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INFLUENCE OF INOCULUM TYPE (ILEAL, CAECAL AND FAECAL) ON THE *IN VITRO* FERMENTATION OF DIFFERENT SOURCES OF CARBOHYDRATES IN RABBITS

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Abstract: Two *in vitro* experiments were performed to analyse the fermentative potential of ileal content, caecal content, soft faeces and hard faeces from adult rabbits. Experiment 1 evaluated 3 doses (0.5, 1.0 and 2.0 g fresh digesta/g substrate dry matter [DM]) of ileal and caecal digesta as inoculum in 28 h-incubations. Two ileal and 2 caecal *inocula* were obtained, each by pooling the ileal or caecal digesta of 2 adult rabbits. Pectin from sugar beet pulp (SBP) and the insoluble residue obtained after a 2-step *in vitro* pre-digestion of SBP and wheat straw were used as substrates. The 0.5 dose produced the lowest ($P<0.05$) amount of gas at 28 h, with no differences ($P>0.05$) between the 1.0 and 2.0 doses (44.9, 51.6 and 53.8 mL/g substrate DM, respectively; values averaged across *inocula* and substrates). Experiment 2 evaluated two doses of ileal inoculum (1 and 1.5 g fresh digesta/g substrate DM) and compared ileal digesta, caecal digesta, soft faeces and hard faeces as inoculum for determining *in vitro* gas production (144-h incubations) of the 3 substrates used in Experiment 1 and wheat starch. Three *inocula* of each type were obtained, each by pooling either digesta or faeces from 3 rabbits. There were no differences ($P>0.05$) between the 2 ileal doses tested in gas production parameters, and therefore the 1.0 dose was selected for further ileal fermentations. Starch and pectin showed similar ($P>0.05$) values of gas production rate and maximal gas production rate when they were fermented with caecal digesta (0.038 *vs*. 0.043%/h, and 13.7 *vs*. 15.2 mL/h, respectively), soft (0.022 *vs*. 0.031%/h, and 9.97 *vs*. 9.33 mL/h) and hard faeces (0.031 *vs*. 0.038%/h, and 13.6 *vs*. 10.8 mL/h), and values were higher than those for SBP and wheat straw; in contrast, values for starch and pectin differed with the ileal inoculum (0.046 *vs*. 0.024%/h, and 18.4 *vs*. 6.60 mL/h). Both ileal and caecal gas production parameters were well correlated with those for hard and soft faeces *inocula*, respectively ($r\geq0.77$; $P\leq0.040$). The ileal inoculum showed a relevant fermentative potential, but lower than that of caecal digesta and soft and hard faeces for all substrates except wheat starch.

Key Words: *In vitro,* gas production, sugar beet pulp, pectin, wheat straw, starch, rabbit.

INTRODUCTION

The *in vitro* gas production technique (Menke *et al*., 1979) has been widely used over the past 3 decades for feed evaluation in ruminants, and more recently has been adapted to nutritional research in non-ruminant animals (Williams *et al.,* 1995; Williams *et al.,* 2001). This technique is relatively simple and inexpensive, and can help to understand the gut physiology and improve intestinal health by selecting feed ingredients that enhance the beneficial gut microbiota (Williams *et al.,* 2001). Although this *in vitro* technique does not quantitatively resemble the *in vivo* process, there is a significant correlation between the *in vivo* and *in vitro* results (Williams *et al.,* 2005). The use of this methodology in rabbits has been mainly focused on caecal fermentation (Piattoni *et al.,* 1997; Calabrò *et al.,* 1999; Bovera *et al.,* 2006), although the procedure still lacks standardisation.

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The digestion of fibre in rabbits primarily occurs at caecal level, although a significant ileal digestion has also been reported in some studies (Gidenne, 1992; Carabaño *et al.,* 2001). Abad-Guamán *et al.* (2015) observed an appreciable digestibility of both soluble and insoluble fibre at ileal level, which contrasts with the low fibrolytic activity found in the ileal digesta (Marounek *et al.,* 1995) and the short retention time of digesta in the small intestine (Gidenne, 1994; García *et al.,* 1999). The observed ileal digestibility could be partly explained by a partial hydrolysis of the fibre (soluble and insoluble), which would prevent the recovering of unfermented fibre in the analysis of ileal digesta. The *in vitro* gas production technique could be used to identify differences in fermentation pattern using substrates differing in their soluble and insoluble fibre content. The first aim of this study was therefore to assess the *in vitro* gas production of substrates with a wide range of fermentability by using ileal digesta from rabbits as inoculum. In addition, the fermentative potential of ileal digesta was compared to that of caecal digesta, soft faeces and hard faeces. As no studies on *in vitro* incubations using rabbit ileal digesta as inoculum were found in the literature and only a low amount of ileal digesta can be obtained from a single animal, a preliminary experiment was carried out to establish an adequate dose of ileal digesta for *in vitro* incubations.

MATERIALS AND METHODS

All procedures involving animals were carried out in accordance with the Spanish guidelines on experimental animal protection (BOE, 2013). Two *in vitro* experiments were conducted using the same basal substrates.

