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In vitro fibre fermentation of feed ingredients with varying fermentable carbohydrate and protein levels and protein synthesis by colonic bacteria isolated from pigs

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ABSTRACT

An *in vitro* experiment was carried out using the gas technique to study the fermentation characteristics of different feed ingredients differing in their fermentable carbohydrate and protein composition by colonic bacteria isolated from pigs. The effect on *in vitro* bacterial protein synthesis was also evaluated. The ingredients used were wheat bran (WB), wood cellulose (Solka-floc®, SF), peas, pea hulls (PH), pea inner fibre (PIF), sugar beet pulp (SBP), flax seed meal (FSM) and corn distillers dried grains with solubles (DDGS). The samples were pre-treated with pepsin and pancreatin and the hydrolyzed substrates were then incubated with pig faeces in a buffered mineral solution. The nitrogen source in the buffer solution (NH_4HCO_3) was replaced by an equimolar quantity of ^{15}N-labeled NH_4Cl, used for the determination of the rate of bacterial protein synthesis. Gas production, proportional to the amount of fermented carbohydrate, was recorded for 48 h and modelled. The fermented product was subjected to short-chain fatty acids (SCFA) analysis. The source of fibre affected the *in vitro* dry matter degradability (*IVDMD*), the fermentation kinetics and the gas production profile ($P<0.05$). The highest ($P<0.001$) *IVDMD* values were observed for peas (0.80) and FSM (0.70), whereas SF was essentially undegraded (0.06). The fractional rate of degradation appeared to be lower ($P<0.001$) for WB and DDGS (0.07 and 0.05 h, respectively) and highest for SBP (0.20 h). Peas started to ferment rapidly (lag time 1.3 h). Half gas production ($T/2$) was achieved sooner for PIF (8.4 h) and was the longest for DDGS (19.8 h). The total gas production was the highest for PH, followed by SF, PIF and peas (276, 266, 264 and 253 ml/g DM incubated, respectively) and the lowest for FSM and WB

Abbreviations: ACE, acetic acid; ADF, acid detergent fibre; AOAC, Association of Official Analytical Chemists; BUT, butyric acid; BCFA, branched-chain fatty acids; BNI, bacterial nitrogen incorporation; CHO, carbohydrates; CP, crude protein; DDGS, corn distillers dried grains with solubles; DF, dietary fibre; DM, dry matter; FSM, flax seed meal; GC, gas chromatography; iCP, indigestible protein; *IVDMD*, *in vitro* dry matter degradability; N, nitrogen; NDF, neutral detergent fibre; NSP, non-starch polysaccharides; PRO, propionic acid; PH, pea hulls; PIF, pea inner fibre; RS, resistant starch; SBP, sugar beet pulp; SF, Solka-floc®; SCFA, short-chain fatty acids; WB, wheat bran.

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(130 and 124 ml/g DM incubated, respectively). There was no difference (P>0.05) in SCFA production after the fermentation of SF, P, PH, PIF and SBP (ranging from 3.8 to 4.5 mmol/g DM incubated) while WB and FSM yielded lowest (P<0.05) SCFA. The bacterial nitrogen incorporation (BNI), both at *T*/2 and after 48 h of fermentation was the highest (P<0.001) for PIF (18.5 and 15.6 mg/g DM incubated, respectively) and the lowest for DDGS and WB. In conclusion, peas and pea fibres had higher rates of fermentability, produced more SCFA and had high bacterial protein synthesis capacity. They thus have the potential to be included in pig diets as a source of fermentable fibre to modulate the gut environment and reduce nitrogen excretion.

1. Introduction

Swine nutritionists are working on strategies to reduce nitrogenous gaseous emission from pig facilities. To deal with this socio-technical issue, different approaches have been investigated (Aarnink and Verstegen, 2007). One of them consists in increasing fermentable dietary fibre (DF) in the pig diets. The non-starch polysaccharides (NSP) and resistant starch (RS) that escape small intestinal digestion are fermented by the caecal and colonic microbiota (Cummings and Englyst, 1987). Increased intestinal fibre fermentation has been reported to reduce the ammonia emission from manure (Mroz et al., 2000; Nahm, 2003) by shifting nitrogen (N) excretion from urine to faeces (Kirchgessner et al., 1994; Zervas and Zijlstra, 2002; Bindelle et al., 2009). This shift in the N excretion pathway is due to an increase in ammonia uptake by bacteria in the large intestine (Mosenthin et al., 1992). In addition, the fermentation of DF in the pig intestines increases the production of short-chain fatty acids (SCFA) (Houdijk et al., 2002), since DF is the main substrate for microbial fermentation in gut. Thus, DF inclusion might be an effective approach to modulate the gut environment and, at the same time, to reduce the footprint of pork production on the environment (Verstegen and Williams, 2002). However, most of the functional properties of DF have been obtained with isolated fibres or with wheat bran and sugar beet pulp, while these characteristics are also affected by the source of fibre and the fibrous matrix (Pieper et al., 2008; Jha et al., 2010). Moreover, the inclusion of isolated fibre in pig diets on a regular basis to reduce ammonia emission or modulate the gut environment is not economically justified. Thus, the use of feed ingredients with desirable properties instead of isolated fibre in pig rations to achieve these functional benefits makes more sense. However, many fibrous ingredients have not been investigated in this perspective yet. Many feed ingredients with highly fermentable DF are also rich in indigestible protein (iCP), which impairs the positive effect of DF fermentation on ammonia emission (Le et al., 2005). Again, limited information is available on the interaction of DF and crude protein (CP) fermentation, especially when it resides in the matrix of feed ingredients.

