : 2 high fibre diets, 2 low fibre diets and low or high levels of soluble dietary fibres within both types of diets.

The aim of the study was to examine the influence of the faeces donor bodyweight and of the diet composition on the fermentation pattern of sugar beet fibre measured *in vitro* through gas production. Experiments 1 and 2 showed that, globally, neither the bodyweight, nor the dietary fibre content of the feed influenced the final gas production (G_f). Potential degradation of sugar beet pulp fibre and total gas production was reached for all inoculum sources after less than 48 h of incubation. However, even if hydrolysed sugar beet pulp is a readily degradable substrate, differences in the kinetics of fermentation were observed. This is consistent with McBurney and Thompson (1989) who noticed that the influence of faecal donors on *in vitro* digestibility of various fibrous feedstuffs diminished with increasing incubation time. It suggests, as mentioned by Awati *et al.* (2005), that the whole microbial community present in the inoculum adapts to the substrate during fermentation and reaches the maximum gas production, whatever the donor, after less than 48 h (Figure 3).

It is known that, compared to growing pigs, adults have more cellulolytic bacteria in the colon and their intestinal flora is more adapted to the digestion of lignocellulosic material (Varel and Yen, 1997). Therefore, the digestibility of dietary fibre increases with bodyweight (Noblet and Le Goff, 2001). Our first experiment showed that faeces from animals differing in their bodyweight yielded similar final gas production when fermenting sugar beet fibre *in vitro*. Nevertheless, the fermentation kinetics measured through the lag times (L) and the fractional rates of substrate degradation ($\mu_{t=T/2}$) slowed down with increasing bodyweight. This last effect was probably accentuated by the increasing content of the diets in easily digestible and rapidly fermentable carbohydrates, concomitant with bodyweight. The fermentation kinetics differences are attributable to the composition of the microbial community present in the collected faeces and to its activity. A lower activity of the inocula in sows is linked to their lower level of feed intake per kg bodyweight, the higher content in starch and soluble fibre of the diet, their slower digestive transit time (Le Goff *et al.*, 2002) and their greater intestinal volume, compared to growing and finishing pigs. Therefore, in adults an almost complete digestion of the fermentable dietary fibres occurs in the hindgut. On

the contrary, with high fibre diets, faeces of young animals still contain an important part of unfermented carbohydrates (Le Goff *et al.*, 2003) which, in our experiment, enhanced the bacterial activity in the inocula. This was confirmed by the remarkable gas production observed in the plastic syringes during the transportation at 39°C of the young pig faeces from farm to laboratory. As the blanks reflect the activity of the inoculum used in the method (Menke and Steingass, 1988), this assumption is also consistent with the higher blanks fermentation recorded for the young pigs. It explains the longer lag times recorded when the animals were heavier.

Beside the transit time, the composition in the intestinal microflora probably also influenced the recorded kinetics. Katouli *et al.* (1997) showed that dietary shifts during the weaning phase but also when passing from growing to fattening or adult diets, coincided with significant changes in metabolic pattern of the faecal flora. These changes are associated to an overall decrease in the ability of pig flora to ferment several carbohydrates when the animals age. Studies realised on children (Lifschitz, 1995) also showed that the establishment of a stabilised microflora in the digestive tract is a remarkably long process. Therefore, in our experiment, we suspect the microbial equilibrium of the species present in the faeces to differ between the 3 categories of animals. The metabolic pathways used by the bacteria to ferment the sugar beet pulp could also differ. Comparing the microbial activities of unweaned and adult pigs faeces, Bauer *et al.* (2001) observed that raw sugar beet pulp was more rapidly fermented by the inoculum prepared from unweaned pigs faeces, yielding higher volatile fatty acids and gas production, while adult faeces induced higher DM loss of the substrate. However, since in our first experiment, all the pigs were fed a fibre-rich diet, great differences in fermentation pathways were less likely to occur.

The results of the second experiment indicate that the composition of the diet also influences the fermentation patterns. Dietary non starch polysaccharides are known to modify the species and also the quantities of micro-organisms found in the large intestine (Williams *et al.*, 2001) and in the faeces (Wang *et al.*, 2004). In Experiment 2, the calculated kinetics parameters, excepted the lag time, varied between the values recorded for two extreme diets (LOW-I and HIGH-S) containing 7.3 and 25.8 g soluble dietary fibre kg^{-1}DM respectively. The intermediate diets (LOW-S and HIGH-I) had

comparable soluble dietary fibre content (18.0 and 15.1 g kg^{-1}DM respectively) and gave similar *b*, *c*, *T/2* and G_f, even if for *T/2* the slight difference was significant (9.9 vs. 10.1 h). Therefore, the results indicate that the main influence of the faeces donors on the *in vitro* fermentation kinetics lies in the diet soluble-fibre fraction. Bach Knudsen *et al.* (1991) showed that the state of energy limitation for microbial fermentation occurs at later stages in the colon, with diets providing larger amounts of fermentable substrates. Soluble dietary fibre which have mostly a high water retention capacity, are generally highly fermentable compared to insoluble dietary fibre (McBurney *et al.*, 1985). They increase the intestinal microbial activity and reduce the faecal transit time (Wenk, 2001). Therefore, the faecal flora used to prepare the inocula was probably more stimulated with the diets enriched in soluble fibre. The lower lag times observed with the LOW-S and HIGH-S diet confirm this explanation.

It can be concluded that, even if the final gas production of hydrolysed sugar beet pulp is not influenced by the bodyweight of the faeces donors and the content in soluble dietary fibre of the diets, these factors have a real impact on the fermentation kinetics measured with the gas production technique, especially during the growth phase of the fermentations.

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CHAPTER V

Article 4:

The source of fermentable carbohydrates influences the in vitro protein synthesis by colonic bacteria isolated from pigs

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Running head: dietary fibre influence intestinal bacteria growth

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1. Abstract

Two *in vitro* experiments were carried out to quantify the incorporation of N by pig colonic bacteria during the fermentation of dietary fibre, including non-starch polysaccharides and resistant starch. In the first experiment, 5 purified carbohydrates were used: starch (S), cellulose (C), inulin (I), pectin (P) and xylan (X). In the second experiment, 3 pepsin-pancreatin hydrolysed ingredients were investigated: potato (Pot), sugar beet pulp (SBP) and wheat bran (WB).

The substrates were incubated in an inoculum, prepared from fresh faeces of sows and a buffer solution providing ^{15}N-labelled NH_4Cl. Gas production was monitored. Bacterial N incorporation (BNI) was estimated by measuring the incorporation of ^{15}N in the solid residue at half-time to asymptotic gas production (*T/2*). The remaining substrate was analysed for sugar content. Short-chain fatty acids (SCFA) were determined in the liquid phase.

In the first experiment, the fermentation kinetics differed between the substrates. P, S and I showed higher rates of degradation ($P<0.001$), while X and C showed a longer lag time and *T/2*. The sugar disappearance reached 0.91, 0.90, 0.81, 0.56 and 0.46, respectively for P, I, S, C and X. Starch and I fixed more N per g substrate ($P<0.05$) than C, X and P (22.9 and 23.2 mg fixed N/g fermented substrate vs. 11.3, 12.3 and 9.8, respectively). Production of SCFA was the highest for the substrates with low N fixation: 562 and 565 mg/g fermented substrate for X and C vs. 290 to 451 for P, I and S ($P<0.01$). In the second experiment, Pot and SBP fermented more rapidly than WB ($P<0.001$). Substrate disappearance at *T/2* varied from 0.17 to 0.50. BNI were 18.3, 17.0 and 10.2 mg fixed N/g fermented substrate, for SBP, Pot and WB, respectively but were not statistically different. SCFA productions were the highest with WB (913 mg/g fermented substrate) followed by SBP (641) and Pot (556) ($P<0.05$).

The differences in N uptake by intestinal bacteria are linked to the partitioning of the substrate energy content between bacterial growth and SCFA production. This partitioning varies according to the rate of fermentation and the chemical composition of the substrate, as shown by the regression equation linking BNI to T/2 and SCFA ($r^2 = 0.91$, $P<0.01$) and the correlation between BNI and IDF ($r = -0.77$, $P<0.05$) when pectin was discarded from the data base.

Keywords: pig, dietary fibre, nitrogen excretion, *in vitro* fermentation.

2. Introduction

Increasing attention has been paid to dietary fibre (DF) fermentation, including non-starch polysaccharides (NSP) and resistant starch (RS), in the large intestine of pigs during the past several years. Indeed, DF lower the energy value of the diet since their digestibility varies from 0.40 to 0.60 compared to the other nutrients (protein, fat, sugars or starch) which are above 0.80 (Noblet and Le Goff, 2001). On the other hand, the short-chain fatty acids (SCFA) produced by intestinal bacteria due to fibre fermentation can be used by the host animal for his own energy supply. This can cover up to 15 % of the maintenance energy requirements in growing pigs and 30 % in sows (Varel and Yen, 1997). The knowledge of the contribution of DF to energy supply is also important for smallholders in the tropics, since the latter feed their pigs with unconventional fibrous ingredients, such as tree leaves (Leterme *et al.*, 2006).

The bulking effect of fibre and the prebiotic influence on some intestinal bacterial strains can also benefit the animals by improving satiety, quietness and intestinal health (Williams *et al.*, 2001). It has been shown, however, that some types of DF may increase diarrhoea in piglets (Montagne *et al.*, 2003).

The bacterial growth supported by DF fermentation induces a shift of N excretion from urea in urine to bacterial protein in faeces and lowers the pH of the latter (Zervas and Zijlstra, 2002; Martinez-Puig *et al.*, 2003). The protein catabolism in the distal part of the colon and the NH_3 emission from the manure are therefore reduced (Nahm, 2003). The relationship between DF fermentability and N excretion shift is still poorly documented although the source of DF is suspected to influence the growth of the bacterial population (Kreuzer *et al.*, 1998; Zervas and Zijsltra, 2002).

The aim of the present study was to determine *in vitro*, the amount of protein synthesis by faecal microbes, when (1) starch and different sources of purified NSP, or (2) ingredients differing in DF content, are available as the energy source for microbial fermentation. The kinetics of fermentation and SCFA production were also measured in order to evaluate their relationship with microbial protein synthesis.

3. Materials and methods

1. Animals and diets

The experiments were carried out using three Belgian Landrace sows (weighing from 226 ± 12 to 257 ± 14 kg) as sources of bacterial inoculum. The animals were kept in one group and received daily, in two meals (8 am and 3 pm), 3 kg of a commercial diet (ZENA-D, Quartes, Deinze, Belgium) with the following chemical composition (g kg^{-1} DM): crude protein, 164; ash, 67; NDF, 253; ADF, 144; ADL, 29; total dietary fibre, 312. The sows had free access to water for the duration of the experiments. The collection of the faeces started after 4 weeks of adaptation to the diet.

2. Substrate

Experiment 1: Fermentation of purified carbohydrates

Five purified sources of fermentable carbohydrates were chosen according to their differences in soluble and insoluble fibre content, constituent sugars and glucosidic bonds (Table 9): potato starch (Fluka 85650), fibrous cellulose (Sigma C-6663), inulin (Fibruline, Cosucra, Warcoing, Belgium), citrus pectin (Sigma P-9135) and xylan from oat spelts (Fluka 95590).