Substrates for in vitro *incubations*

Four sources of carbohydrates differing in their rate and extent of fermentation were selected. The substrates used were wheat starch (99%; SIGMA S-5127; Sigma–Aldrich Quimica, S.A, Alcobendas, Spain), sugar beet pulp (SBP; Fipec®, Nordic Sugar, Copenhagen, Denmark), pectin from sugar beet pulp (Betapec RU 301, Herbstreith & Fox, Neuenbürg, Germany) and wheat straw (Pagran, PITE S.A., Tordesillas, Spain). Most chemical constituents of sugar beet pulp, pectin and wheat straw cannot be digested by endogenous enzymes of rabbits, but starch can be digested and is also a rapid and completely fermentable substrate, and was therefore included as a reference. The chemical composition of the substrates is shown in Table 1. Both SBP and wheat straw were subjected to a predigestion procedure to simulate the digestion in the stomach and small intestine, as described by Abad *et al.* (2013) and using ANKOM filter bags (F57; 25 µm pore size; Ankom Technology, New York, USA). Briefly, samples were first incubated in a pepsin solution (2000 FIP-Units/g protein, Merck n7190, Merck, Darmstadt, Germany; 25 mg pepsin/mL 0.2 *M* HCl; pH=2.0) at 40°C for 1.5 h, and then in a pancreatin solution (Grade VI, Sigma 1750, Sigma–Aldrich Quimica,

Table 1: Chemical composition of the raw ingredients and the substrates (pectin and residue from predigestion of sugar beet pulp and wheat straw) used for *in vitro* fermentations (g/kg DM).

	Raw materials[1]			Residue from predigestion	
	Sugar beet pulp	Pectin	Wheat straw	Sugar beet pulp	Wheat straw
DM (g/kg)	935	905	951	1000	1000
Ash	51.0	42.0	79.1	40.7	16.5
Total dietary fibre	646	934	785	-	-
aNDFom-cp	369	6.4	748	484	857
ADFom	244	1.5	425	-	-
Lignin (sa)	9.14	0	46.5	-	-
Total soluble fibre[2]	278	928	37.2	-	-
Crude protein	86.3	53.1	28.0	52.9	33.5

DM: dry matter; aNDFom-cp; neutral detergent fibre analysed using a thermostable amylase, without sodium sulphite added and expressed exclusive of ash and protein; ADFom: acid detergent fibre expressed exclusive of ash and protein; lignin (sa): analysed by the gravimetric sulphuric acid lignin method.

[1] Sugar beet pulp (SBP; Fipec®, Nordic Sugar, Copenhagen, Denmark); Pectin from sugar beet pulp (Betapec RU 301, Herbstreith & Fox, Neuenbürg, Germany); Wheat straw (Pagran, PITE S.A., Tordesillas, Spain).

[2] Calculated as total dietary fibre - aNDFom-cp.

S.A, Alcobendas, Spain; 100 mg pancreatin/mL phosphate buffer; pH=6.8) for 40°C for 3.5 h. The indigestible residue was used as substrate for the *in vitro* incubations. This approach has been used previously in other studies (Bindelle *et al.,* 2007; Rodríguez-Romero *et al.,* 2011; Abad *et al.,* 2013).

Experiment 1: Dose of ileal and caecal inoculum

The objective of this experiment was to assess different doses of ileal and caecal digesta as inoculum for the *in vitro* incubations. The substrates were pectin and the insoluble and indigestible residue obtained after the pre-digestion procedure of both SBP and wheat straw. The ileal and caecal content were obtained from four adult New Zealand White×Californian rabbits (4.1±0.39 kg body weight). Rabbits were fed *ad libitum* a commercial diet (Cunilactal, NANTA, S.A., Madrid, Spain) containing 158 g crude protein, 357 g aNDFom-cp (neutral detergent fibre analysed using a thermostable amylase, without sodium sulphite added and expressed exclusive of ash and protein) and 161 g starch per kg of dry matter (DM). Animals were slaughtered at 9:00 h by concussion, their total digestive tracts were removed and the ileal and caecal contents were collected. Two ileal and 2 caecal *inocula* were obtained by mixing the ileal and caecal content, respectively, of 2 rabbits (pooled digesta), and each substrate was incubated with all *inocula*. Samples of ileal and caecal digesta were taken to determine their DM content.

Samples of each substrate (200 mg DM) were weighed into 60 mL serum vials. Each inoculum was mixed with Goering and Van Soest buffer solution (1970; no trypticase added) in 3 different proportions: 0.5, 1.0 and 2.0 g per 100 mL to achieve ratios of 0.5, 1.0 and 2.0 g of fresh digesta per g substrate DM, respectively. The mixtures were homogenised with a blender for 2 min and 20 mL were added into each vial using a peristaltic pump (Watson-Marlow 520UIP31; Watson-Marlow Fluid Technology Group, Cornwall, United Kingdom). Vials were sealed with rubber stoppers and incubated at 39°C for 28 h. Preparation of the buffer solution, its mixture with the *inocula*, and vials filling were conducted at 39°C under continuous flushing with CO_2. A total of 72 vials with substrate (2 types of inoculum×3 inoculum:buffer ratios×2 replicates×3 substrates×2 vials/substrate) and 24 vials without substrate (blanks; 2 vials for each combination of type of inoculum, inoculum:buffer ratio and replicates) were incubated.