Bindelle et al. (2009) recently concluded that *in vivo* N excretion shifts observed with graded concentrations of fermentable DF in pig diets were correlated with enhanced bacterial N uptake in the large intestine, as measured by an *in vitro* fermentation technique. They suggested that the *in vitro* technique can be used to predict the N excretion shift from urine to faeces by measuring the bacterial nitrogen incorporation (BNI). *In vitro* models also provide information on different fermentation variables for a large number of ingredients in relatively short time. They are also cost-effective, easy to develop and non-invasive (Coles et al., 2005). In this method, part of the soluble CHO is lost during the filtration process which is practically inevitable in such an *in vitro* system and is considered to be the limitation of the method (Bindelle et al., 2007a,b, 2009). However, a significant part of the S-NSP is not washed out during the filtration step. This fraction is probably embedded in the insoluble fibre matrix. Nevertheless the model can generate valuable information as has been confirmed by results obtained with the method being comparable to data obtained *in vivo* (Bindelle et al., 2009). The present study was carried out to evaluate the fermentation characteristics of feed ingredients with varying fermentable fibre and indigestible protein content. It also aimed at evaluating their possible influence on the intestinal environment and nitrogen excretion, as indicated by the BNI, using an *in vitro* technique. The investigation was based on the hypotheses that feed ingredients differing in their content and type of fermentable fibre and indigestible protein affect the fermentation kinetics and their end products profile as well as bacterial protein synthesis in the pig's gastrointestinal tract.

2. Materials and methods

The animal handling for the study was performed in accordance with the recommendations of the Canadian Council on Animal Care (CCAC, 1993) as specified in the Guide to the Care and Use of Experimental Animals and the Standard Animal Care Protocol (No. 970019) approved by the University of Saskatchewan Committee on Animal Care and Supply.

2.1. Ingredients

Eight feed ingredients with potentially high levels of fermentable fibre content were studied: wheat bran (WB), wood cellulose (Solka-floc®, SF), peas, pea hulls (PH), pea inner fibre (PIF), sugar beet pulp (SBP), flax seed meal (FSM) and corn distillers dried grains with solubles (DDGS). They were selected based on their diversity of fibre and protein content. Their

Table 1
Chemical composition of the ingredients and the hydrolyzed substrates used in the experiment (g/kg DM).

Ingredient	Ingredient								Hydrolyzed substrates		
	DM	Ash	EE	CP	NDF	ADF	tNSP	S	CP	tNSP	S
Wheat bran	884	62	57	221	382	125	183	186	103	347	7
Solka-floc®	953	2	4	16	995	901	234	NA	NA	487	NA
Peas	879	31	10	257	214	81	81	441	50	254	80
Pea hulls	922	32	14	176	400	350	228	184	17	380	31
Pea inner fibre	890	16	3	48	213	134	151	540	12	245	71
Sugar beet pulp	913	117	5	97	425	271	196	NA	32	286	NA
Flax seed meal	906	59	107	381	245	165	130	NA	72	222	NA
Corn DDGS	883	56	134	271	366	177	136	59	147	215	21
Feed[a]	887	56	38	220	172	80	NA	384	NA	NA	NA

Abbreviations: DM, dry matter; EE, ether extract; tNSP, total non-starch polysaccharides; S, total starch; NA, not analyzed.
[a] Feed sample (commercial grower feed, devoid of antibiotics) of pig used as donor of faeces used as inoculum for *in vitro* fermentation.

chemical composition, along with the feed of the pigs used as faeces donor for the *in vitro* fermentation, is presented in Table 1.

2.2. Enzymatic hydrolysis

Prior to the fermentation, the substrates, ground to pass a 1 mm screen (Retsch ZM1 Mill, Newton, PA, USA), underwent an *in vitro* pepsin and pancreatin hydrolysis following the first steps of the protocol of Boisen and Fernandez (1997) in order to mimic digestion occurring in the upper gastro-intestinal tract. Briefly, 2 g sample 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 1 M HCl or 1 M NaOH and 2 ml of a chloramphenicol (Sigma C-0378) solution (0.5 g 100/ml ethanol) was added to prevent bacterial growth during hydrolysis. Fresh pepsin solution (4 ml, 20 g/l, porcine pepsin: Sigma P-0609) was added and the flasks were placed in a water-bath at 39 °C for 2 h under gentle agitation (50 rpm).

Afterwards, 40 ml phosphate buffer (0.2 M, pH 6.8) and 20 ml of a 0.6 M NaOH was added into the solution. The pH was adjusted to 6.8 with 1 M HCl or 1 M NaOH. Fresh pancreatin solution (2 ml, 100 g/l pancreatin, Sigma P-1750) was added and hydrolysis was continued for 4 h under the same conditions.

