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

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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 four dilution levels in a buffer solution (0.025, 0.05, 0.1 and 0.2 g ml^{-1}) with two 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 *versus* 8.0 h, $P<0.02$), but the 2 inocula yielded similar fractional rates of degradation (0.16h^{-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

1. 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.

2. Materials and methods

2.1. 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.

2.1.1. 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.

2.1.2. 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 1.

2.1.3. Inocula

In order to reduce variation between animals, the inocula were prepared by mixing the intestinal content or the faeces of the three pigs. The samples were collected directly in plastic bags saturated with CO_2 and immediately placed in a water-bath at 39 °C. 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 s to a mechanical pummelling with a Stomacher Lab Blender 400 (Seward Medical, Norfolk, UK) to suspend fibre-associated bacteria in the liquid (Merry and MacAllan, 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).

2.1.4. In vitro fermentation

Table 1. *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	-

Two hundred milligrams 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 h of incubation. Three syringes per inoculum containing just inoculum (blanks) were systematically included for each run.

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

2.1.5. Chemical analysis

The substrates were analyzed for their content in dry matter (105 °C for 24 h), ash (550 °C for 8 h), nitrogen (Kjeldahl method, crude protein = 6.25 × N content), ether extract (Soxhlet method, using ether), neutral (using Na_2SO_3 and Termamyl: 120KNU/g, Novo Nordisk, Bagsværd, Denmark) and acid detergent fibres and lignin, using the Fiber-cap system (Foss Electric, Bagsvaerd, Denmark). Starch was determined, after grinding the samples through a 0.5mm-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).

2.1.6. Calculations and statistical analysis

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

$$V_{(t)}^{\mathrm{corr}} = \frac{V(t) - V_0 - B(t) \times V_0}{W}$$

where $V_{(t)}^{\mathrm{corr}}$ (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):

$$G = \begin{cases} 0 & \text{if } 0 < t < L \\ G_{\mathrm{f}}(1 - \exp\{-\langle b(t-L) + c(\sqrt{t} - \sqrt{L})\rangle\}) & \text{if } t \geq L \end{cases}$$

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:

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

In addition to the kinetics parameters G_{f} and L, the *T/2* (time to half asymptote when $G = G_{\mathrm{f}}/2$) 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:

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

where Y is the result, α the mean, S_{i} the fixed effect of the substrate (i=l, 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.

2.2. Experiment 2: enzymatic hydrolysis

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

2.2.1. Animals and diets

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

2.2.2. Substrates

The following feedsuffs were tested: maize (*Zea mays*), wheat bran (*T. aestivum*), peas (*Pisum sativum*), sugar-beet pulp (*B. 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 Section 2.1. Their composition is detailed in Table 1.

2.2.3. 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 0.5ml 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 no. 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 ml × 10 ml 95 % ethanol) and acetone (2 ml × 10 ml 99.5% acetone), dried for 24 h at 60 ± 1 °C and weighed. Each substrate was tested 24 times (8 replicates × 3 periods).

2.2.4. 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 two periods. For each period, six syringes per substrate (+3 blanks) were incubated at the same time, three containing the non-hydrolysed substrate, three containing the hydrolysed substrate.

2.2.5. Calculations and statistical analysis

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

$$\mathrm{dDM} = \frac{\text{weight of the sample before hydrolysis} - \text{weight of the residue}}{\text{weight of the sample before hydrolysis}}$$

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.

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

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

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).