Experiment 2: Fermentation of sources of fermentable carbohydrates

Sugar beet pulp (*Beta vulgaris*), raw potato (*Solanum tuberosum*) and wheat bran (*Triticum aestivum*), ground to pass a 1 mm-mesh screen by means of a Cyclotec 1093 Sample Mill (FOSS Electric A/S, Hilleroed, Denmark), were used as substrates for the *in vitro* fermentation.

Prior to fermentation, the substrates underwent an *in vitro* pepsin-pancreatin hydrolysis following the protocol of Boisen and Fernández (1997). In this method, 2 g samples were weighed in conical flasks. A phosphate buffer solution (100 ml, 0.1 M, pH 6.0) and an HCl solution (40 ml, 0.2 M) were poured into the flasks. The pH was adjusted to 2.0 with 1M HCl or 1M NaOH and 2 ml of a chloramphenicol solution (Sigma C-0378, 0.5 g 100 ml^{-1} ethanol) was added. Finally, a solution of pepsin (4 ml, 25 g l^{-1}, porcine pepsin : 2000 FIP-U/g, Merck n°7190) was added to

the mixture. The flasks were closed with a rubber stopper and placed for 2 h under gentle agitation in a water-bath at 39 ± 0.5°C.

Table 9. Chemical composition of the purified carbohydrate sources (Expt. 1) and the raw and the pepsin-pancreatin hydrolysed substrates[1] (Expt. 2) (g kg^{-1}DM)

	Experiment 1					Experiment 2					
	Purified carbohydrates					Raw substrates			Hydrolysed substrates		
	Starch	Cellulose	Inulin	Pectin	Xylan	Sugar beet pulp	Wheat bran	Potato	Sugar beet pulp	Wheat bran	Potato
DM (g kg^{-1}diet)	911	958	949	924	957	917	920	944	924	933	909
Ash	2	0	0	48	125	73	55	53	82	10	8
Crude protein	2	1	0	33	2	79	179	100	40	96	7
Fat	-	-	-	-	-	5	33	9	-	-	-
Starch	-	-	-	-	-	-	166	672	-	0	810
NDF	0	874	0	0	115	409	467	93	534	866	111
ADF	0	881	0	0	1	223	124	39	323	270	66
ADL	0	82	0	0	0	16	34	8	85	76	9
Insoluble dietary fibre	0	1000	9	3	328	531	408	99	705	849	101
Total dietary fibre	0	1000	34	893	795	682	491	128	896	904	173
Rhamnose	-	-	-	-	-	-	-	-	22	1	2
Fucose	-	-	-	-	-	-	-	-	1	0	0
Arabinose	-	0	-	-	58	-	-	-	185	127	7
Xylose	-	2	-	-	515	-	-	-	15	257	4
Mannose	-	1	-	-	0	-	-	-	13	4	2
Galactose	-	0	-	-	10	-	-	-	48	10	31
Glucose	768	858	38	-	43	-	-	-	237	181	10
Fructose	-	-	961	-	-	-	-	-	-	-	-
Galacturonic acid	-	-	-	672	-	-	-	-	111	-	-

[1] Hydrolysed substrates are the residues of the raw substrates after they have undergone a pepsin-pancreatin hydrolysis according to Boisen and Fernandez (1997).

After the pepsin hydrolysis, 40 ml of a phosphate buffer solution (0.2 M, pH 6.8) and 20 ml of a NaOH solution (0.6 M) were added. The pH was adjusted to 6.8

with 1M HCl or 1M NaOH and a solution of pancreatin (2 ml, 100 g l^{-1}, pancreatin: Sigma P-1750) was added. The flasks were then closed with a rubber stopper and placed for 4 h under gentle agitation in a water-bath at 39 ± 0.5°C. After hydrolysis, the residues were collected by filtration on a Nylon cloth (42 µm), washed with ethanol (2 x 25 ml 95 % ethanol) and acetone (2 x 25 ml 99.5 % acetone), dried for 24 h at 60 ± 1°C and weighed. The enzymatic hydrolysis was performed from 40 to 51 times (8 replicates x 6 or 7 periods, according to the substrate). The hydrolysis residues from the different replicates and periods were pooled for subsequent *in vitro* fermentation.

The dry matter disappearance (*dDM*) during the pepsin-pancreatin hydrolysis was calculated as follows:

$$(14) \qquad dDM = \frac{\text{weight of the sample before hydrolysis - weight of the residue}}{\text{weight of the sample before hydrolysis}}$$

The chemical compositions of the raw and hydrolysed substrates are detailed in Table 9.

In vitro fermentation

In vitro fermentation was performed using the gas test method described by Menke and Steingass (1988) and adapted to the pig by Bindelle *et al.* (2007). Briefly, an inoculum was prepared from fresh faeces of the three experimental sows. Faeces (50 g l^{-1}) were mixed to a buffer solution composed of salts and minerals (Menke and Steingass, 1988). The N source in the buffer solution (NH_4HCO_3) was replaced by an equimolar quantity of ^{15}N-labelled NH_4Cl (2% of enrichment, ISOTEC n°T85-70216, Miamisburg, Ohio, USA). The fermentation at 39 °C started when 200 mg of one of the substrates and 30 ml of the inoculum were introduced into 100 ml-glass syringes.

The experimental scheme was as follows:

- for Experiment 1: 5 substrates × 9 replicates + 3 blanks (containing only inoculum), repeated over 2 periods;
- for Experiment 2: 3 substrates × 9 replicates + 3 blanks, repeated over 3 periods.

The gas volumes of 3 syringes per substrate were recorded at regular intervals until 72 h. The 6 remaining syringes were stopped by quenching in an iced water-bath for 20 min, at half-time to asymptotic gas production, *T/2* according to the model of

France *et al.* (1993) presented below. At this moment, half of the final gas volume shown on Table 10 and Table 11 was produced in the syringes. . This time was determined during a preliminary fermentation run since it differed according to the substrate. At the half-time to asymptotic gas production, the rate of gas production and bacterial growth is in a linear phase, near its maximum.

The syringes were subsequently emptied and rinsed with distilled water (2 x 5 ml). The fermentation residue of three syringes were pooled and freeze-dried for further determination of residual sugars. The content of the 3 other syringes were centrifuged (12,000 g, 20 min, 4°C). An aliquot of the supernatant (approx. 10 ml) was taken for short-chain fatty acid (SCFA) analysis and the rest was discarded. The pellet was suspended in distilled water (30 ml) to dilute traces of ^{15}N-labelled NH_4Cl originating from the buffer, centrifuged (12,000 g, 20 min, 4°C) and the supernatant was discarded. The resulting pellet concentrating the bacteria and the undigested substrate was freeze-dried, weighed and analysed for total N and ^{15}N-enrichment. For each period, 3 samples of the inoculum were also taken, centrifuged for further ^{15}N and SCFA analysis.

Kinetics of gas production

Gas accumulation curves recorded during the 72 h of fermentation were modelled according to France *et al.* (1993) :

$$(15) \quad G = 0, \qquad \text{if } 0 < t < L$$

$$= G_f\left(1 - \exp\left\{-\left\langle b(t-L) + c\left(\sqrt{t} - \sqrt{L}\right)\right\rangle\right\}\right), \qquad \text{if } t \geq L$$

where G (ml g^{-1}DM) denotes the gas accumulation at time (t), G_f (ml g^{-1}DM) the maximum gas volume for $t = \infty$ and L (h) the lag time before the fermentation starts. The constants b (h^{-1}) and c ($h^{-1/2}$) determine the fractional rate of degradation of the substrate μ (h^{-1}), which is postulated to vary with time as follows:

$$(16) \quad \mu = b + \frac{c}{2\sqrt{t}}, \quad \text{if } t \geq L$$

The kinetics parameters (G_f, L, $\mu_{t=T/2}$ and $T/2$) were compared in the statistical analysis. $T/2$ is the half-time to asymptotic gas production when $G = G_f/2$. The syringes that suffered an accidental leakage of gas were discarded.

Measurement of SCFA production at half-time to asymptotic gas production

Supernatants prepared as described above were filtered using 0.2µm Nylon 13 mm HPLC Syringe Filter N°2166 (Alltech Associates Inc., Deerfield, IL, USA) and analysed for SCFA with a Waters 2690 HPLC system (Waters, Milford, MA, USA ; 30°C, with iso-caproic acid as the internal standard) fitted with a HPX 87 H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210 nm).

Measurement of N incorporation into microbial cells

Total N and ^{15}N-enrichment in the freeze-dried pellets were measured by means of an elemental analyser coupled to an isotope-ratio mass spectrometer (Europa Scientific Ltd, Crewe, UK). Bacterial N incorporation (BNI corresponding to N in the pellet incorporated from the buffer solution into the bacteria), per amount of incubated substrate at *T/2* was calculated as follows:

$$\text{BNI}\,(\text{mg g}^{-1}\text{DM}) = \left(\frac{\left(\frac{^{15}\text{N} \times \text{N} \times M_{\text{pellet}}}{0.003663} \right) - \text{N} \times M_{\text{pellet}}}{\left(\frac{0.02}{0.003663} - 1 \right) \times W} \right) - \text{BNI}_{\text{inoculum}} \times \frac{V_0}{W} \qquad (17)$$

where N (g g^{-1}) denotes the concentration of N in the pellet, M_{pellet} (mg) the dry weight of the pellet, 0.003663 the natural enrichment in ^{15}N of the substrates and the faeces used to prepare the inoculum, 0.02 the enrichment of the mineral buffer in ^{15}N, ^{15}N (g g^{-1}) the concentration of ^{15}N in total N of the pellet, V_0 (ml) the volume of inoculum transferred in the syringe at the start of the fermentation and W (g DM) the amount of substrate placed in the syringe.

Chemical analysis

The raw and hydrolysed substrates and the diet, ground to pass a 1 mm-mesh screen by means of a Cyclotec 1093 Sample Mill (FOSS Electric A/S, Hilleroed, Denmark), were analysed for their content in dry matter (105 ºC for 24 h), ash (550 ºC for 8 h), nitrogen (Kjeldahl method, crude protein = 6.25 × N content), ether extract (Soxhlet method, using ether), NDF (using Na_2SO_3 and Termamyl, Novo Nordisk, Bagsværd, Denmark) and ADF and lignin, using the Fibercap system (Foss Electric, Bagsvaerd, Denmark). Starch was determined using amyloglucosidase according to the method of Faisant *et al.* (1995). Total and soluble dietary fibre contents were measured by means of the AOAC 991.43

method (AOAC International, 1995), after grinding the samples through a 0.5 mm-mesh screen. Constituent sugars of cellulose and xylan were determined as alditol acetates by gas-liquid chromatography (Englyst *et al.*, 1992). The glucose and fructose contents of inulin and the uronic acid content of pectin were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex DX500 chromatrography system (Dionex Corp., Sunnyville, CA, USA) after enzymatic hydrolysis with endo and exo-inulinase (50°C, 24h) and Viscozyme (Realco, Louvain-la-Neuve, Belgium, 50°C, 15h) respectively.

Statistical analysis

Statistical analyses were performed using the MIXED procedure of the SAS 8.02 software (SAS, 1999) using the following general linear model :

- for Experiment 1 :

$$(18) \qquad Y = \alpha + S_i + P_j + \varepsilon$$

where Y is the result, α the mean, S_i the fixed effect of the substrate (i = 1, ..., 5), P_j the random effect of the period (j = 1, 2) and ε the error term.

- for Experiment 2 :

$$(19) \qquad Y = \alpha + S_i + P_j + \varepsilon$$

where Y is the result, α the mean, S_i the fixed effect of the substrate (i = 1, 2, 3), P_j the random effect of the period (j = 1, 2, 3) and ε the error term.