After hydrolysis, the residues were collected by filtration on a nylon cloth (42 μm), washed with ethanol (2× 25 ml 95% ethanol) and acetone (2× 25 ml 99.5% acetone), dried for 24 h at 60 °C and weighed. The enzymatic hydrolysis was repeated from 6 to 26 times according to the degradability of the ingredients, in order to yield enough hydrolyzed residues for the *in vitro* fermentation and their characterisation (N, NSP and starch content). Hydrolyzed residues from the different replicates and periods were pooled for subsequent analyses (N, NSP and starch content) and for the *in vitro* fermentation.

2.3. In vitro fermentation

The rate of fermentation of the hydrolyzed ingredients was assessed *in vitro*, using a cumulative gas production technique adapted to the pig by Bindelle et al. (2007a) as follows. The 200 mg-substrates were incubated at 39 °C (in a shaking water-bath with 50 rpm) in a 125 ml-glass bottle with 30 ml buffer solution containing macro- and micro-minerals (Menke and Steingass, 1988) and a faecal inoculum. The N source in the buffer solution (NH_4HCO_3) was replaced by an equimolar quantity of ^{15}N-labeled 2% NH_4Cl (ISOTEC, Miamisburg, OH, USA, CAS 39466-62-1), which was used for the determination of BNI. Three growing pigs (6–8 weeks age) from the herd of Prairie Swine Centre (Saskatoon, SK, Canada) were fed a standard commercial diet devoid of antibiotics (Table 1) and used as donors of faecal inoculum. Faecal samples were collected directly from the rectum, immediately placed in air tight plastic syringes and kept in a waterbath at 39 °C until further use. It never exceeded 1 h. The inoculum was prepared by diluting faeces 20 times in the buffer solution and filtering through a 250 μm screen. The inoculum was transferred into bottles with the fermentation substrates and the bottles were sealed with a rubber stopper and placed for incubation. An anaerobic environment was maintained throughout time, from inoculum preparation until the incubation step by flushing with CO_2 gas. The gas produced during fermentation was measured at 0, 2, 5, 8, 12, 18, 24, 36 and 48 h by means of a pressure transducer (GP:50, SIN-54978, Grand Island, NY) (Mauricio et al., 1999), fitted with digital data tracker (Tracker 211, Intertechnology Inc., Ontario, Canada). The bottles were vented after every measurement and fermentation was stopped at 48 h by quenching the bottles in iced water.

The experimental scheme was as follows: 8 ingredients × 6 bottles + 6 blanks (containing only inoculum) repeated over 4 runs (batch).

Before the experimental runs, one fermentation run (6 replicates of each substrate) was carried out until 48 h and the accumulated gas was used to determine the half-time ($T/2$) of total gas production, using the mathematical model of France et al. (1993). The incubation of 4 bottles from each ingredient was stopped at half-time of the asymptotic gas production ($T/2$), time varying according to the ingredient (Table 3), by quenching the fermentation bottles in an iced water-bath for 20 min. The remaining 2 bottles of each ingredient were stopped at 48 h. When the bottles were stopped, they were subsequently

emptied and rinsed with distilled water (2× 5 ml). The fermentation broth of 2 bottles stopped after production of half-asymptote gas volume, $T/2$ h according to the mathematical model of France et al. (1993) described below, were pooled and freeze-dried for further determination of total carbohydrates (CHO, NSP + starch) content in the residue. The content of the 2 other bottles stopped at $T/2$ and the 2 bottles stopped after 48 h of fermentation were centrifuged (12,000 × *g*, 20 min, 4 °C) and an aliquot of the supernatant was taken for SCFA analysis. The pellet was suspended in distilled water (30 ml), centrifuged (12,000 × *g*, 20 min, 4 °C) and the supernatant was discarded. The resulting pellet containing the bacteria and the undigested substrate was freeze dried, weighed and analyzed for total N, ^{15}N-enrichment and CHO (NSP + starch) content. For each period, 6 samples of the inoculum were also taken for N, ^{15}N, SCFA and CHO determination. The blanks were used to subtract the gas produced due fermentation of substrates available in the inoculums.

2.4. Chemical analyses

All the ingredients and the diet were ground in a laboratory mill (Restch ZM1, Newton, PA, USA) to pass a 1 mm screen and chemical analysis (Table 1) were performed according to the Association of Official Analytical Chemists standard procedures (AOAC, 2007): dry matter (135 °C for 2 h, AOAC 930.15), nitrogen (AOAC 968.06; using an elemental analyzer LECO FP528, St. Joseph, MI, USA; crude protein = N × 6.25), ether extract using Soxhlet apparatus and petroleum ether (AOAC 920.39), ash (AOAC 942.05), ADF (AOAC 973.18), NDF (AOAC 2002.04) and gross energy (PARR 1281 calorimeter, Moline, IL, USA).