Gas production was measured using a pressure transducer (Wide Range Pressure Meter; Sper Scientific LTD, Scottsdale, AZ, USA) and a plastic syringe at 2, 6, 10, and 28 h. At each measurement time, the gas in the headspace of the vials was removed using the syringe until pressure was 0 and released. After 28 h of incubation, vials were opened and the pH was measured using a pH-meter basic 20 (Crison Instruments, Alella, Barcelona, Spain). Then vials were placed in iced water to stop fermentation and their content was transferred to previously weighed filter crucibles (pore size 100-160 µm) and filtered under vacuum. The residue of the incubation was washed with 10 mL of distilled water and dried at 103°C for 24 h to determine the DM disappearance (DMD) of the substrates. The residue was then analysed for ash to calculate the organic matter disappearance (OMD) after 28 h of incubation.

Experiment 2. Influence of inoculum type

The 3 substrates used in Experiment 1 and wheat starch were used to compare the *in vitro* fermentative activity of ileal, caecal and soft and hard faeces. Ileal and caecal digesta, soft faeces and hard faeces were obtained from 9 New Zealand White×Californian fattening rabbits of 70 d of age (2.2±0.23 kg body weight) fed *ad libitum* the same diet used in Experiment 1. At 9:00 h, hard faeces were taken from a tray which had been placed below the cage the previous day at 21:00 h. Rabbits were then slaughtered by concussion, the digestive tracts were removed and the ileal and caecal contents and soft faeces in the rectum were collected. Three different *inocula* of each type (ileal, caecal, soft faeces and hard faeces) were obtained by pooling the digesta from 3 rabbits, and samples were taken to determine their DM content. Caecal digesta, soft faeces and hard faeces were diluted with Goering and Van Soest buffer solution (1970; no trypticase added) in ratios of 1.0, 0.755 and 0.415 g of fresh digesta per 100 mL buffer solution, respectively, and homogenised with a blender for 2 min. The amounts of inoculum were selected to supply 0.050 g DM of each inoculum per vial based on previous results on DM content of caecal digesta, soft faeces and hard faeces (Carabaño *et al.,* 1988; García *et al.,* 2000). Ileal digesta was diluted with the same buffer in 2 proportions: 1.0 and 1.5 g of fresh digesta per 100 mL and homogenised with a blender for 2 min. These proportions were selected from the results of Experiment 1. The incubation procedure was as described in Experiment 1, but the vials were incubated for 144 h. Gas production was recorded as before described at 1, 2, 3, 4, 6, 10,

12, 16, 21, 26, 36, 48, 60, 72, 96, 120, and 144 h. This long incubation time was chosen to reach the potential gas production for all substrates. At the end of fermentation, vials were processed as described in Experiment 1 to determine final pH, DMD and OMD. A total of 120 vials with substrate (5 combinations of type and dose of inoculum×3 replicates×4 substrates×2 vials/substrate) and 30 vials without substrate (blanks; 5 combinations of type and dose of inoculum×3 replicates×2 vials/replicate) were incubated.

Chemical analyses

The AOAC procedures (2000) were used to determine DM (method 934.01), ash (method 942.05), crude protein (method 968.06), and total dietary fibre (method 985.29). The filter bag system (ANKOM Technology, Macedon, NY, USA) was used to analyse aNDFom-cp according to Mertens *et al.* (2002) using a thermostable amylase and without sodium sulphite added. Values were corrected for ash and protein. Dietary ADFom (acid detergent fibre expressed exclusive of ash and protein) and lignin (analysed by the gravimetric sulphuric acid lignin method [sa]) were analysed according to the AOAC (2000; method 973.187) and Van Soest *et al.* (1991), respectively. The soluble fibre content was calculated as the difference between total dietary fibre and aNDFom-cp, both corrected for ash and protein.

Calculations and statistical analysis

The values of gas produced at each measurement time were corrected for the amount of gas produced in the corresponding blanks. Values measured in the 2 vials incubated for each inoculum type, substrate and dose were averaged before statistical analysis.

In Experiment 1, the values of accumulative gas produced at different times of incubation were analysed using a mixed model for repeated measurements (SAS, 2011). The model included as a fixed source of variation the type of inoculum (ileal *vs.* caecal), dose (0.5, 1 and 2 g), substrate (pectin, SBP and wheat straw), time (3, 6, 10 and 28 h), and their interactions. A compound symmetry structure was fitted because it showed the lowest value of the Schwarz Bayesian criterion (Littell *et al.,* 1998). The model for pH, DMD and OMD included as a fixed source of variation the type of inoculum, dose, substrate (pectin, SBP and wheat straw), and their interactions. In all cases, the pooled sample of digesta used as inoculum was the replicate and it was included as a random variable. The data are presented as least squared means, and when a significant effect ($P<0.05$) was detected, means were compared using a protected t-test.