4. Results

1. Experiment 1

The gas accumulation curves recorded during the fermentation of the purified carbohydrates are illustrated in Figure 7 and fermentation kinetics parameters, BNI and SCFA productions of the 5 carbohydrates are shown in Table 10. With the lowest lag (*L*) and half-time to asymptotic gas production (*T/2*), inulin, starch and pectin were the most rapidly fermented substrates ($P<0.001$). Inulin and cellulose showed lower fractional rates of degradation, compared to starch, pectin and xylan.

The final gas production (G_f) also differed between the substrates (P<0.001): pectin yielded the highest production and xylan the lowest.

Sugar disappearance ranged from 0.8 to 0.9 for inulin, pectin and starch. This indicates that almost all the substrate initially present in the syringe was fermented at *T/2* for these fibre sources. For cellulose and xylan, only half of the substrate had been fermented when the fermentation was stopped at *T/2*. Bacterial N incorporations (BNI) measured at *T/2* were higher (P<0.05) for inulin and starch when compared to xylan, cellulose and pectin. The SCFA production for starch and inulin was higher compared to that of pectin, xylan and cellulose (P<0.025). However, cellulose and xylan produced more SCFA per g of fermented sugars compared to starch and inulin. Pectin yielded the lowest SCFA per g of fermented sugars. The molar ratio of SCFA showed that a higher proportion of acetate was produced for pectin and xylan compared to the other carbohydrates.

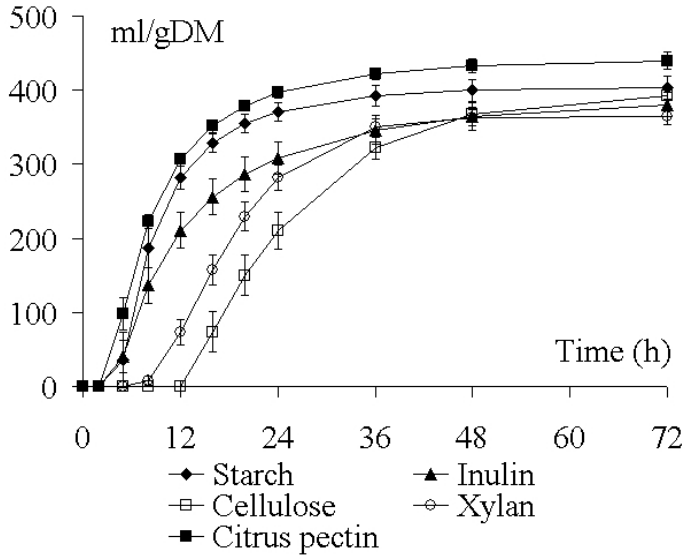

Figure 7. Mean values and standard deviations of the gas production curves recorded during the fermentation of purified carbohydrates incubated with sow faecal inoculum (Experiment1).

Table 10. Kinetics parameters of the gas accumulation curves recorded for the purified carbohydrates incubated with sows faecal inoculum and sugars disappearance, bacterial nitrogen incorporation (BNI), total short chain fatty acid (SCFA) production and molar ratios at half-time to asymptotic gas production (Expt.1).

	Substrates					*Source of variation* Substrate		*Variance parameter estimates*	
	Starch	Inulin	Cellulose	Xylan	Citrus pectin	d.f.[1]	P-values	Period	Residual
Kinetics parameters									
N[2]	6	6	6	5	6				
L[3] (h)	4.5 c[4]	4.1 cd	12.7 a	6.5 b	3.6 d	4	***	0.77	0.39
$T/2$[5] (h)	8.5 d	11.1 c	23.0 a	17.3 b	8.0 d	4	***	1.52	2.00
$\mu_{t=T/2}$ [6](h^{-1})	0.153 a	0.081 d	0.074 d	0.104 c	0.133 b	4	***	1.1E-5	16.0E-5
G_f [7](ml g^{-1}DM)	405 b	393 c	396 bc	365 d	443 a	4	***	184.0	76.2
N	2	2	2	2	2				
Sugar disappearance[8]	0.809 a	0.901 a	0.562 b	0.458 b	0.909 a	4	**	0	14.3
N	6	6	6	6	6				
BNI (mg g^{-1}DM incubated)	18.5 a	20.7 a	5.7 b	5.8 b	8.8 b	4	**	1.59	4.15
N	2	2	2	2	2				
BNI (mg g^{-1} fermented substrate)	22.9 a	23.2 a	11.3 b	12.3 b	9.8 b	4	*	2.99	9.03
N	6	6	6	6	6				
SCFA (mg g^{-1}DM incubated)	365 a	369 a	285 b	257 b	263 b	4	*	0	618
N	2	2	2	2	2				
SCFA (mg g^{-1} fermented substrate)	451 b	411 b	565 a	562 a	290 c	4	**	0	942
Molar ratio of acetic/propionic/butyric	50:45:5	47:47:5	44:55:1	63:32:5	81:18:1				

[1] d.f., degrees of freedom; [2] Number of observations; [3] L, lag time (h); [4] For one parameter, means followed by different letters in the columns differ at a significance level of 0.05; [5] $T/2$, half-time to asymptotic gas production (h); [6] $\mu_{t=T/2}$, fractional rate of degradation at t = $T/2$ (h^{-1}); [7] G_f, maximum gas volume (ml g^{-1} DM); [8] Proportion of sugars disappeared from the syringe content at half-time to asymptotic gas production.

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2. *Experiment 2*

The gas accumulation curves recorded during fermentation of the hydrolysed feedstuffs are shown in Figure 8 and kinetics parameters, BNI and SCFA productions are given in Table 11.

Potato yielded the greatest final gas volume ($P<0.001$) followed by sugar beet pulp and wheat bran. The latter showed a lower fractional rate of degradation compared to potato and sugar beet pulp ($P = 0.008$) and had the shortest lag and the earliest half-time to asymptotic gas production ($P<0.001$).

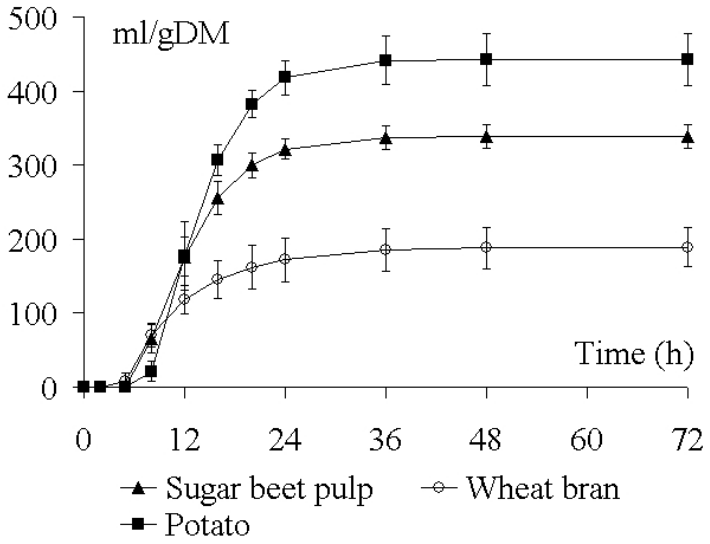

Figure 8. Mean values and standard deviations of the gas production curves recorded during the fermentation of pepsin-pancreatin hydrolysed feedstuffs incubated with sow faecal inoculum (Experiment 2).

The rate of sugar disappearance at *T/2* ranged from 0.17 for wheat bran to 0.50 for potato. BNI expressed per g of incubated substrate was the highest for potato and sugar beet pulp compared to wheat bran ($P<0.01$). When BNI was expressed per g of fermented sugars, the difference observed between wheat bran, potato and sugar beet pulp became insignificant due to a reduction in the number of observations. Conversely, in Experiment 1, there was a difference in BNI between the substrates, even when expressed per g of fermented sugars. SCFA productions per g of incubated substrate were the highest for potato followed by sugar beet pulp and wheat bran ($P<0.001$).

Wheat bran yielded higher SCFA per g fermented sugars, compared to sugar beet pulp and potato ($P<0.05$). The molar ratio also differed between the substrates: potato yielded more butyrate, and sugar beet pulp and wheat bran produced more acetate. Branched SCFA were not detected during SCFA analysis

Table 11. Dry matter disappearance (dDM) during the pepsin-pancreatin hydrolysis, kinetics parameters of the gas accumulation curves recorded for the hydrolysed feedstuffs incubated with sows faecal inoculum and sugars disappearance, bacterial nitrogen incorporation (BNI), total short chain fatty acid (SCFA) production and molar ratios at half-time to asymptotic gas production (Expt. 2).

	Substrates			*Source of variation* Substrate		*Variance parameter estimates*	
	Potato	Sugar beet pulp	Wheat bran	d.f.[1]	P-values	Period	Residual
N[2]	51	43	40				
dDM	0.46 b[3]	0.38 c	0.57 a	2	***	0.673	7.720
Kinetics parameters							
	6	6	6				
L[4] (h)	7.3 a	5.1 b	4.7 b		***	2.6	1.6
$T/2$[5] (h)	13.9 a	12.0 b	10.6 c		***	0.67	0.29
$\mu_{t=T/2}$[6] (h^{-1})	0.166 a	0.152 a	0.128 b		***	0.00E-4	5.23E-4
G_f[7] (ml g^{-1}DM)	443 a	338 b	189 c		***	215	545
N	3	3	3				
Sugar disappearance[8]	0.50 a	0.40 a	0.17 b		**	0	34.7
N	9	9	9				
BNI (mg g^{-1}DM incubated)	8.3 a	7.4 a	1.7 b		**	6.43	1.75
N	3	3	3				
BNI (mg g^{-1} fermented substrate)	17.0	18.3	10.2		NS	39.9	30.8
N	9	9	9				
SCFA (mg g^{-1}DM incubated)	275 a	249 b	151 c		***	26	113
N	3	3	3				
SCFA (mg g^{-1} fermented substrate)	556 b	641 b	913 a		*	0	8,56
Molar ratio of acetic/propionic/butyric	48:22:30	66:31:03	60:36:04				

[1] d.f., degrees of freedom; [2] Number of observations; [3] For one parameter, means followed by different letters in the columns differ at a significance; [4] L, lag time (h) level of 0.05; [5] $T/2$, half-time to asymptotic gas production (h); [6] $\mu_{t=T/2}$, fractional rate of degradation at t = $T/2$ (h^{-1}); [7] G_f, maximum gas volume (ml g^{-1} DM); [8] Proportion of sugars disappeared from the syringe content at half-time to asymptotic gas production.

3. Correlation and regression

Correlation and regression were calculated throughout Exp. 1 and 2. When pectin was discarded from the database, BNI (mg g^{-1} sugar fermented) was correlated to the half-time of asymptotic gas production (*T/2*) (r = -0.61, P = 0.143) and to SCFA (mg g^{-1} sugar fermented) (r = -0.72, P = 0.068) with levels approaching significance. BNI was also correlated to sugar disappearance (r = 0.80, P = 0.029), to insoluble dietary fibre (IDF) (Table 9) (r = -0.77, P = 0.043) and to soluble dietary fibre (SDF; starch included in SDF) content of the substrates (r = 0.79, P = 0.039). A regression equation was calculated linking BNI to SCFA and to *T/2*. The equation is : BNI (mg g^{-1} sugar fermented) = 40.7 – 0.71 T/2 (h) – 0.247 SCFA (mg g^{-1} sugar fermented) (r^2 = 0.91, P = 0.004).