Samples were ground to pass through a 0.5 mm-mesh screen and analyzed using commercial test kits (Megazyme International Ltd., Ireland) to determine total starch (AOAC 996.11). Samples were analyzed for their CHO (NSP + starch) by gas chromatography (GC) along with individual sugars content (Englyst et al., 1994), without enzymatic digestion to retain the starch content of the sample.

The SCFA were analyzed by GC (Agilent 6890 system, Germany) fitted with a flame ionization detector and a fused-silica capillary column (ZB-FFAP, Phenomenex, USA), using trimethyl acetic acid as internal standard. Before injection, fermented samples were acidified by adding 25% metaphosphoric acid to make pH ~2.2. The following column conditions were used: initial temperature, 100 °C; initial hold time, 1 min; and final temperature, 200 °C with gradient, 8 °C/min; carrier gas, helium (flow rate, 1.9 ml/min). To obtain the final SCFA concentrations in the sample, gas chromatography readings were corrected for dilution. Branched-chain fatty acids (BCFA) were calculated as the sum of the iso-butyric and iso-valeric acids.

Total N and ^{15}N-enrichment in the freeze-dried pellets were measured by means of an elemental analyzer coupled with a Tracermass mass spectrometer (Europa Scientific of Crewe, UK) (Bindelle et al., 2007b).

2.5. Calculations and statistical analysis

2.5.1. In vitro degradability

The *in vitro* dry matter degradability (*IVDMD*) during the pepsin and pancreatin hydrolysis was calculated as follows:

$$IVDMD = \frac{\text{dry weight of the sample before hydrolysis} - \text{dry weight of the residue}}{\text{dry weight of the sample before hydrolysis}} \quad (1)$$

The disappearance of the other nutrients was calculated using the degradability coefficient of DM and the relative content of individual nutrients in the ingredients and hydrolyzed substrates. Degradability co-efficient of DM was used as reference for other nutrients as using any marker is not practical like the *in vivo* system.

2.5.2. Kinetics of gas production

Gas pressure measurements were converted into gas volume (G, g^{-1} DM) using to the ideal gas law, assuming an atmospheric pressure of 101,325 Pa and a temperature of 312.15 K. Gas accumulation curves recorded during the 48 h of fermentation were modelled according to France et al. (1993):

$$\begin{aligned} G \ (\text{ml g}^{-1}\ \text{DM}) &= 0, && \text{if } 0 < t < L \\ &= G_f(1 - \exp\{-\langle b(t-L) + c(\sqrt{t} - \sqrt{L})\rangle\}), && \text{if } t \geq L \end{aligned} \quad (2)$$

where G 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:

$$\mu = b + \frac{c}{\sqrt[2]{t}}, \quad \text{if } t \geq L \quad (3)$$

The kinetics parameters (G_f, L, $\mu_{t=T/2}$ and $T/2$) were compared in the statistical analysis. $T/2$ is the time to half-asymptote when $G = G_f/2$.

2.5.3. Measurement of N incorporation into microbial cells

Bacterial nitrogen incorporation (BNI, corresponding to N in the pellet incorporated from the buffer solution into the bacteria) per g of incubated hydrolyzed residue (DM) and per g of actually fermented polysaccharides (CHO, NSP + Starch) was calculated from total N and ^{15}N content according to the equations of Bindelle et al. (2007b), as follows:

Table 2
Degree of enzymatic hydrolysis of the ingredients.

Ingredient	N[a]	Degree of hydrolysis			
		IVDMD	Crude protein[b]	NSP[b,c]	Starch[b]
Wheat bran	13	0.63[d]	0.83	30	0.99
Solka-floc®	6	0.06[g]	NA	NA	NA
Peas	26	0.80[a]	0.96	36	0.96
Pea hulls	12	0.55[e]	0.96	25	0.92
Pea inner fibre	12	0.55[e]	0.89	26	0.94
Sugar beet pulp	10	0.48[f]	0.82	23	NA
Flax seed meal	18	0.70[b]	0.94	48	NA
Corn DDGS	16	0.67[c]	0.82	47	0.88
SEM		0.004			
P-value		<0.001			

Abbreviations: IVDMD, in vitro dry matter degradability; NSP, non-starch polysaccharides; NA, not available. Means with different superscript within the column are significantly different (P<0.05).

[a] Number of replicates of enzymatic hydrolysis.

[b] Values from pooled samples of different replicates of enzymatic hydrolysis, thus statistical analysis could not be conducted.

[c] Unrecovered non-starch polysaccharides.

$$\text{Bacterial Nitrogen Incorporation (BNI) (mg/g incubated DM)} = \left[\left(\frac{^{15}\text{N} * \text{N} * \text{Mpellet}}{0.003663} - \text{N} * \text{Mpellet}\right) / \left(\left(\frac{0.02}{0.003663} - 1\right) * W * \frac{V_{\text{skip}}}{G_f * 2}\right)\right] - \text{BNI inoculum} * \frac{V_0}{W} \quad (4)$$

where N (g/g) denotes the concentration of N in the pellet, Mpellet (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) the concentration of ^{15}N in total N of the pellet, V_0 (ml) the volume of inoculum transferred in the bottle at the start of the fermentation and W (gDM) the amount of substrate placed in the bottle and V_{skip} the volume of gas when fermentation was stopped at $T/2$.