In Experiment 2, gas production values were fitted to the logistic model described by Schofield *et al.* (1994):

$$Y_t = \frac{V_f}{1 + e^{[2-4k(t-L)]}}$$

where Y_t is the gas produced (mL/g DM) at time t, V_f is the asymptotic gas production, k is the fractional rate of gas production, and L is the initial delay in the onset of gas production. The parameters V_f, k and L were estimated by an iterative least squares procedure (Marquardt algorithm) using the NLIN procedure of SAS (SAS, 2011). The maximum gas production rate (μ_m) and the time when μ_m is reached (t_i) were calculated according with Schofield *et al.* (1994) as:

$$\mu_m = k \times V_f$$

$$t_i = L \times \frac{V_f}{2 \times \mu_m}$$

Data on gas production parameters, pH and DMD and OMD were analysed as a mixed model. The fixed sources of variation were inoculum type (ileum, caecum, soft faeces and hard faeces), substrate (starch, pectin, SBP and wheat straw), and their interactions. To analyse the 2 doses tested of ileal inoculum, the dose (1.0 and 1.5 g fresh ileal digesta/g DM substrate) was included in the model previously described as a fixed source of variation. In all cases, the pooled sample of digesta used as inoculum was the replicate, and it was included as a random variable. Means comparisons were carried out as in Experiment 1. Correlations between gas parameters obtained either with different doses of inoculum or with different types of inoculum were assessed by Pearson correlation analysis using the PROC CORR of SAS (2011).

RESULTS

Experiment 1

The average DM content of ileal and caecal digesta was 170 and 250 g/kg fresh matter, respectively. Accordingly, the 0.5, 1.0 and 2.0 doses supplied 0.017, 0.034 and 0.068 g of ileal digesta DM per vial, respectively (0.085, 0.17 and 0.34 g ileal digesta DM/g substrate DM), and 0.025, 0.050 and 0.10 g caecal digesta DM per vial (0.125, 0.25 and 0.50 g caecal digesta DM/g substrate DM).

Gas production kinetics of substrates incubated with ileal and caecal *inocula* is shown in Figure 1. The 0.5 dose resulted in lower ($P<0.05$) accumulated gas production values at 28 h incubation, with no differences ($P>0.05$) between the 1.0 and 2.0 doses (45.0, 51.7 and 53.7 mL/g substrate DM for 0.5, 1.0 and 2.0 g fresh digesta/g DM substrate, respectively; values averaged across *inocula* and substrates). No inoculum type×dose interaction was detected ($P=0.38$), but a dose×substrate×time interaction ($P=0.052$) was observed.

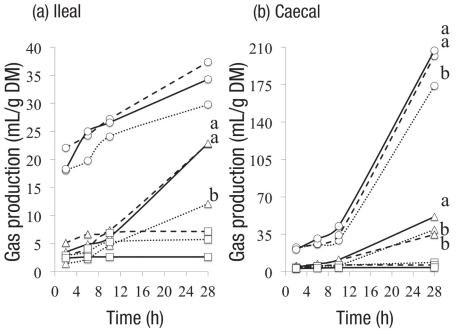

Figure 1: Experiment 1. Gas production kinetics obtained by incubating 3 substrates [pectin (○), sugar beet pulp (△), and wheat straw (□)] with different doses [0.5 (···), 1.0 (---) and 2.0 g (—) of fresh digesta/g substrate dry matter] with ileal (a) or caecal digesta (b) from rabbits.

[a-b]Within each substrate, lines not sharing a common letter differ ($P<0.05$). Standard error of mean values for inoculum, substrate, dose and time effects were 0.851, 1.04, 1.04 and 0.878, respectively. Significant ($P<0.05$) effects were detected for dose, type of inoculum, substrate, time and the type of inoculum×substrate, type of inoculum×time, substrate×time, and type of inoculum×substrate×time interactions. Two different *inocula* were used for each experimental treatment, and each inoculum was a pooled content (either ileal or caecal) from 2 rabbits. Raw materials defined in Table 1.

The caecal inoculum resulted in higher ($P<0.001$) gas production values compared with the ileal inoculum, but inoculum type×substrate and inoculum type×time interactions were detected ($P<0.001$). Differences between ileal and caecal inoculum were more marked for SBP and pectin than for wheat straw, and became more pronounced as incubation time increased. Differences among doses of inoculum over the incubation time appeared for SBP and pectin, but this was not observed for wheat straw, leading to a trend to a dose×substrate×time interaction ($P=0.052$). Increasing the amount of inoculum from 1 to 2 g fresh digesta/g substrate DM did not increase the final accumulated gas production for pectin and wheat straw, but an increase was observed for SBP with the caecal inoculum.