5. Discussion

The results clearly indicate that the source of dietary carbohydrate influences bacterial protein synthesis during colonic fermentation in pigs. The primary function of microbial carbohydrate fermentation is to release ATP, which is required for bacterial cell basal and growth metabolism (Karsli and Russell, 2001). BNI will thus depend on the efficiency of ATP generation from the substrate and on the use of this ATP for the maintenance or growth of the bacteria. The rate of fermentation of carbohydrates depends on their composition and on their structure. Generally, solubility is also linked to rapid fermentation (Hoover and Strokes, 1991) since the swelling and the high water binding capacity of SDF increase the surface area accessible to bacteria. This is highlighted in this study by the trends towards negative and positive correlation linking BNI to IDF and to SDF (starch included in SDF), respectively.

Two major steps are involved: the hydrolysis of the polysaccharide and the fermentation of the released oligo- and monosaccharides.

BNI is firstly a consequence of the hydrolysis kinetics. As indicated by the negative correlation between BNI and *T/2,* high BNI were found among the substrates with high fermentation rates as measured by the low *L* and *T/2* values and the high $\mu_{t=T/2}$ (Table 10 and Table 11) : inulin and starch in Experiment 1 and sugar beet pulp and potato in

Experiment 2. Cellulose, xylan, and wheat bran had lower rates of fermentation and yielded lower BNI values. Hydrolysis is the rate-limiting step of microbial fermentation for cellulose (Noike *et al.*, 1985; Lynd *et al.*, 2002), xylans and arabinoxylans (Strobel and Russell, 1986; Hopkins *et al.*, 2003), conversely to starch, inulin and pectin. When hydrolysis is slow, more energy is required for cell maintenance per unit of time and less energy is available for growth.

The differences in the use of the energy coming from fermentation of substrates for bacterial growth is also reflected by the amount of SCFA produced per g of fermented substrate (Blümmel *et al.*, 1997). When pectin was discarded from the database, BNI values tended to be negatively correlated to SCFA production ($r = -0.72$, $P = 0.068$). Higher SCFA productions for cellulose and xylan compared to inulin and starch indicate that a lower proportion of the energy content of the substrate was used for growth, since this energy is found in the SCFA. This observation is also applicable when sugar beet pulp, potato and wheat bran are compared in Experiment 2.

Once the polysaccharides are hydrolysed, the chemical composition of the substrates also influences the pathways of SCFA production, ATP generation and, as a consequence, the BNI. The released monosaccharides support microbial growth with little differences in efficiency (Hoover and Stokes, 1991) since the intestinal bacteria use mainly the Embden-Meyerhof-Parnas pathway, also known as glycolysis, that degrades glucose to pyruvate via glucose-6-phosphate (Prescott *et al.*, 1996), for ATP production (Miller and Wolin, 1996). However, xylans and pectins are first metabolised by Pentose-phosphate pathway (Macfarlane and Macfarlane, 2003) starting from the pentose to fructose-6-phosphate and glyceraldehyde-3-phosphate via xylulose-5-phosphate (Prescott *et al.*, 1996). This reduces the bacterial growth rates as reported by Crittenden *et al.* (2002) who compared xylose and xylo-oligosaccharides to glucose. It explains, beyond the rate of hydrolysis, the lowest BNI values observed for xylan (515 g xylose kg^{-1}DM) and pectin (672 g glucuronic acid kg^{-1}DM) in Experiment 1 and wheat bran (251 g xylose kg^{-1}DM) in Experiment 2.

The SCFA profiles confirm that different fermentation strategies were used by the bacteria. The production of acetate and butyrate yields 34 mmol ATP g^{-1} SCFA compared to 40 mmol ATP g^{-1} of propionate (Blümmel *et al.*, 1997). Acetate is typical

of fermentation under C-limiting conditions (Macfarlane and Macfarlane, 2003). The high production of acetate observed with pectinolytic and xylanolytic bacteria indicate that they recover less energy from the substrate compared to bacteria fermenting starch or fructans which use faster and more energy efficient cycles, leading to higher propionate production. In the case of xylan and wheat bran, high acetate levels are a consequence of low hydrolysis rates that limit the release of monosaccharides. For pectin, this is due to the production of extracellular pectinolytic enzymes (Drochner *et al.*, 2004). The extracellular release of oligosaccharides benefit microbial competitors and reduce the growth of pectinolytic bacteria. On the contrary, *Bacteroides*, the most abundant saccharolytic genus in the intestine, bind starch to their outer membrane prior to hydrolysis, which prevents any competition with other bacteria (Hooper *et al.*, 2002). The low BNI yield recorded for pectin, despite a low *T/2*, a high $\mu_{t=T/2}$ and low SCFA production, was an exception compared to the other substrates. This is due to the high degree of methoxylation of the pectin used in this study (Sigma-Aldrich, 2006). Methoxylation decreases the growth rate of pectin-fermenting bacteria (Olano-Martin *et al.*, 2002). The methyl group can be esterified with a release of methanol (Drochner *et al.*, 2004) but, unlike glucuronic acid, the methyl group is unusable by pectinolytic bacteria (Hall and Herejk, 2001) for ATP generation and growth.

The N content of pectin (32.5 g crude protein kg^{-1}DM) and that of the hydrolysed wheat bran (96.0 g kg^{-1}DM) decreased the mineral N uptake from the buffer solution compared to the other fast-fermenting substrates. Intestinal bacteria use ammonia as a major source of nitrogen (Younes *et al.*, 1995). However, microbes that ferment substrates with high levels of crude protein recycle the nitrogen content of the substrate protein (Hoover and Stokes, 1991; Cone *et al.*, 2005).

Finally, the *in vitro* classification of potato, sugar beet pulp and wheat bran in terms of potential impact on urinary vs. faecal N excretion patterns should be combined with *in vivo* data such as the retention time of the feed in the gastrointestinal tract. In this study, fermentation was stopped at T/2. This time differs from *in vivo* residence time in the large intestine.

It can be concluded that the rate of fermentation of the carbohydrates and the chemical composition, the soluble fibre content, the constituent sugars and the crude protein

content, were the major factors that affected bacterial energy production, BNI and SCFA production by the colonic bacteria of pigs. These *in vitro* observations need to be confirmed through *in vivo* experiments.

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CHAPTER VI

Article 5:

Influence of source and levels of dietary fiber on in vivo nitrogen excretion pathways in pigs and in vitro fermentation and protein synthesis by fecal bacteria[1]

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Running head: Intestinal bacteria growth and N excretion in pigs

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Abbreviations:
DF, dietary fiber; HF-S, high fiber, soluble diet; INS, insoluble fiber diet; INT, intermediate fiber diet; MF, medium fiber diet; NDF, neutral detergent fiber; OH, oat hulls; SBP, sugar beet pulp; SD, standard diet; VHF, very high fiber diet.

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1. Abstract

The inclusion of fermentable dietary fiber (DF) in diets is a way to reduce ammonia emission in pig barns because it contributes to shift N excretion from urine to feces. However, it is unclear whether this shift is due or not to an increase in bacterial fecal biomass. This study compared *in vitro* bacterial protein synthesis to *in vivo* N excretion shift with diets differing in DF levels and types. A first experiment measured the effect of graded levels of sugar beet pulp (SBP) (0, 10, 20 and 30%) in corn-soybean meal-based diets on *in vivo* N excretion partitioning between urine and feces. A second experiment studied the consequences of different ratios of SBP and oat hulls (OH), used as sources of fermentable and non-fermentable carbohydrates, respectively, in the diets. In parallel, the characteristics of fermentation of the indigestible carbohydrates was evaluated in an *in vitro* gas test, based on the incubation with colonic microbiota, using a mineral buffer solution enriched with ^{15}N. Mineral N incorporation in the bacterial proteins (BNI) was measured when half of the final gas volume was produced (*T/2*: 8.5 to 14.5 h of fermentation) and after 72 h of fermentation. Short-chain fatty acids (SCFA) were determined in the liquid phase. The N retention in pigs was not influenced by the diet ($P>0.597$). In the first experiment, the inclusion of SBP decreased urinary N excretion from 0.285 to 0.215 g urinary N/g N ingested ($P<0.01$) and urinary-N:fecal–N excretion ratio from 2.171 to 1.177 ($P<0.001$) linearly. In the second experiment, urinary and fecal N excretions remained unchanged by the incorporation of OH ($P>0.05$). Unlike SCFA production, BNI was higher at *T/2* than at 72 h of fermentation. SBP enhanced BNI ($P<0.001$): 2.01, 2.06 and 2.35 mg N g^{-1} dietary N with 10, 20 and 30 % SBP, respectively, as compared to 1.51 mg for the control diet. OH inclusion in the diet decreased BNI ($P<0.01$). BNI was correlated to fermentation kinetics, i.e. *T/2* ($r = -0.937$, $P = 0.006$) and fractional rates of degradation ($r = 0.844$, $P = 0.025$). *In vitro* kinetics parameters and BNI were correlated to *in vivo* N excretion parameters. Bacterial N resulting from fermentation accounted for 40 to 46 % of the fecal N excretion with SBP-based diets. This proportion dropped to 22 % when OH was the main DF source. In conclusion, highly fermentable DF in pig rations

contributes to increase bacterial protein excretion in feces at the expense of urinary N excretion.

Keywords: pig, dietary fiber, nitrogen excretion, fermentation, bacterial protein synthesis.

2. Introduction

Increasing attention is currently paid to dietary fiber (DF) fermentation in the pig large intestine for its capacity to reduce ammonia emission from pig barns (reviewed by Bindelle *et al.*, 2008) and because fibrous feedstuffs are now incroporated in swine rations (Leterme *et al.*, 2006). Non-digestible carbohydrates are the major energy source for gut bacteria and their fermentation induces a shift of N excretion from urea in urine to feces (Mosenthin *et al.*, 1992; Zervas and Zijlstra, 2002), a reduction in protein catabolism in the distal part of the colon (Pastuszewska *et al.*, 2000) and an acidification of the feces consecutive to the short-chain fatty acid (SCFA) production (Canh *et al.*, 1998). Ammonia emission during manure storage and handling are thereby reduced (Nahm, 2003). The N excretion shift also improves N recycling by plants in integrated livestock-cropping systems (Rufino *et al.*, 2006).

However, the relationship between DF nature and intake, the shift in N excretion and bacterial protein synthesis in the colon is still unclear. A previous *in vitro* fermentation experiment with colonic inoculum showed that bacterial protein synthesis is enhanced by the rate of DF fermentation and the level of soluble DF in the diet (Bindelle *et al.*, 2007). The present study aimed to confirm these observations *in vivo* and to estimate the contribution of bacterial protein synthesis to total fecal N excretion.

3. Materials and methods

1. Total Tract in vivo Digestibility

Animals

A total of 32 Belgian Landrace x Piétrain castrated pigs, weighing on average 32 ± 2 kg at the beginning of the study and from 46 to 54 ± 3 kg at the beginning of the second period of the experiment, were used. They were divided in two equal groups, corresponding to two experiments. The protocols were approved by the Ethical Committee of the Belgian Council for Laboratory Animal Science (Lab reference: LA19000-46, protocol: CRA06/01).

Diets

In Experiment 1, 4 diets were formulated in order to contain graded levels of SBP (0, 10, 20 and 30%) at the expense of corn and soybean meal (Table 12). In Experiment 2, 4 diets were prepared in order to contain similar proportions of total DF but different insoluble:soluble ratios, by means of SBP (source of soluble DF) and oat hulls (OH, insoluble DF) (Table 12).