The BNI was measured when half the final gas volume was produced ($T/2$) and after 48 h of fermentation.

2.5.4. Statistical analyses

IVDMD during hydrolysis, gas production kinetics and fermentation metabolites production were analyzed using the mixed procedure of SAS 9.1 software (SAS, 2003) with the ingredient as fixed factor and batch as random factor, using the following general linear model:

$$Y = \alpha + S_i + B_j + \varepsilon_{ij} \quad (5)$$

where Y is the parameter to be tested, α the mean, S_i the effect of the ingredient ($i = 1,\ldots,8$), B_j the random effect of the batch ($j = 1,\ldots,4$) and ε_{ij} the error term. Means were separated using Tukey method with a significance level of 0.05.

Pearson's correlation calculations between different variables were performed using CORR procedure of SAS 9.1 software (SAS, 2003).

3. Results

3.1. Degradability of nutrients during enzymatic hydrolysis

The *IVDMD* and degree of enzymatic hydrolysis of the ingredients are presented in Table 2. The *IVDMD* varied from one ingredient to another (P<0.001). The highest values were observed for peas (0.80), followed by FSM (0.70), while SF was almost indigestible (0.06). No statistical analysis could be performed on CP and starch degradability, because the samples were pooled from different batches of hydrolysis. Nevertheless, the CP degradability was similar in pattern as *IVDMD*, the highest values being obtained for peas and PH (0.96 each), followed by FSM (0.94), WB, SBP and DDGS (0.82–0.83). The results for starch degradability were different: WB had the highest starch degradability (0.99) followed by peas and PIF (0.96 and 0.94, respectively). The latter also showed the highest starch content in the hydrolyzed residues (80 and 71 g/kg DM, respectively).

Table 3
Fitted kinetics parameters (means) on the gas accumulation recorded for the different hydrolyzed substrates incubated with pig faecal inoculums.

Ingredient	Per g DM incubated					Per g CHO fermented		
	N^a	L^b	$T/2^c$	μ^d	G_f^e	N^f	μ^d	G_f^e
Wheat bran		2.83^a	12.4^b	0.07^c	124^f	8	0.07^d	639^{de}
Solka-floc®	8	NA	NA	NA	266^b	8	NA	608^e
Peas	8	1.33^d	10.23^b	0.10^b	253^c	8	0.11^c	1040^b
Pea hulls	8	3.59^a	12.85^a	0.10^b	276^a	8	0.11^c	713^{cde}
Pea inner fibre	8	1.46^{cd}	8.38^d	0.11^b	264^b	8	0.14^b	1050^b
Sugar beet pulp	8	2.91^b	9.39^c	0.20^a	237^d	8	0.16^a	834^c
Flax seed meal	8	1.75^c	10.66^b	0.10^b	130^f	8	0.11^c	753^{cd}
Corn DDGS	8	1.2^b	19.75^a	0.05^d	158^e	8	0.05^e	1199^a
SEM		0.289	0.519	0.006	1.9		0.003	32.6
P-value		<0.001	<0.001	<0.001	<0.001		<0.001	<0.001

Abbreviations: DM, dry matter; CHO, carbohydrates (non-starch polysaccharides + starch). NA, data not presented as these parameters did not fit in fermentation kinetics model used. Means with different superscripts within the columns are significantly different (P<0.05).

[a] Number of observations in fermentation.
[b] L, lag time (h).
[c] $T/2$, half-time to asymptote (h).
[d] μ, fractional rate of degradation (h^{-1}) at $t = T/2$.
[e] G_f, maximum gas volume (ml per g DM incubated or CHO fermented).
[f] Number of observations in pooled fermented substrates.

3.2. Kinetics of gas production

In general, the fermentation parameters varied according to the ingredient (P<0.05) (Table 3). Due to very slow fermentation at the beginning, followed by a sharp increase of the gas production curves, SF did not fit the mathematical model used and was excluded from the pool of statistical analysis for fermentation kinetics parameters. When expressed per g incubated DM, the fractional rate of degradation appeared to be lower (P<0.001) for WB and DDGS (0.07 and 0.05/h, respectively) and higher for SBP (0.20/h), with no difference between the other ingredients (0.10/h on average). The final gas production also differed between ingredients (P<0.001) with the highest values obtained for PH, SF and PIF (276, 266 and 264 ml/g DM incubated, respectively) and the lowest (P<0.05) for DDGS, FSM and WB (158, 130 and 124 ml/g DM incubated, respectively). However, the values changed markedly when expressed per g fermented CHO. In that case, the DDGS accounted for the highest gas volume (1199 ml/g CHO fermented), followed by PIF and peas (1050 and 1040 ml/g CHO fermented, respectively). The lowest value was obtained for SF (608 ml/g CHO fermented). Peas started fermentation sooner (lag time 1.3 h) while the half gas production was achieved sooner for PIF (8.4 h). DDGS took the longest time to reach $T/2$ for total gas produced (19.8 h) and PH had the highest lag time (3.6 h). Interestingly, SF showed one of the slowest fermentation but produced one of the highest final gas volumes by the end of fermentation time.