The accumulated gas production at 28 was 4.2 times greater for the caecal inoculum compared with the ileal inoculum ($P<0.001$; 80.9 *vs.* 19.4 mL/g DM; values averaged across substrates). However, differences between the 2 *inocula* varied with the incubated substrate, as indicated by the type of inoculum×substrate interaction ($P<0.001$). The amount of gas at 28 h was 5.7, 2.2 and 1.2 times greater for the caecal inoculum than for the ileal one for pectin, SBP and wheat straw, respectively. In addition, an inoculum type×substrate×time ($P<0.001$) was detected; whereas gas production kinetics of wheat straw followed a similar evolution with both *inocula*, a greater accumulated gas production at 28 h was observed with the caecal inoculum for pectin, and to a lesser extent for SBP, than with the ileal inoculum. As expected, gas production was higher for pectin than for SBP ($P<0.05$; 47.7 *vs.* 11.9 mL/g substrate DM; values averaged across type of inoculum, dose and time), and both values were higher than that for wheat straw ($P<0.05$; 4.51 mL/g substrate DM; $P<0.05$).

As shown in Table 2, there was no effect of either inoculum type or dose on DMD and OMD degradation, despite the differences in the gas production observed between the 2 *inocula* and among doses. As expected, there were differences among substrates, with pectin having the greatest ($P<0.05$) DMD and OMD values and wheat straw showing the lowest ($P<0.05$). Final pH (data not shown) was influenced ($P<0.05$) by both the inoculum type and substrate, but was not affected ($P=0.93$) by inoculum dose. Caecal inoculum reduced final pH compared to the ileal inoculum (6.75 *vs.* 7.25; $P=0.046$), and final pH decreased successively for wheat straw, SBP and pectin (7.35,

Table 2: Experiment 1. Effect of the inoculum type (ileal and caecal digesta from rabbits, inoculum dose (0.5, 1.0 and 2.0 g fresh digesta/g substrate dry matter) and substrate on dry matter (DMD) and organic matter disappearance (OMD) after 28-h *in vitro* incubations.

Inoculum type[1]	Substrate[2]	Inoculum dose	DMD (g/g)	OMD (g/g)
Ileal	Pectin	0.5	0.988	0.984
		1.0	0.972	0.978
		2.0	0.954	0.976
	Sugar beet pulp	0.5	0.212	0.238
		1.0	0.201	0.244
		2.0	0.207	0.248
	Wheat straw	0.5	0.00	0.003
		1.0	0.00	0.004
		2.0	0.00	0.010
Caecal	Pectin	0.5	0.972	0.979
		1.0	0.957	0.971
		2.0	0.965	0.973
	Sugar beet pulp	0.5	0.208	0.237
		1.0	0.197	0.253
		2.0	0.268	0.293
	Wheat straw	0.5	0.00	0.014
		1.0	0.00	0.009
		2.0	0.00	0.00
SEM				
Dose			0.879	0.765
Substrate			0.879	0.765
Type of inoculum			0.75	0.65
P-value[3]				
Dose			0.56	0.72
Substrate			<0.001	<0.001
Type of inoculum			0.68	0.54

[1]n=2. Each inoculum was a pooled content (either ileal or caecal) from 2 rabbits.

[2]Substrates: raw material defined in Table 1.

[3]Dose×substrate, dose×type of inoculum, and dose×substrate×type of inoculum interactions were not significant (P=0.26 to 0.88).

SEM: standard error of mean.

7.11 and 6.55; respectively; P<0.05). There were no dose×substrate, dose×inoculum type, substrate×inoculum type, or dose×substrate×inoculum type interactions on final pH, DMD and OMD (P=0.26 to 0.88).

Experiment 2

The first objective of this experiment was to confirm the results observed in Experiment 1 by comparing 2 doses of ileal inoculum (1 and 1.5 g fresh digesta/g substrate DM), and the obtained results are shown in Table 3. There were no differences on any fermentation parameter between the 2 tested doses (P=0.27 to 0.71), but a dose×substrate interaction was detected for the gas production rate (P=0.003). Compared with the 1.0 dose, the 1.5 dose resulted greater (P<0.05) gas production rates for the highly fermentable substrates (1.4 and 1.3 times greater for starch and pectin, respectively), but for SBP and wheat straw the values for the 1.5 dose were 0.76 and 0.56 of those obtained with the 1.0 dose, respectively. Despite these differences, the gas production kinetics was quite similar for both doses of inoculum (Figure 2). This is in agreement with the positive correlations observed between the values of the gas parameters obtained with the 2 doses (r≥0.90 for V_f, μ_m and k, and r≥0.72 for L and t_i; P<0.05; n=12). The inoculum

Table 3: Experiment 2. Effect of 2 doses of ileal inoculum (1.0 and 1.5 g of fresh digesta/g substrate DM) from rabbits and substrate on the *in vitro* gas production parameters in 144 h incubations[1].