All the diets were formulated to contain similar levels of digestible energy, net energy, crude protein and ileal digestible protein to the pigs.

Methodology

The same methodology was used for Experiments 1 and 2.

The experimental scheme was as follows: 4 pigs × 4 diets × 2 periods.

After a 2 week-adaptation period to the diet, the pigs (4 per diet) were placed in individual metabolism cages designed to collect urine (using 1 M H_2SO_4) and feces separately.

During the 5 d-collection period, the animals received 90 g $kg^{-0.75}$ d^{-1} in two meals (8 am and 3 pm), mixed with an equal amount of fresh water. Feces and urine were totally collected every day and weighed. An aliquot representing 0.1 of the daily excretion was kept at -18°C. The refusals were also collected, weighed and kept at -18°C.

At the end of the experiment, the animals were randomly assigned to another experimental diet and the procedure was repeated.

Analyses

Refusals, diets and feces were freeze-dried, ground to pass through a 1 mm-mesh screen by means of a Cyclotec 1093 Sample Mill (FOSS Electric A/S, Hilleroed, Denmark) and analyzed for their content in DM (105 ºC for 24 h, AOAC 967.03 method), ash (550 ºC for 8 h AOAC 923.03), nitrogen (Kjeldahl method, crude protein = 6.25 × N content; AOAC 981.10), ether extract (Soxhlet method, using diethyl ether; AOAC 920.29) and NDF (using Na_2SO_3 and Termamyl, Novo Nordisk, Bagsværd, Denmark; Van Soest *et al.*, 1991) using the Fibercap system (Foss Electric, Bagsvaerd, Denmark). Urine was analyzed for nitrogen content (Kjeldahl method).

The diets were also analyzed for ADF and ADL (Van Soest *et al.*, 1991), starch (Faisant *et al.*, 1995), total, soluble and insoluble DF contents (AOAC 991.43 method after grinding the samples through a 0.5 mm-mesh screen).

Calculations and Statistics.

The apparent digestibilities and N retentions were calculated by difference between the amount of nutrients ingested and excreted.

The statistical comparison of the treatments was performed by means of the MIXED procedure of the SAS 8.02 software (SAS, 1999), using the following general linear model:

$$Y = \alpha + S_i + P_j + S_i \times P_j + A_k + \varepsilon \quad (20)$$

where Y is the result, α the mean, S_i the fixed effect of the diet (i = 1, ..., 4), P_j the fixed effect of the period (j = 1, 2), A_k the random effect of the pig (k = 1,...,16) and ε the error term. The weight of the pig was used as covariable in the model.

Classification of means was performed using the LSMEANS option and orthogonal contrasts in the MIXED procedure.

Table 12. Composition (g kg^{-1} diet) and analysis of the diets (g kg^{-1}DM).

	Standard (SD)	Medium fibre (MF)	High fibre, soluble (HF-S)	Very high fibre (VHF)	Intermediate (INT)	Insoluble (INS)
	Expt. 1, 2	Expt. 1	Expt. 1, 2	Expt. 1	Expt. 2	Expt. 2
Composition						
Maize	752.0	630.0	500.0	340.0	455.0	375.1
Sugar beet pulp	-	100.0	200.0	300.0	105.0	-
Oat hulls	-	-	-	-	105.0	225.0
Soybean meal	198.7	190.0	185.0	175.0	200.0	195.0
Soybean oil	-	5.0	25.0	42.0	50.0	65.0
Skimmed milk powder	-	30.0	50.0	100.0	45.0	100.0
Mineral and vitamin premix[1]	20.0	20.0	20.0	20.0	20.0	20.0
Salt	3.0	3.0	3.0	3.0	3.0	3.0
Calcium carbonate	6.3	3.0	-	-	-	-
Dicalcium phosphate dihydrate	19.8	18.8	16.8	20.0	16.8	16.8
L-Tryptophan	0.2	0.2	0.2	0.01	0.02	0.01
Analysis						
DM (g kg^{-1}diet)	890.8	895.8	902.6	903.3	901.3	904.1
Ash	72.4	76.3	77.5	88.2	75.3	76.8
Crude protein	174.7	174.6	171.3	171.3	170.2	169.9
Fat	38.6	43.3	65.8	84.4	87.9	116.5
Starch	525.0	441.5	360.7	257.7	321.1	280.3
NDF	71.1	102.9	120.3	152.4	156.8	204.6
ADF	29.6	50.8	68.6	91.9	89.3	111.0
ADL	2.4	3.6	5.8	7.7	8.4	14.6
Total dietary fibre	107.7	163.1	215.5	281.5	233.1	235.4
Insoluble dietary fibre	99.1	133.8	168.3	208.2	207.1	226.7
Soluble dietary fibre	8.6	29.3	47.2	73.3	26.0	8.7

[1] Mineral and vitamin premix, 2507 VAPOR 220 LMT GREEN (Trouw Nutrition, Ghent , Belgium).

2. In vitro enzymatic Hydrolysis and Fermentation

The diets tested in Experiments 1 and 2 were treated simultaneously during the *in vitro* part of this study. The method has been described in detail by Bindelle *et al.* (2007). Briefly, samples were treated with pepsin and pancreatin according to the method of Boisen and Fernandez (1997). The dry matter disappearance (dDM_{vitro}) during the enzymatic hydrolysis was calculated. Subsequently, the residue was incubated in glass syringes using a bacterial inoculum prepared with a buffer solution (Menke and

Steingass, 1988) and a mix of feces of 3 pigs fed with the INT diet, containing SBP and OH (Table 12). ^{15}N-labeled NH_4Cl (2% of enrichment, ISOTEC n°T85-70216, Miamisburg, Ohio, USA) was used as sole N source in the buffer solution. In a preliminary step, the incubation lasted for 72 h. The half time to asymptotic gas production (*T/2*), corresponding to the time required to produce 50% of the total gas production, was then estimated. The procedure was repeated for 3 different periods and the incubation was stopped at T/2 (from 8.5 to 14.5h, depending on the diets (see Results)) and 72 h, according to the syringe.

The experimental scheme was as follows: (9 syringes × 6 diets + 3 blanks) × 3 periods. Per diet and per period, 6 syringes were stopped at *T/2* by quenching in an iced water-bath for 20 min. The 3 syringes remaining were stopped after 72 h and their gas volumes were recorded at regular intervals.

When the syringes were stopped, they were subsequently emptied and rinsed with distilled water (2 x 5 ml). The fermentation broth of 3 syringes stopped after *T/2* h of fermentation were pooled and freeze-dried for further determination of starch and total DF in the residue. The content of the 3 other syringes stopped at *T/2* and the 3 syringes stopped after 72 h of fermentation were centrifuged (12,000 g, 20 min, 4°C) and an aliquot of the supernatant was taken for short-chain fatty acid (SCFA) analysis. The pellet was suspended in distilled water (30 ml), centrifuged (12,000 g, 20 min, 4°C) and the supernatant discarded. The resulting pellet concentrating the bacterial and the undigested substrate was freeze-dried, weighed and analyzed for total N and ^{15}N-enrichment.

For each period, 6 samples of the inoculum were also taken for ^{15}N, SCFA and starch and total DF analysis.

Kinetics of Gas Production

Gas accumulation curves recorded during the 72 h of fermentation were modeled according to France *et al.* (1993):

$$(21) \quad G = 0, \qquad \text{if } 0 < t < L$$

$$= G_f\left(1 - \exp\left\{-\left\langle b(t-L) + c\left(\sqrt{t} - \sqrt{L}\right)\right\rangle\right\}\right), \qquad \text{if } t \geq L$$

where G denotes the gas accumulation, G_f (ml g^{-1}DM) the maximum gas volume for $t = \infty$ and L (h) the lag time before the fermentation starts. The constants b (h^{-1}) and c ($h^{-1/2}$) determine the fractional rate of degradation of the substrate μ (h^{-1}), which is postulated to vary with time as follows:

$$(22) \qquad \mu = b + \frac{c}{2\sqrt{t}}, \quad \text{if } t \geq L$$

The kinetics parameters (G_f, L, $\mu_{t=T/2}$ and $T/2$) were compared in the statistical analysis (Figure 9).

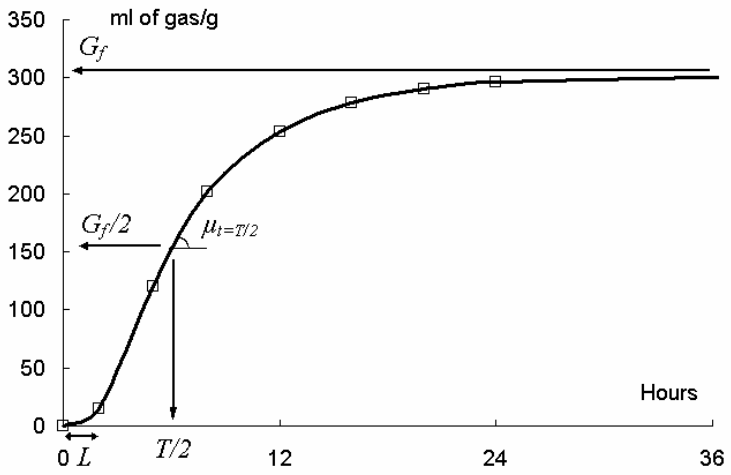

Figure 9. Representation of the kinetics parameters of the gas accumulation curves modeled according to France *et al.* (1993)

$T/2$ is the half-time to asymptotic gas production when $G = G_f/2$. At this time, the rate of gas production is in a linear phase, near its maximum. The syringes that suffered an accidental leakage of gas were discarded.

Measurement of SCFA Production

Supernatants prepared as described above were filtered using 0.2 µm Nylon 13 mm HPLC Syringe Filter N°2166 (Alltech Associates Inc., Deerfield, IL, USA) and analyzed for SCFA with a Waters 2690 HPLC system (Waters, Milford, MA, USA ; 30°C, with iso-caproic acid as the internal standard) fitted with a HPX 87 H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210 nm).

Measurement of N Incorporation into Microbial Cells

Total N and ^{15}N-enrichment in the freeze-dried pellets were measured by means of an elemental analyser coupled to an isotope-ratio mass spectrometer (Europa Scientific Ltd, Crewe, UK). Bacterial N incorporation (BNI corresponding to N in the pellet incorporated from the buffer solution into the bacteria) per g of diet and per g of actually fermented polysaccharides was calculated from total N and ^{15}N content according to the equations of Bindelle *et al.* (2007).

The bacterial N incorporation in feces consecutive to the fermentation of the fibrous fraction of the diet was estimated as follows:

$$(23) \qquad \text{Fecal bacterial N (g g}^{-1}) = \frac{BNI \times 6.25}{(1 - dCP) \times CP}$$

where *BNI* (g g^{-1}DM) denotes the bacterial N incorporation per g of diet, 6.25 the conversion factor of N into crude protein, *dCP* the apparent fecal digestibility of the crude protein and *CP* the crude protein content of the diet.

Statistical Analysis

Statistical analyses were performed using the MIXED procedure of the SAS 8.02 software (SAS, 1999) using the following general linear model:

$$(24) \qquad Y = \alpha + S_i + P_j + \varepsilon$$

where Y is the result, α the mean, S_i the effect of the substrate (i = 1, ..., 6), P_j the random effect of the period (j = 1, 2, 3) and ε the error term.