3. Results

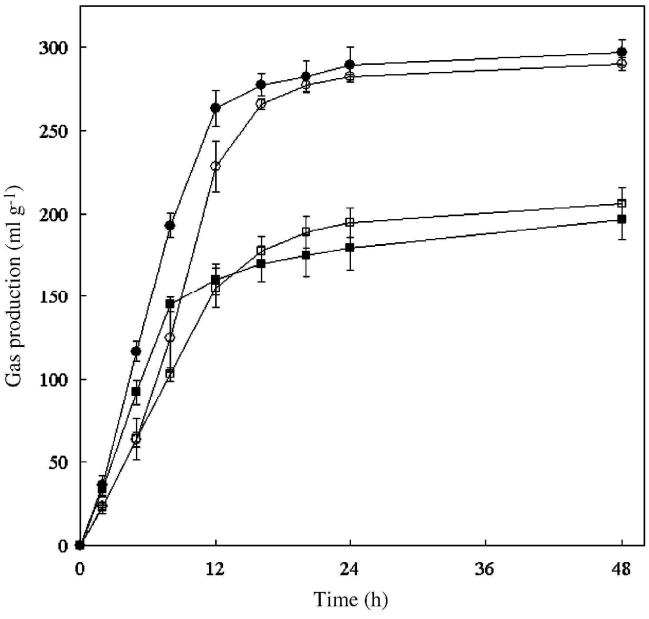
3.1. Experiment 1

The gas accumulation curves ($V_{(t)}^{corr}$) 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 Fig. 1. 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 2. 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).

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.

Fig. 1. *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 (O), wheat bran incubated with large intestine content (■) and wheat bran incubated with faecal inocula (□) (0.05 g ml^{-1} buffer).*



3.2. 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 3. 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).

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.

Table 2. *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[a]	L[b]	$\mu_{t=T/2}$[c]	$T/2$[d]	G_f[e]
Inocula	Large intestine	47	1.5 NS[f]	0.17 NS	5.5 b[g]	250 NS
	Faeces	45	1.3 NS	0.14 NS	8.0 a	253 NS

Main effects		N[a]	L[b]	$\mu_{t=T/2}$[c]	**Substrates**		G_f[e]
					Sugar-beet pulp	**Wheat bran**	
Dilutions	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	2.1a	0.13 b	9.0 a	8.5 a	247 b

	d.f.[h]	**P-values**			
Source of variation					
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 × inoculum	1	0.081	0.259	0.861	0.052
Substrate × dilution	3	0.291	0.491	0.029	0.198
Inoculum × dilution	3	0.356	0.656	0.081	0.676
Substrate × inoculum × dilution	3	0.991	0.800	0.914	0.383
Variance parameter estimates					
Period × inoculum		0.157	0.0004	0.120	29.8
Residual		0.445	0.0009	0.389	112.2

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

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; hemi-cellulose: r = -0.899, P<0.05).

Table 3. *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[a]	**dDM**	N_2[b]	L[c]	$\mu_{t=T/2}$[d]	$T/2$[e]	G_f[f]
Non-hydrolysed	Lupins		-	6	7.3 a[g]	0.11 c	13.7 a	331 a
	Maize		-	5	7.1 a	0.16 b	11.1b	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[h]	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
	P-value		**d.f.**[h]	**P-values**				
Source of variation								
Hydrolysis		-	1	<0.001	<0.001		<0.001	0.108
Substrate	<0.001		5	<0.001	<0.001		<0.001	<0.001
Substrate × 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

[a] *N_1, number of observations for the hydrolysis.*
[b] *N_2, number of observations for the fermentation.*
[c] *L, lag time (h).*
[d] *μ_t=T/2, fractional rate of degradation at t = T/2(h^{-1}).*
[e] *T/2, half-time to asymptote (h).*
[f] *G_f, maximum gas volume (mlg^{-1}).*
[g] *For one parameter, averages followed by different letters in the columns differ at significance level of 0.05.*
[h] *d.f., degrees of freedom.*

4. 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 (7-10%) and stabilised at around 3-4% after 20 h (Fig. 1).

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 2). 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 fermentations 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 0.025g ml^{-1} compared to the three other dilutions (0.2, 0.1 and 0.05g ml^{-1}), *i.e.*, 1.37 *versus* 2.22 ml g^{-1} for faecal samples and 2.39 *versus* 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 3). 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 3) can be explained by their lower content in rapidly fermentable components such as free sugars or soluble fibre (Macfarlane and Macfarlane, 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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