Treatment	V_f (mL)	k (%/h)	L (h)	μ_m (mL/h)	t_i (h)
Dose					
1.0	256	0.021	30.7	6.26	58.4
1.5	234	0.023	27.3	7.01	60.4
SEM	27.8	0.002	4.09	0.84	7.18
Substrate[2]					
Starch	419^{d}	0.041^{c}	14.7^{a}	17.0^{c}	27.4^{a}
Pectin	298^{c}	0.021^{b}	29.5^{b}	6.08^{b}	55.1^{b}
Sugar beet pulp	217^{b}	0.015^{a}	38.9^{b}	2.90^{a}	76.6^{c}
Wheat straw	46.0^{a}	0.012^{a}	32.9^{b}	0.52^{a}	78.5^{c}
SEM	31.8	0.003	4.68	1.06	8.17
Substrate×dose					
Starch-1.0	447	0.035^{A}	15.9	15.7	30.2
Starch-1.5	391	0.046	13.6	18.4	24.7
Pectin-1.0	322	0.017^{A}	30.1	5.57	60.1
Pectin-1.5	274	0.024	28.9	6.60	50.0
Sugar beet pulp-1.0	217	0.017	39.6	3.23	73.0
Sugar beet pulp-1.5	217	0.013	38.3	2.57	80.2
Wheat straw-1.0	37.8	0.016^{A}	37.4	0.57	70.2
Wheat straw-1.5	54.3	0.009	28.4	0.47	86.9
SEM	38.7	0.003	5.69	1.40	9.86
P-value					
Dose	0.34	0.27	0.30	0.43	0.71
Substrate	<0.001	<0.001	<0.001	<0.001	<0.001
Dose×Substrate	0.59	0.003	0.80	0.58	0.34

^{a,b,c}Within each parameter, substrate means with different superscripts differ (P<0.05).

^{A}Indicates a difference between inoculum doses (P<0.05) within each substrate.

[1]n=3; Each inoculum was a pooled ileal content from 3 rabbits.

[2]Raw materials defined in Table 1 and wheat starch (99%; SIGMA S-5127; Sigma–Aldrich Quimica, S.A, Alcobendas, Spain).

SEM: standard error of mean, V_f: asymptotic gas production (mL), k: fractional gas production rate (%/h), L: initial delay in the onset of gas production (h), μ_m: maximum gas production rate (mL/h), t_i: time when μ_m is reached (h).

dose did not affect DMD (P=0.22) and OMD (P=0.19) after 144 h of incubation, although final pH (data not shown) tended to be lower (P=0.095) for the 1.5 than for the 1.0 dose (6.64 and 6.77, respectively; values not shown).

As expected, starch has the lowest (P<0.05) L and t_i values and the highest (P<0.05) V_f and k values (Table 3 and Figure 2). In contrast, SBP and wheat straw had the highest (P<0.05) t_i values and the lowest (P<0.05) k and μ_m values. Even though there were no differences (P>0.05) in L among SBP, pectin and wheat straw, pectin had greater (P>0.05) gas production rate than the others, yielding a higher gas production (P<0.05). The low fermentation of wheat straw led to a higher (P<0.05) final pH than that observed for the other substrates (7.32, 6.48, 6.44 and 6.59 for wheat straw, starch, pectin and SBP, respectively; values not shown), but it was lower (P<0.05) than that observed in the blanks (7.71).

The second objective of this experiment was to compare the fermentative activity of the four *inocula* (ileal, caecal, soft faces and hard faeces) when a similar DM amount of all of them were used (0.050 g digesta DM per vial; i.e. 0.20 g inoculum DM per g substrate DM). The average DM content of the ileal digesta, caecal digesta, soft and hard faeces was 13.5, 25.8, 23.4 and 53.2%, respectively. Consequently, the different *inocula* provided, per vial, 0.041, 0.052, 0.035 and 0.044 g DM for ileal digesta (1.5 dose), caecal digesta, soft faeces and hard faeces, respectively. The amount of DM provided by the high-dose of ileal digesta, soft faeces and hard faeces was slightly lower than the

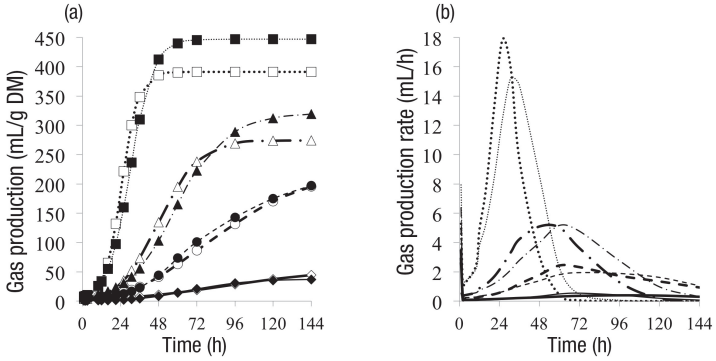

Figure 2: Experiment 2. Gas production kinetics (a) and gas production rate (b) obtained by fermenting different substrates (starch, pectin, sugar beet pulp and wheat straw) with 2 doses of ileal digesta (1 and 1.5 g of fresh digesta/g of substrate dry matter). Starch 1 (···■···), starch 1.5 (•••□•••), pectin 1 (—·▲·—), pectin 1.5 (— •△• —), sugar beet pulp 1 (—●—), sugar beet pulp 1.5 (—○—), wheat straw 1 (—◆—) and wheat straw 1.5 (—◇—). Three different *inocula* were used for each experimental treatment, and each inoculum was a pooled ileal content from three rabbits. Raw materials defined in Tables 1 and 3.

expected value of 0.050 g digesta DM per vial. However, it must be taken into account that bacterial concentrations in each inoculum were not measured and probably differed among *inocula*.