3.3. Profile in fermentation end-products of the different substrates

There was no difference (P>0.05) in SCFA production after fermentation of SF, peas, PH, PIF and SBP (ranging from 3.8 to 4.5 mmol/g DM incubated) while less (P<0.05) SCFA was produced with WB and FSM (2 and 2.4 mmol/g DM incubated, respectively) (Table 4). When expressed per g fermented CHO, no difference (P>0.05) in SCFA production was observed between ingredients.

Table 4
Concentration of metabolites (mmol/g DM incubated or CHO fermented) from different fibre sources after 48 h fermentation.

Ingredient	mmol/g DM incubated						mmol/g CHO fermented	
	N^a	SCFA	ACE[b]	PRO[b]	BUT[b]	BCFA[b]	N^a	SCFA
Wheat bran		2.0^b	0.65^c	0.20^{cd}	0.11^b	0.020^{ab}	8	17
Solka-floc®	8	3.9^a	0.80^a	0.10^e	0.09^{cd}	0.000^e	8	11
Peas	8	4.0^a	0.61^{de}	0.22^b	0.13^a	0.017^{bc}	8	35
Pea hull fibre	8	4.5^a	0.70^b	0.19^d	0.08^d	0.005^{de}	8	19
Pea inner fibre	8	4.4^a	0.60^e	0.26^a	0.11^b	0.011^{cd}	8	34
Sugar beet pulp	8	3.8^a	0.69^b	0.21^{bc}	0.07^e	0.010^{cd}	8	21
Flax seed meal	8	2.4^b	0.62^d	0.27^a	0.07^e	0.017^{bc}	8	29
Corn DDGS	8	3.3^{ab}	0.66^c	0.20^d	0.10^c	0.027^a	8	37
SEM		0.30	0.004	0.004	0.003	0.0025	8	7.4
P-value		<0.001	<0.001	<0.001	<0.001	<0.001		<0.001

Abbreviations: DM, dry matter; CHO, carbohydrates (non-starch polysaccharides + starch); SCFA, short-chain fatty acids; ACE, acetic acid; PRO, propionic acid; BUT, butyric acid; BCFA, branched-chain fatty acids (sum of iso-butyric and iso-valeric acids). Means with different superscripts within the columns are significantly different (P<0.05).

[a] Number of observations in fermentation.
[b] Molar ratio of the individual SCFA.

In terms of SCFA profile, SF produced the highest acetic acid (ACE) and one of the lowest butyric acid (BUT) concentration (0.80 and 0.09, respectively). Peas and PIF had the lowest ACE concentration (0.61 and 0.60, respectively) and the highest propionic acid (PRO) and BUT concentrations (0.13 and 0.11, respectively). The highest concentration of BCFA was found in DDGS and WB (0.027 and 0.02, respectively) while no BCFA was detected in the fermented solutions of SF.

3.4. Bacterial nitrogen incorporation

Bacterial nitrogen incorporation both at $T/2$ and after 48 h of fermentation was the highest for PIF (18.5 and 15.6 mg/g DM incubated, respectively) and the lowest for DDGS and WB, both at $T/2$ and 48 h of fermentation. A similar trend was found when BNI was expressed per g fermented CHO, with the highest value obtained for PIF both at $T/2$ and 48 h of fermentation. The lowest BNI were obtained for SF at $T/2$ and for WB at 48 h of fermentation.

3.5. Correlations between carbohydrate components, fermentation parameters and bacterial nitrogen incorporation

There was a strong negative correlation observed between the ADF and NDF content and *IVDMD* ($r = -0.94$ and -0.93, respectively, $P<0.001$). A similar relationship was found between the NDF content and CP degradability ($r = -0.54$, $P<0.001$) and between ADF and NDF content with starch degradability ($r = -0.58$ and $r = -0.45$, respectively, $P<0.001$). A positive correlation was obtained between the ADF and NDF content of the ingredients and the lag time of fermentation ($r = 0.62$ and $r = 0.63$, respectively, $P<0.001$).

There was also a positive correlation obtained between the total amount of gas produced and BNI (per g DM incubated), both at $T/2$ and 48 h of fermentation ($r = 0.58$ and 0.80, respectively, $P<0.001$), and a similar relationship was found between the SCFA concentration and BNI, both at $T/2$ and 48 h of fermentation ($r = 0.45$ and $r = 0.62$, respectively, $P<0.001$).

4. Discussion

This study aimed to explore the fermentation characteristics, with a special focus on bacterial nitrogen incorporation of some fibrous ingredients with potential to be incorporated in pig diets using an *in vitro* model. The ingredients varied widely in composition, especially in their fibre fractions and indigestible protein content, which affected the studied parameters like *IVDMD*, fermentation kinetics and metabolites produced, as well as the bacterial protein synthesis.