Classification of means was performed using the LSMEANS option and orthogonal contrasts in the MIXED procedure. Pearson's correlations and linear regressions calculations were performed using the CORR and the REG procedures of the SAS 8.02 software (SAS, 1999).

4. Results

1. Total Tract In vivo Digestibility

The pigs behaved normally and remained in good health during both experiments, with the exception of two pigs that were discarded for leg problems.

The digestibilities of the diets, the N retentions and N excretions obtained in Experiments 1 and 2 are detailed in Table 13 and Table 14.

The N retention values were not affected by the treatment ($P = 0.597$). However, in Experiment 1, the N excretion balance was modified: N excretion in urine ($P = 0.005$) and the urinary-N:fecal-N excretion ratio ($P<0.001$) decreased linearly ($r^2 = 0.87$, $P<0.05$) when graded levels of SBP were added to the diets. In Experiment 2, the OH content in the diets had no influence as compared to the standard diet on N excretion in urine ($P = 0.436$) and urinary-N:fecal-N excretion ratio ($P = 0.164$), unlike SBP which did ($P<0.01$). When pooling the data of both experiments, linear equations linking the N excretion in urine as well as the urinary-N:fecal-N excretion ratio to the SBP content of the diets were calculated ($r^2 = 0.84$, $P<0.011$).

Table 13. Digestibility and N retention and excretion pathways of the diets with increasing sugar beet pulp content (Experiment 1).

Item	Diets				SEM	Source of variation			
	Standard (SD)	Medium fibre (MF)	High fibre (HF-S)	Very high fibre (VHF)		Diet	Period	Diet x Period	Weight
n[1]	8	8	7	8					
dDM_{vivo}	0.870[a2]	0.852[b]	0.842[bc]	0.832[c]	4.18E-3	0.003	0.080	0.468	0.028
dOM	0.899[a]	0.882[b]	0.872[bc]	0.865[c]	3.57E-3	0.001	0.091	0.641	0.026
dEE	0.510[b]	0.550[b]	0.654[a]	0.673[a]	1.71E-2	0.025	0.460	0.195	0.286
dNDF	0.509[d]	0.640[c]	0.707[b]	0.789[a]	2.00E-2	0.001	0.362	0.592	0.145
dCP	0.865[a]	0.831[b]	0.818[b]	0.815[b]	6.61E-3	0.002	0.138	0.308	0.023
N-retention (g retained g-1 diet)	0.580	0.571	0.592	0.566	7.70E-3	0.597	0.612	0.660	0.772
Urinary-N excretion (g in urine g-1 diet)	0.285[a]	0.261[a]	0.223[b]	0.215[b]	6.79E-3	0.005	0.105	0.646	0.106
Urinary-N : faecal-N (g in urine g-1 in faeces)	2.171[a]	1.646[b]	1.243[c]	1.177[c]	9.61E-2	< 0.001	0.020	0.292	0.007

[1] n, number of observations.
[2] Within a row, means without common superscript letter differ ($P < 0.05$).

Table 14. Digestibility and N retention and excretion pathways of the diets with differing oat hulls/sugar beet pulp ratio (Experiment 2).

Item	Diets				SEM	Source of variation			
	Standard (SD)	Insoluble (INS)	Intermediate (INT)	High fibre (HF-S)		Diet	Period	Diet x Period	Weight
n[1]	8	8	7	8					
dDM_{vivo}	0.867[a2]	0.731[d]	0.788[c]	0.842[b]	1.00E-2	<0.001	0.630	0.464	0.938
dOM	0.896[a]	0.751[d]	0.813[c]	0.870[b]	1.06E-2	<0.001	0.949	0.409	0.797
dEE	0.595[c]	0.844[a]	0.759[b]	0.646[c]	2.32E-2	<0.001	0.344	0.001	0.809
dNDF	0.507[b]	0.191[d]	0.369[c]	0.708[a]	3.63E-2	<0.001	0.211	0.478	0.353
dCP	0.857[a]	0.861[a]	0.829[b]	0.810[b]	5.11E-3	0.002	0.485	0.379	0.241
N-retention (g retained g-1 diet)	0.554	0.569	0.544	0.574	1.16E-2	0.898	0.916	0.625	0.879
Urinary-N excretion (g in urine g-1 diet)	0.303[a]	0.285[a]	0.250[ab]	0.224[b]	9.29E-3	0.027	0.467	0.771	0.459
Urinary-N : faecal-N (g in urine g-1 in faeces)	2.178[a]	2.076[a]	1.477[b]	1.198[b]	1.05E-1	0.008	0.857	0.565	0.648

[1] n, number of observations.
[2] Within a row, means without common superscript letter differ ($P < 0.05$).

2. *In vitro Digestion and Fermentation*

DM disappearance after pepsin-pancreatin hydrolysis (dDM_{vitro}) was negatively affected by the inclusion of SBP and OH in the diet (P<0.001) (Table 15).

The kinetics parameters of the gas accumulation curves (France *et al.*, 1993) recorded during the fermentation of the hydrolyzed diets are given in Table 15. The fermentation rates were positively influenced by the presence of SBP in the diet and negatively by the incorporation of OH as indicated by the decreasing half-time to asymptotic gas production (*T/2*) and increasing fractional rates of degradation ($\mu_{t=t/2}$) recorded when passing from SD to MF, HF and finally VHF (P<0.001). *T/2* increased and $\mu_{t=t/2}$ decreased when passing from HF to INT and INS. OH also decreased the final gas production (P<0.001) but the inclusion of SBP had no influence on this parameter (P>0.05).

The bacterial nitrogen incorporation (*BNI*), SCFA productions, polysaccharide disappearance in the syringes after *T/2* h of fermentation are detailed in Table 16. *BNI*, expressed per g of diet, was higher when fermentation was stopped at *T/2* than when it was after 72 h (P<0.001). On the contrary, the SCFA production was higher after 72 h of fermentation, as compared to *T/2* (P<0.001). BNI was positively influenced by the presence of SBP in the diets (P<0.001) but the incorporation of OH had a negative effect on BNI (P<0.01). When expressed per g of fermented polysaccharides, BNI was also positively influenced by the presence of SBP (P = 0.02) and negatively by the presence of OH (P<0.01). The polysaccharides disappearance at half-time to asymptotic gas production was negatively influenced by the presence of OH in the diet (P<0.001).

Table 15. Dry matter disappearance (dDM_{vitro}) during the pepsin-pancreatin hydrolysis and kinetics parameters of the gas accumulation curves recorded during the fermentation of the hydrolyzed diets.

		Diets						SEM	Source of variation
	Item	Standard (SD)	Medium fibre (MF)	High fibre, soluble (HF-S)	Very high fibre (VHF)	Intermediate (INT)	Insoluble (INS)		Diet
Pepsin-pancreatin hydrolysis	n[1]	20	20	23	20	22	23		
	dDM_{vitro}	0.765 a[2]	0.745 b	0.752 a	0.714 d	0.722 c	0.719 c	2.36E-3	< 0.001
	n	9	9	9	8	9	9		
Gas production kinetics	*L* (h)	1.3 c	1.1 c	1.2 c	3.5 a	1.7 c	2.9 b	1.51E-1	< 0.001
	T/2 (h)	10.1 b	9.2 cd	8.7 d	8.5 d	9.7 bc	14.5 a	3.03E-1	< 0.001
	$\mu_{t=T2}$ (h^{-1})	0.088 d	0.100 c	0.122 b	0.150 a	0.090 d	0.047 e	4.47E-3	< 0.001
	Gf (ml g^{-1}DM)	302 a	304 a	301 a	307 a	238 b	158 c	7.77E+0	< 0.001

[1] n, number of observations.
[2] Within a row, means without common superscript letter differ ($P < 0.05$).

Table 16. Short chain fatty acid (SCFA) production, molar ratios, bacterial nitrogen incorporation (BNI) and polysaccharides disappearance measured after T/2 and 72 h fermentation.

Time stop (h)	Diet	n[1]	SCFA (mg g^{-1} DM incubated)	Acetate (%)	Propionate (%)	Butyrate (%)	BNI (mg g^{-1} diet)	n	BNI (mg g^{-1} fermented polysaccharides)	Polysaccharide disappearance[1]	Faecal bacterial N
T/2	Standard diet (SD)	9	156^{a}	68^{b}	23	8^{a}	1.51^{b}	3	11.0^{b}	0.597^{a}	0.389
	Medium fibre (MF)	8	161^{a}	67^{b}	26	7^{a}	2.01^{a}	3	14.5^{ab}	0.561^{a}	0.426
	High fibre, soluble (HF-S)	8	151^{a}	72^{ab}	24	4^{b}	2.06^{a}	3	16.4^{a}	0.539^{a}	0.404
	Very high fibre (VHF)	8	154^{a}	74^{a}	23	3^{b}	2.35^{a}	3	15.5^{a}	0.526^{a}	0.463
	Intermediate (INT)	9	139^{a}	71^{ab}	23	6^{ab}	2.17^{a}	3	17.4^{a}	0.437^{b}	0.466
	Insoluble (INS)	8	73^{b}	68^{b}	27	4^{b}	0.82^{c}	3	10.8^{b}	0.265^{c}	0.217
72	Standard diet (SD)	8	295^{ab}	63^{b}	27	10^{a}	0.87^{a}				
	Medium fibre (MF)	9	301^{ab}	68^{ab}	23	9^{ab}	0.93^{a}				
	High fibre, soluble (HF-S)	9	335^{a}	69^{a}	24	8^{ab}	0.89^{a}				
	Very high fibre (VHF)	8	318^{ab}	70^{a}	23	7^{ab}	0.98^{a}				
	Intermediate (INT)	9	266^{b}	71^{a}	22	7^{ab}	0.92^{a}				
	Insoluble (INS)	9	164^{c}	71^{a}	23	6^{b}	0.40^{b}				
SEM			9.73E+0	6.07E-1	4.95E-1	3.12E-1	7.88E-2		1.24	2.87E-2	
Source of variation	Diet		< 0.001	0.021	0.401	< 0.001	< 0.001		0.035	< 0.001	
	Time stop		< 0.001	0.174	0.415	< 0.001	< 0.001				
	Diet x Time stop		0.150	0.317	0.235	0.613	0.005				

[1] n, number of observations.
[2] Proportion of polysaccharides (dietary fibre and starch) disappeared from the syringe content at half-time to asymptotic gas production.
[3] Within a column for one same time stop, means without common superscript letter differ ($P < 0.05$).

The OH content of the diet also decreased the SCFA production (P<0.001). The SBP content had no influence on this parameter (P>0.05). The presence of SBP increased the proportion of acetate and decreased the proportion of butyrate (P<0.05). OH had no influence on the proportion of acetate in the molar ratio but decreased the proportion of butyrate (P<0.001).

3. Correlations and Integration of In vitro to In vivo Data

BNI, measured *in vitro* and expressed per g diet, were used to calculate the bacterial N incorporation in feces consecutive to the fermentation of the fibrous fraction of the diet (Table 16). Table 17 presents the Pearson's correlations between the *in vivo* digestibility coefficients, the fiber content of the diets, the *in vitro* pepsin-pancreatin hydrolysis and the fermentation parameters.

The CP apparent digestibility (*dCP*), the urinary-N excretion and the urinary-N:fecal-N excretion ratio were negatively correlated to soluble DF content of the diets (|r|>|-0.88|, P<0.05), the fractional rate of degradation ($\mu_{t=t/2}$) (|r|>|-0.82|, P<0.05), the proportion of acetate in the molar ratio (|r|>|-0.83|, P<0.05) and the *BNI* (|r|>|-0.82|, P<0.05). *T/2* appeared to be highly correlated to the proportion of N originating from the bacteria in the feces (r = -0.94, P = 0.006).