As shown in Table 4 and Figure 3, the type of inoculum (ileal, caecal, soft and hard faeces) had no marked effect on gas production parameters when data of all substrates were analysed together, and only a trend (P=0.085) was observed for gas production rate. However, inoculum type×substrate interactions were detected for the gas production rate (k; P=0.009) and the maximum gas production rate (μ_m; P=0.022). Differences between substrates were more marked for the ileal inoculum than for the rest of the *inocula*. Thus, for ileal inoculum starch showed the highest (P<0.05) k and μ_m values, followed by pectin, SBP and wheat straw. For the rest of the *inocula*, starch and pectin had the highest (P<0.05) k and μ_m values and both SBP and wheat straw the lowest ones (P<0.05). Gas production parameters of the ileal inoculum were positively correlated with those obtained for the faecal inoculum (r=0.95, 0.82, and 0.77, for V_f, μ_m, and t_l respectively; P<0.005). In addition, gas production rate (k) for the ileal inoculum was positively correlated with that for the caecal inoculum (r=0.72; P=0.008), but no other correlation of gas production parameters for ileal inoculum was found. In contrast, all gas production parameters for caecal, soft faeces and hard faeces inoculum were positively correlated among them (r≥0.77; P<0.005).

Whereas the gas production rate (k) and the maximum gas production rate (μ_m) of starch and pectin were lower (P<0.05) for the soft faeces than for the caecal inoculum (P<0.05), there were no differences (P>0.05) between the 2 *inocula* for these parameters for SBP and wheat straw. This meant that the gas production rate tended to be lower (P=0.085) for the soft faeces compared with the caecal inoculum. However, gas production parameters for the soft faeces and caecal inoculum were positively correlated (r=0.95, 0.77, 0.77, 0.81 and 0.93 for V_f, k, L, μ_m, and t_l, respectively; P<0.005). The lack of differences (P=0.27) among *inocula* in the asymptotic gas production (V_f) is consistent with the absence of differences in the DMD, although the caecal and soft faeces *inocula* resulted in greater (P<0.05) OMD values than the ileal and hard faeces *inocula* (Table 5). The DMD and OMD from the starch and pectin was 1.0 g/g with all *inocula*, so these values were not included in the statistical analysis. There were no differences among *inocula* in final pH (values not shown), but pH values were affected by the incubated substrate, with wheat straw having greater (P<0.001) values than starch, pectin and SBP, but lower (P<0.001) than those measured in the blanks (7.79; value averaged across *inocula*).