Unlike previous research where fermentation is generally stopped after 72 h, we ceased fermentation after 48 h. This time period may have been too short to permit the fermentation of some slow-fermenting substrates, however, the purpose for choosing 48 h was to mimic conditions found in the pig gastrointestinal tract. According to Wilfart et al. (2007), the average transit time of a substrate in the pig gastrointestinal tract is around 44 h, including the proximal part, which leaves more or less 40 h in the large intestine. As expected, while mimicking the upper gut enzymatic digestion *in vitro*, *IVDMD* was largely influenced by the type and amount of fibre, especially the ADF and NDF content of the ingredients. The high *IVDMD* observed for peas and the low *IVDMD* obtained for SF can be ascribed to their respective insoluble fibre content, which is reflected by the strong negative correlation observed between the ADF and NDF content and *IVDMD* ($r = -0.94$ and -0.93, respectively, $P<0.001$). However, the variation in the ADF and NDF content of different ingredients can, to some extent, affect the *IVDMD*. A similar relationship was found between the NDF and CP degradability ($r = -0.54$, $P<0.001$) and between ADF and NDF with starch degradability ($r = -0.58$ and $r = -0.45$, respectively, $P<0.001$). These negative relationships between fibre content and the degradability of nutrients is probably due to the presence of hulls, which minimises the accessibility of the enzymes to their substrates (Bach Knudsen, 2001).

These differences in enzymatic hydrolysis and resulting *IVDMD* induced differences in kinetics, metabolite profiles and BNI during fermentation. This variation can be ascribed to their NSP content, as well as to the amount of RS and CP available for fermentation, which might be as important as the NSP fraction. The starch embedded in the fibre matrix might be inaccessible to the pig's digestive enzymes. Amylose is also more resistant to enzymatic hydrolysis. The starch of WB is well digested by the porcine enzymes (Bach Knudsen and Canibe, 2000) while that of peas is less digestible, due to the higher level of amylose (Le Guen et al., 1995; Wiseman, 2006). Thus, more RS is available from peas for microbial fermentation, as evidenced in this study.

Conversely to starch, the CP enzymatic digestibility of the cereal products (WB and DDGS) was lower than that of pea products. This is a consequence of the high proportion of protein entrapped within the aleuronic layer of the grains' bran. In corn DDGS, the high indigestible protein content might also be due to the Maillard reactions occurring during the distillation process, which binds the free sugars that were not fermented to the proteins (Pierce and Stevenson, 2008).

In the *in vitro* method, the rates of fermentation of the indigestible ingredients and the subsequent SCFA and gas production are reflected by the gas production measurements. The pea NSP were previously found to be more fermentable than those of WB (Goodlad and Mathers, 1991). The difference in the fermentation kinetics and products found between ingredients can be ascribed to the nature of their respective fibre fractions. Fermentation kinetics derived from the *in vitro* gas production technique may inform on the site of fibre fermentation in the gastrointestinal tract of animals. For example, the degradation of the CHO fraction of peas occurs throughout the large intestine, as the soluble and pectic polysaccharides of the cotyledon are readily available to the microbiota present in the distal small intestine and in the proximal segments of the large intestine while slowly degradable fractions are available at the distal segments. On the other hand, acidic xylans

Table 5
Bacterial nitrogen incorporation obtained for different fibre sources measured after one-half the final gas volume was produced (*T*/2) and 48 h of fermentation.

Cultivars	*T*/2			48 h		
	N[a]	mg/g DM incubated	mg/g CHO fermented	*N*[a]	mg/g DM incubated	mg/g CHO fermented
Wheat bran		6.5^{d}	52.9^{cd}	4	4.1^{d}	22.2^{d}
Solka-floc®	8	10.9^{c}	30.6^{d}	4	12.7^{b}	29.1^{cd}
Peas	8	11.9^{c}	106.0^{b}	4	9.5^{c}	39.6^{bc}
Pea hull fibre	8	14.4^{b}	60.0^{c}	4	13.6^{ab}	36.0^{bc}
Pea inner fibre	8	18.5^{a}	133.9^{a}	4	15.6^{a}	62.6^{a}
Sugar beet pulp	8	14.7^{b}	79.3^{bc}	4	12.2^{b}	43.4^{b}
Flax seed meal	8	7.6^{d}	72.2^{c}	4	6.3^{d}	37.0^{bc}
Corn DDGS	8	5.6^{d}	78.3^{bc}	4	5.2^{d}	40.1^{bc}
SEM		0.86	7.53		0.82	2.98
P-value		<0.001	<0.001		<0.001	<0.001

Abbreviations: DM, dry matter; CHO, carbohydrates (non-starch polysaccharides + starch). Means with different superscripts within the columns are significantly different (P<0.05).

[a] Number of observations in fermentation.

and cellulose from pea hulls are fermented more distally (Canibe et al., 1997). As shown in this experiment by the lower rates of fermentation, these types of fibre are slowly depolymerised and become available to microbiota for fermentation at the distal part of the large intestine. This is consistent with the positive correlation obtained here between the ADF and NDF content of the ingredients and the lag time of fermentation ($r = 0.62$ and $r = 0.63$, respectively, P<0.001) as well as with the slow fermentation and lower level of SCFA produced per g CHO fermented for SF.