Table 17. Pearson's correlation coefficients between *in vivo* digestibility coefficients and N excretion pathways, fibre content of the diet, *in vitro* pepsin-pancreatin hydrolysis and fermentation parameters at half-time to asymptotic gas production (n = 6).

	Starch and fibre content of the diet						*In vitro* pepsin-pancreatin hydrolysis	*In vitro* fermentation parameters									
	Starch	NDF	ADF	IDF[1]	SDF[2]	TDF[3]	dDM_{vitro}	*L*	*T/2*	$\mu_{t=T/2}$	*Gf*	*BNI*[4]	*PSD*[5]	SCFA	%Acet	%Prop	%But
dDM_{vivo}	$0.69^{NS,6}$	-0.94^{**}	-0.87^{*}	-0.83^{*}	0.29^{NS}	-0.56^{NS}	0.71^{NS}	-0.52^{NS}	$-0.80^{\dagger}$	0.62^{NS}	0.96^{**}	0.55^{NS}	0.99^{***}	0.93^{**}	0.06^{NS}	-0.52^{NS}	0.45^{NS}
dOM	0.66^{NS}	-0.92^{**}	-0.85^{*}	$-0.81^{\dagger}$	0.33^{NS}	-0.52^{NS}	0.69^{NS}	-0.49^{NS}	-0.82^{*}	0.65^{NS}	0.97^{***}	0.58^{NS}	0.99^{***}	0.93^{**}	0.09^{NS}	-0.53^{NS}	0.42^{NS}
dNDF	0.11^{NS}	-0.53^{NS}	-0.38^{NS}	-0.33^{NS}	$0.81^{\dagger}$	0.06^{NS}	0.24^{NS}	-0.08^{NS}	-0.88^{*}	0.94^{**}	0.91^{*}	$0.77^{\dagger}$	$0.80^{\dagger}$	0.82^{*}	0.52^{NS}	-0.45^{NS}	-0.17^{NS}
dCP	0.44^{NS}	-0.04^{NS}	-0.20^{NS}	-0.28^{NS}	-0.88^{*}	-0.58^{NS}	0.29^{NS}	-0.08^{NS}	$0.74^{\dagger}$	-0.82^{*}	-0.49^{NS}	-0.87^{*}	-0.32^{NS}	-0.51^{NS}	$-0.74^{\dagger}$	0.34^{NS}	0.49^{NS}
N-urine[7]	0.48^{NS}	-0.05^{NS}	-0.22^{NS}	-0.30^{NS}	-0.95^{**}	-0.62^{NS}	0.28^{NS}	-0.22^{NS}	0.71^{NS}	-0.89^{*}	-0.50^{NS}	-0.82^{*}	-0.33^{NS}	-0.47^{NS}	-0.89^{*}	0.46^{NS}	0.62^{NS}
N-ratio[8]	0.50^{NS}	-0.09^{NS}	-0.26^{NS}	-0.34^{NS}	-0.90^{*}	-0.64^{NS}	0.33^{NS}	-0.15^{NS}	0.70^{NS}	-0.82^{*}	-0.45^{NS}	-0.85^{*}	-0.27^{NS}	-0.45^{NS}	-0.83^{*}	0.39^{NS}	0.57^{NS}
Fecal bacterial N[9]	0.13^{NS}	-0.49^{NS}	-0.35^{NS}	-0.25^{NS}	0.59^{NS}	0.04^{NS}	0.08^{NS}	-0.24^{NS}	-0.94^{**}	$0.77^{\dagger}$	$0.77^{\dagger}$	0.96^{**}	$0.73^{\dagger}$	0.88^{*}	0.50^{NS}	$-0.75^{\dagger}$	0.17^{NS}

[1] IDF, insoluble dietary fibre.
[2] SDF, soluble dietary fibre.
[3] TDF, total dietary fibre.
[4] *BNI*, bacterial nitrogen incorporation (g g^{-1} diet).
[5] *PSD*, polysaccharides disappearance at half time to asymptotic gas production.
[6] ***, P<0.001; **, P<0.01; *, P<0.05; † ; P<0.10 ; NS, not significant.
7 N-urine, urinay-N excretion.
[8] N-ratio, urinary-N:fecal-N.
[9] Fecal bacterial N, fraction of the fecal N resulting from the bacterial growth consecutive to the fermentation of the fibrous fraction of the diet.

5. Discussion

Similar N retention in pigs fed diets differing in SBP and/or OH content confirms that the ileal protein digestibilities were not affected by DF intake. The differences in N excretion can thus be ascribed to DF fermentation in the large intestine, as also confirmed by the correlations observed between *in vitro* BNI and the *in vivo* N excretion.

The rate of fermentation plays a major role on both protein synthesis by intestinal bacteria and N excretion shift. The presence of highly fermentable DF in the SBP, as highlighted in Experiment 1 by the linear increase in NDF apparent digestibility with increasing SBP content in the diets, did not influence the total N excretion but shifted N excretion from urine to feces. These observations are consistent with Canh *et al.* (1997) and Zervas and Zijlstra (2002) who observed increased fecal N output and lower N excretion in pigs fed with SBP and soybean hulls. Unlike SBP, the addition of poorly fermentable OH had no influence on the fecal and the urinary N excretions. The *in vitro* relationships between *BNI* and $\mu_{t=T/2}$ (r = 0.84, P = 0.035) or *T/2* (r = -0.99, P<0.001) observed in this study confirm previous observations showing that fast fermenting substrates yield higher protein synthesis by isolated colonic bacteria in pigs (Bindelle *et al.,* 2007). Diets with high *in vitro* fractional rates of degradation ($\mu_{t=T/2}$) induced lower CP apparent digestibility, urinary-N excretion and urinary-N:fecal-N excretion ratio in pigs. As a consequence, the bacterial N incorporation in feces consecutive to the fermentation of the fibrous fraction of the diet can be doubled when using DF source with high rates of fermentation instead of poorly fermentable DF.

The use of some DF sources yielding high SCFA production to induce a shift in N excretion can limit the detrimental impact of the energy value of increasing the fibrous content of the diet, as indicated by the relationship between SCFA production and the fecal bacterial N. SCFA contribute to the host energy supply, but the efficiency of energy utilization reaches 0.82, approximately 5 to 10 % lower than starch digested and absorbed in the small intestine (Jørgensen *et al.*, 1997). Furthermore, fermentation gases and bacterial biomass in feces are also a loss of energy for the pig.

The decrease in *BNI* between *T/2* and 72 h of fermentation ($P<0.001$) suggests that the N shift is influenced by the intestinal transit time. Unlike the SCFA, the bacterial biomass does not accumulate continuously in the syringes. After the linear phase of gas production, when the fermentation broth becomes depleted in carbohydrates, a death phase occurs and the number of viable cells declines (Prescott *et al.*, 1996) and *BNI* decreases. In animals with long transit times such as adult sows (Le Goff *et al.*, 2002), when the intestinal content passes from the proximal to the distal colon, it becomes depleted in fermentable carbohydrates. In this case, after a phase of bacterial protein synthesis, proteolysis occurs, with a release of ammonia and its elimination in urine. However, in growing pigs (>35 kg) fed diets with similar DF content, transit can be too fast to reach the depletion in fermentable carbohydrates (Le Goff *et al.*, 2002), reducing the proteolysis. Growing pigs are responsible of 50 to 64 % of NH_3 emission of intensive pig barns (Hayes *et al.*, 2006).

Some DF sources also accelerate the intestinal transit (Wenk, 2001). This is a consequence of the bulking effect of DF and their physico-chemical properties. DF with high water-holding capacity are more efficient in increasing the digesta flow (Varel and Yen, 1997). A short transit time combined to a high rate of DF fermentation maintains high bacterial activity throughout the entire large intestine and decreases proteolysis in the distal colon. This was certainly the case here with diets high in SBP (HF-S and VHF). The combination of DF sources differing in their rate of fermentation in the INT diet (OH and SBP) was also effective to maintain high bacterial activity in the colon despite a lower content of fermentable DF, as compared to HF-S and VHF diets. Poorly fermentable DF also acts on transit time and displace the fermentation site from the proximal to the distal part of the colon (Govers *et al.*, 1999).

Finally, it is worth mentioning that the *in vitro* technique was able to predict *in vivo* N excretion shift parameters through simple regression equations linking the CP apparent digestibility, the N excretion in urine and the N-urine:N-fecal excretion ratio to *BNI* and $\mu_{t=T/2}$. However, the use of these equation for the prediction of N excretion shifts in pigs requires a broader database with different DF sources.

It can be concluded that N excretion shifts consecutive to graded levels of DF in swine rations is caused by enhanced bacterial protein synthesis in the large intestine. DF

sources highly influence the intestinal bacteria growth. The gas test fermentation appears to be a valuable tool to characterize the DF contribution to N excretion shift.

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CHAPTER VII

General discussion and future prospects

The research carried out in this thesis demonstrates that the gas production technique is a useful tool to characterise some aspects and parameters related to fibre fermentation in the pig intestines.

Inoculum preparation

The first section of the experimental work concerned the development of an *in vitro* method in order to study the fate of DF during fermentation. Comparing the fermentation of wheat bran and SBP incubated with colonic content and faeces, it firstly was showed, in Chapter III, that the use of the latter induces only a sligth delay in the gas production kinetics. Both flora have similar ability to ferment a same substrate since final gas production was not affected by the inoculum source. The microbial inoculum can be thus prepared from fresh faeces, making the method easier and ethically acceptable since no intestinal or caecal cannula is required.

Secondly, the enzymatic hydrolysis with pepsin and pancreatin prior to fermentation in order to simulate digestion occuring in the upper gastro-intestinal tract was shown to be an important step of the protocol. It modifies the composition and significantly affects the fermentation patterns of the substrates. The ranking order of the 6 substrates that were investigated in the framework of the experiment presented in Chapter III differed whether they had undergone the hydrolysis prior to the fermentation or not. The rate of degradation decreased when peas, lupins, maize and wheat bran were hydrolysed but it increased with soybean meal and SBP. Final gas production increased with peas and soybean meal, remained unchanged for lupins and decreased for the other substrates. Nevertheless, the enzymatic hydrolysis induces a loss of soluble DF which *in vivo* are fermented by intestinal bacteria and, in Chapter V, soluble DF was shown to support high BNI.

Finally, as observed in Chapter IV, the bodyweight of the faeces donors and the soluble DF content of their diets influence significantly the fermentation kinetics measured with the gas production technique, especially during the growth phase of the fermentation. Final gas production remained unaffected, but lag times increased with bodyweigth while the rates of degradation decreased. It was also stated that the

presence of soluble fibre in the diets of the faeces donors decreased lag and half-times to asymptote. Inoculum prepared from pigs fed low fibre diets gave also slower fermentation. When studying a topic related to a specific category of animal, e.g. weaning piglets, growing pigs or adult sows, it is thus recommended to use animals from the same category as faeces donors to prepare the inoculum. The use of donors fed diets high in soluble DF to maximise the activity of the bacterial population during the initial phase of the fermentation is also important to reduce the lag time and to induce high fermentation rates, especially in animals with long transit times. However, due to the influence of the DF source on the microbial equilibrium (Williams *et al.*, 2001) of the faeces and, as a consequence, of the inoculum, it is also important to favour the development, in the donor's intestines, of bacterial species susceptible to ferment the substrates. This can be reached by giving the animals a combination of DF sources related to the substrates. If a wide variety of DF types are studied, different sources of DF are recommended in the pig's diet. The results presented in Chapter VI with the diet containing SBP and OH, indicate that diversity in DF sources and high bacterial concentration in faeces can be achieved simultaneously.