Differences in the concentration of individual SFCA reflect the type of substrate fermented as well as microbial diversity. According to Macfarlane and Macfarlane (2003), more propionate and less ACE is produced in presence of a diverse microbiota and high substrate availability. Higher butyrate and lower ACE concentration obtained after fermentation suggests that the fibrous residues of pea fibres enhanced bacterial proliferation, as indicated by their BNI (Table 5). This might have yielded a more diverse community, probably linked to the level of soluble NSP fractions in the residues (Pieper et al., 2009). A reverse scenario of substrate type available for fermentation explains the higher acetate and lower butyrate production obtained for SF fibre. There was a high bacterial nitrogen uptake found with high SCFA producing fibre sources like peas and pea fibres along with SBP, which is similar to the findings of Bindelle et al. (2009), suggesting that, in presence of higher levels of CHO substrate for fermentation, the resident microbiota in the large intestine retain more N for their own growth. This finding was also supported by the positive correlation obtained between the total amount of gas produced and BNI (per g DM incubated), both at *T*/2 and 48 h of fermentation ($r = 0.58$ and 0.80, respectively, P<0.001) and also by the similar relationship obtained between the SCFA concentration and BNI, both at *T*/2 and 48 h of fermentation ($r = 0.45$ and $r = 0.62$, respectively, P<0.001). However, the efficiency of bacterial protein synthesis is not only affected by the degree of fibre fermentation, but also by the sugar components available for fermentation, as was evidenced by the difference in ranking of the total gas and SCFA produced by different ingredients when expressed per g DM incubated or fermented. This is supported by the findings by Kirchgessner et al. (1994), where an increase in excretion of all the nitrogenous fractions was reported with different levels of NSP degradation, without significant changes in the faecal N composition.

Our results show that DF has a major impact on BNI. However, there was a clear impact of the amount of indigestible protein in the substrates as well. Moreover, other N sources from protein degradation might also have been utilized by bacteria. There was higher BNI, both at *T*/2 and 48 h of fermentation, with the peas, pea fibres and SBP. All these ingredients have a relatively low CP:NSP ratio. However, a high level of fermentable DF will not necessarily result in an increased N uptake by intestinal bacteria. Meanwhile, the DDGS contained high levels of iCP which were not well fermented but contributed to the N excretion. This is in the line with previous findings of Canh et al. (1998) and Sutton et al. (1999) that the source of DF in the diet can significantly affect N excretion. However, there is an interaction effect of DF and iCP, as shown by our study. Some fibrous feedstuffs of this study have a high indigestible CP content, as CP is partially bound to the fibrous matrix and becomes unavailable for enzymatic digestion. It also plays a role in N excretion pathways, which is similar with the findings of Le et al. (2008). Fibrous feed ingredients may thus bring more indigestible N to the diet, which will eliminate the advantage gained by the presence of fermentable DF. Thus, the advantage of fermentable DF on BNI and ammonia concentration will depend on the overall composition of the feed ingredients, especially the ratio of fermentable DF and iCP.

Ingredients with low fermentable fibre and high CP residues after enzymatic hydrolysis (WB, DDGS) yielded lower BNI and a higher proportion of BCFA, which can be ascribed to the shift of fermentation to protein (Macfarlane and Macfarlane, 2003), when there is depletion of carbohydrate substrate available for microbiota (Piva et al., 1996) or when the ratio between carbohydrate and protein substrates available to microbes as energy source is unbalanced in the medium. Thus, it becomes important to stimulate carbohydrate fermentation to minimise the growth of potential protein fermenting bacteria (Macfarlane and Macfarlane, 1995) which can lead to the production of toxic substances like ammonia, amines, short-chain phenols and indoles (Macfarlane et al., 1992).

When expressed per g CHO fermented, the DDGS ranked in the top for both the total gas and the SCFA productions. It is basically due to the artifact that DDGS contain high levels of indigestible protein. The latter is fermented and yields carbon

dioxide and SCFA, which account for gas production, but it is not considered in the fermented CHO. The same was true for the high BNI expressed per g CHO fermented found with DDGS. Conversely, there is a decrease in protein fermentation when the level of NSP for microbial fermentation is increased (O'Connell et al., 2005), as was found in the peas and pea fibres during this study. There was higher BNI with these ingredients, containing higher levels of soluble fibre. At the energy balance state, resident bacteria obtain energy from fermentable fibre and N from dietary protein residue as well as endogenous nitrogen to synthesise their biomass in the gut. Thus, not only the fibre fraction but also the ratio of available CHO and protein source become important for bacterial protein synthesis.

In conclusion, the fermentation kinetics, the profile in metabolic products and the bacterial protein synthesis were influenced by the amount and type of fibre fractions and iCP content in the ingredients. Peas and pea fibres were highly fermentable, which produced higher amounts of SCFA and had high bacterial protein synthesis capacity, as indicated by bacterial nitrogen incorporation. They thus have the potential to be included in pig diets as a source of fermentable fibre to modulate the gut environment and reduce nitrogen excretion.

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