Prediction of N excretion shifts

In the second section of the experimental work, the use of ^{15}N-labelled NH_4Cl instead of $(NH_4)HCO_3$ as sole N source in the buffer solution of Menke and Steingass (1988) successfully allowed the observation of differences in bacterial protein synthesis according to the fermented substrate. This bacterial protein synthesis was clearly correlated to *in vivo* N excretion shifts from urine to faeces. Predicting equations for the *in vivo* N excretion shift parameters were calculated using *in vitro* parameters, *BNI*, $\mu_{t=T/2}$ and *T/2*, and the soluble DF content of the diets in the last experiment (Table 13, Table 14, Table 15 and Table 16). The results of the experiments presented in Chapters V and VI showed indeed that these parameters have a major influence on *in vitro* bacterial protein synthesis and on *in vivo* N excretion shifts from urine to faeces. Substrates and diets with high soluble DF content are associated with rapid fermentation: low *T/2* and high $\mu_{t=T/2}$. As discussed in Chapter V, they support high *BNI* since the efficiency of ATP generation from the substrate and the use of this ATP

for the growth is higher compared to slower fermenting substrates. *In vivo*, diets high in SBP and, as a consequence, with high soluble DF content, induced a significant shift in N excretion pathways. As shown in Table 18, for some parameters, stronger relationships were obtained when introducing the butyrate molar ratio (*but*, %) in the equations, *BNI*, $\mu_{t=T/2}$ and *T/2* being independent from *but* (r = -0.090, -0.334 and –0.093 and P = 0.865, 0.518 and 0.861, respectively).

Table 18. Equations for the prediction of *in vivo* N excretion pathways from *in vitro* fermentation data (N=6).

Equation	r² or R²	P	RMSE
Urinary-N:faecal-N $= 2.77 - 0.626 \times BNI$	0.65	0.033	0.25
Urinary-N:faecal-N $= 2.62 - 9.90 \times \mu_{t=T/2}$	0.60	0.043	0.26
Urinary-N:faecal-N $= 2.13 - 0.0154 \times SDF$	0.77	0.014	0.20
Urinary-N:faecal-N $= 2.14 - 0.593 \times BNI + 0.106 \times but$	0.94	0.007	0.10

Let us focus now on the applicability of these equations. Before being generalised, they should be strengthened with a broader range of fibrous ingredients. Indeed, the presence of soluble DF in the experimental diets was almost exclusively influenced by the sugar beet pulp content. Other kinds of soluble DF may behave differently, although the experiment using purified DF sources (Chapter V) highlighted the general positive influence of fast fermenting substrates such as starch and soluble DF on bacterial protein synthesis. Furthermore, a general equation predicting with accuracy the N excretion ratio of all ingredients or diets is probably not imaginable. It seems more adequate to develop specific equations to different substrates categories as is it the case for ruminant studies.

Secondly, the improvement of equation accuracy using butyrate (Table 18) may not necessarily be transposed to all kinds of diets. In the experiments of Chapter VI, butyrate accumulation was due to the presence of RS in maize. Starch is known as a butyrogenic carbohydrate in the large intestine of single-stomached animals (Sajilata *et al.*, 2006). This assumption was confirmed by measuring the starch content in the hydrolysed diets, which increased with the level of maize and decreased with the level

of DF in the diet. This is consistent with Martinez-Puig *et al.* (2003) and Bednar *et al.* (2001) who measured significant RS content in maize, approximately 25 % of the total starch. In Chapter V, we showed that starch supports high *in vitro* bacterial growth. It therefore enhanced the *in vitro* BNI values of the diets containing high levels of maize, especially low fibre diets such as SD and MF. *In vivo*, the influence of this resistant starch on bacterial growth and the consecutive N faecal excretion was lower, as compared to *in vitro* data. Indeed, a significant part of RS, between 40 and 80% according to the starchy source, is already fermented in the distal small intestine (Martinez-Puig *et al.*, 2003) and this fermentation is less likely to contribute to N excretion shift. Furthermore, the results of Chapter IV, comparing the fermentation kinetics using donors fed diets differing in DF content (Table 8), showed that SBP enhanced bacterial activity in faeces. Unlike SBP, RS has a minor influence on water-holding capacity and transit time (Ferguson *et al.*, 2000). With longer transit times, RS is totally fermented before the distal colon (Martinez-Puig *et al.*, 2003) and proteolysis is more likely to happen in the large intestine.

Results of Chapter VI raise the question of the choice of the time necessary to stop the fermentation. BNI was indeed shown to increase in the initial phase and to decrease as fermentation reached extinction (Table 16). The BNI accumulation and depletion curve with time in such gas-test studies and its relationship to transit time require further investigation. The use of parameters such as the water-holding capacity of DF and the bodyweight of the animals to take the influence of the intestinal transit time into account is possibly an easy way to improve the predicting equations.

The mathematical models available in the literature to interpret gas production kinetics do not have a variable reflecting bacterial N incorporation and release in the glass syringe (France *et al.*, 1993; Groot *et al.*, 1996; Dhanoa *et al.*, 2000). The integration of such a variable describing bacterial accumulation and auto-consumption in an existing mathematical model is a possible alternative to the use of ^{15}N and isotope mass spectrometry to estimate bacterial protein synthesis *in vitro*. Some attempts were made in this sense on the edge of this research (Noon, 2005) and they deserve further attention.

Finally, this thesis focussed on the influence of DF source on protein synthesis by intestinal bacteria and the consequences on N excretion pathways. The main application field lies in the reduction of the pollution of intensive pig production and the savings of crop fertilisers for small-holders in the tropics. Growing pigs are the target population for such strategies since they are responsible of at least 50 % of the NH_3 losses of intensive barns (Hayes *et al.*, 2006). However, as exposed in Chapter I, DF sources have other numerous functionnalities. They are related to microbial flora equilibrium in piglets through the prebiotic or detrimental effects of some oligo- and polysaccharides (Williams *et al.*, 2001; Montagne *et al.*, 2003), to animal health as butyrate is known to promote intestinal health (Kien *et al.*, 2007), animal welfare and stereotypies in sows (Meunier-Salaun, 1999; Courboulay *et al.*, 2001) and energy and protein supply (Ogle, 2006). The development of models for such multi-criteria assessment of fibrous feedstuffs potentially available for feeding pigs is a challenge since it should integrate nutritional, environmental, health and welfare data. It should integrate economical data and be specific to the production environment (intensive vs. extensive, tropical vs. temperate) and the animal category (piglets vs. growing pigs or sows) that is considered.

Future possible applications of the in vitro method

Beside the quantification of the consequences of DF types and levels in the diets on the N excretion pathways in pigs, the *in vitro* method has other possible applications.

First of all, numerous studies have highlighted the potential of fermentable DF to reduce ammonia emission from pig facilities (Nahm, 2003) and, in the tropics, the role of faecal N to favour the recycling of manure N by crops (Rufino *et al.*, 2006). This thesis clearly pointed out, on animal level, the role of fermentable DF and the enhanced intestinal bacteria accumulation on the N excretion shifts from urine to faeces. However, in order to determine the conditions in which the use of fibrous ingredients in the rations could be recommended to the farmers, the influence of this practise on the environmental load and the economical balance of intensified and extensive tropical pig farms should be assessed. The use of *in vitro* data, as developed in this research, as model for the animal sub-system in a holistic bio-economical mathematical farm

simulation model is certainly a rapid and low cost alternative to *in vivo* methods for the assessment of the influence of fibrous ingredients at the farm level.

This research focussed on the relationships between DF fermentation and N excretion. Nevertheless, in Chapter I, the influence of DF on energy digestibility and the contribution of SCFA production to energy supply was briefly presented. The *in vitro* method could also be used for investigation on this topic. Using the gross energy content of the SCFA (acetate: 3487 kcal kg^{-1}, propionate: 4968 kcal kg^{-1}, butyrate: 5947 kcal kg^{-1}), the metabolizable energy (ME) available for the pig from fermentation (Livesey and Elia, 1995) can be calculated from *in vitro* fermentation data, with the exception of the released heat. In the case of purified DF sources (Table 10), ME varied from 1813 kcal kg^{-1} fermented OM for inulin to 2489 for cellulose. Substrates supporting less bacterial protein synthesis yielded higher SCFA, as discussed in Chapter V. Moreover, fast growing bacteria, producing less SCFA are also suspected, during the growth phase, when BNI were actually measured (*T/2*), to stock carbon in their cells for later energy or biosynthesis requirements. Indeed, for fast fermenting substrates, starch, inulin and pectin, sugars disappearance exceeded 80 % while only half of the final gas volume was produced (*T/2*) (Table 10). These inclusion bodies are made of lipid droplets, glycogen or even SCFA such as poly-ß-hydroxybutyrate (Prescott *et al.*, 1996). When the broth becomes depleted in carbohydrates and the fermentation reaches extinction, these carbon reservoirs are released by the bacteria and contribute finally to gas and SCFA production. Nevertheless, inulin was shown to have a higher fermentability compared to cellulose and the ME expressed per kg of fermented DF does not take the proportion of DF that will be really fermented in the intestines into account. When feeding pigs, the unfermented DF reduces the ME truly available from fermentation per kg diet. This explains why when pigs were fed diets with increasing levels of SBP, from 0 to 30 %, the ME from fermentation passed from 295 to 373 kcal kg^{-1} diet, between 9.4 and 12 % of the ME content of the diet. This energy dropped to 6 % of the diet (188 kcal kg^{-1} diet) when oat hulls were used as the fibre source (Table 16). The possible use of the *in vitro* method to predict digestible energy content of forages for single-stomached animals is also confirmed by the results of Bindelle *et al.* (2007b) in Guinea pigs who obtained high correlations and

regressions between *in vivo* organic matter and energy digestibility and *in vitro* pepsin-pancreatin and gas fermentation parameters.

Pursuing with other single-stomached animals than the pig, *in vitro* measurements of intestinal bacterial growth should also be interesting in rabbits (Lavrenčič, 2007) or Guinea pigs (Bindelle *et al.*, 2007a) studies, as proteins from intestinal bacteria are recycled for nutritional purpose during caecotrophy.

Finally, as showed by Awati *et al.* (2005), *in vitro* fermentation methods can also highlight the effect of the DF source on the microbial composition. In same idea, the potential prebiotic effect of non-digestible oligo-saccharides in human nutrition was recently studied *in vitro* (Palframan et al., 2003; Vulevic et al., 2004; Ghoddusi et al., 2007). Nevertheless, such potentialities of *in vitro* fermentation methods to investigate the interactions between diet, comensal and pathogenic flora in the intestines of pigs, have, until now, been underexploited because of a lack of proper *in vivo* validation of such studies results, despite the opportunities in weaning piglets.

It can be concluded that this research demonstrated the influence of fermentable DF on N excretion shifts from urine to faeces through enhanced intestinal bacterial growth. The *in vitro* gas test that was developed was very useful for the study of N excretion pathways. It has certainly a great potential to investigate the other functionalities of DF in pig nutrition, especially in these times of competition for cereals with biofuel production which will require to valorise more and more fibrous by-products in single-stomached animals.

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