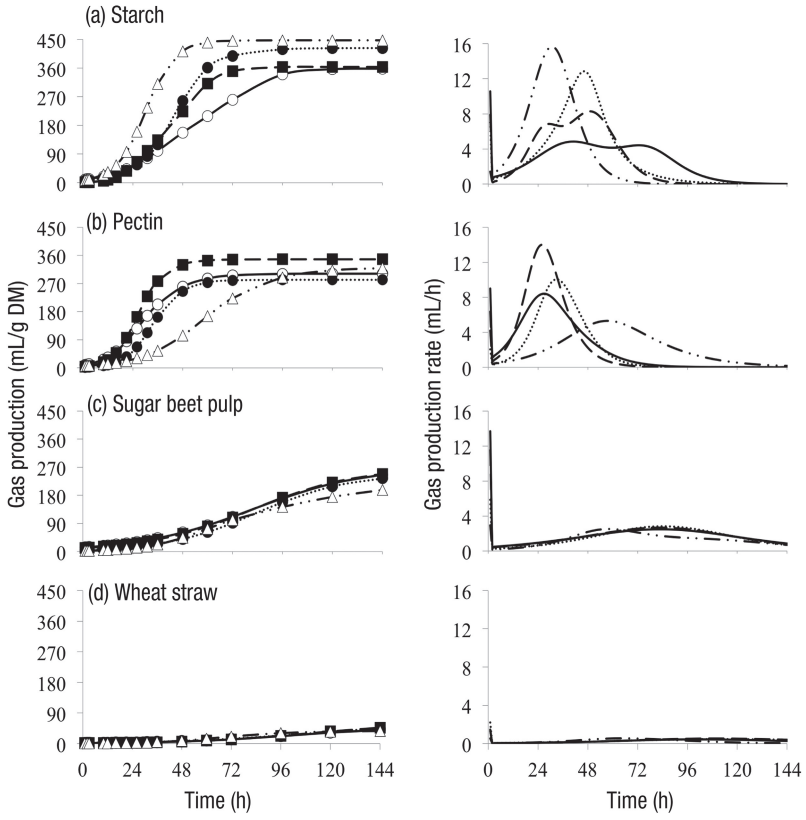

Figure 3: Experiment 2. Gas production kinetics and gas production rate obtained by fermenting different substrates (starch, pectin, sugar beet pulp and wheat straw) with different *inocula* [ileal digesta (— •△• —), caecum digesta (—■—), soft faeces (—○—), and hard faeces (•••●•••)]. Three different *inocula* were used for each experimental treatment, and each inoculum (ileal, caecal, soft faeces or hard faeces) was a pooled ileal content from 3 rabbits. Raw materials defined in Tables 1 and 3.

DISCUSSION

As no previous *in vitro* study had been conducted with ileal digesta of rabbits, the ileal doses tested in our study were selected to be within the range used in a study with ileal digesta of dogs (Murray *et al.,* 2001), although they were lower than those used for *in vitro* studies with rabbit caecal inoculum (Piattoni *et al.,* 1997; Calabrò *et al.,* 1999; Bovera *et al.,* 2006). The lower gas production obtained with the lowest dose of ileal digesta (0.5 g fresh digesta/g substrate DM) might be explained by the smaller amount of microorganisms supplied by this dose. Consequently, substrate fermentation was reduced, as the volume of gas produced is proportional to the amount of substrate fermented (Menke *et al.,* 1979). However, this was not reflected in either final pH values or the DM and OM degradability. The lack of differences in pH values was attributed to the high buffer capacity of the Goering and Van Soest (1970) medium, which prevented a pH drop in the cultures.

The greater gas production and lower final pH observed for the caecal inoculum compared with the ileal in Experiment 1 is in agreement with the higher concentration of microorganisms in the caecum than in the ileum

Table 4: Effect of different *inocula* (ileal, caecal, soft faeces and hard faeces) from rabbits and substrate on *in vitro* gas production parameters in 144 h incubations (Experiment 2)[1].

	V_f (mL)	k (%/h)	L (h)	μ_m (mL/h)	t_i (h)
Inoculum					
Ileal	234	0.023	27.3	7.01	60.4
Caecal	263	0.025	33.8	8.04	68.1
Soft faeces	246	0.018	29.5	5.08	67.2
Hard faeces	259	0.022	35.9	6.98	71.7
SEM	18.2	0.0021	4.27	1.093	6.14
Substrate[2]					
Starch	384^{a}	0.034^{b}	24.0^{a}	13.4^{c}	40.9^{a}
Pectin	301^{b}	0.034^{b}	19.8^{a}	10.5^{b}	35.6^{a}
Sugar beet pulp	253^{c}	0.011^{a}	34.4^{b}	2.68^{a}	82.2^{b}
Wheat straw	64.2^{d}	0.009^{a}	48.3^{c}	0.52^{a}	109^{c}
SEM	18.2	0.002	4.27	1.09	6.14
Inoculum×Substrate					
Ileal					
Starch	391	0.046^{C}	13.6	18.4^{C}	24.7
Pectin	274	0.024^{B}	28.9	6.60^{B}	50.0
Sugar beet pulp	217	0.013^{AB}	38.3	2.57^{AB}	80.2
Wheat straw	54.3	0.009^{A}	28.4	0.47^{A}	86.9
Caecal digesta					
Starch	364	0.038^{B}	28.6	13.7^{B}	42.0
Pectin	348	0.043^{B}	15.7	15.2^{B}	27.8
Sugar beet pulp	271	0.010^{A}	31.0	2.67^{A}	82.1
Wheat straw	71.3	0.009^{A}	60.0	0.60^{A}	121
Soft faeces					
Starch	359	0.022^{B}	28.8	7.97^{B}	52.8
Pectin	301	0.031^{B}	13.6	9.33^{B}	30.2
Sugar beet pulp	272	0.009^{A}	27.6	2.53^{A}	81.7
Wheat straw	50.0	0.009^{A}	48.1	0.47^{A}	104
Hard faeces					
Starch	423	0.031^{B}	25.1	13.6^{B}	44.1
Pectin	282	0.038^{B}	21.0	10.8^{B}	34.6
Sugar beet pulp	251	0.012^{A}	40.9	2.93^{A}	84.9
Wheat straw	81.2	0.008^{A}	56.6	0.57^{A}	123
SEM	26.8	0.0060	7.49	1.881	10.5
P-value					
Type of inoculum	0.27	0.085	0.32	0.14	0.45
Substrate	<0.001	<0.001	<0.001	<0.001	<0.001
Type of inoculum×Substrate	0.24	0.009	0.11	0.022	0.19

^{a,b,c}Within each parameter, substrate means with different superscripts differ (P<0.05).

$^{A, B, C}$Within each parameter and inoculum type, substrate means with different superscripts differ (P<0.05).

[1]n=3; Each inoculum was a pooled content (ileal, caecal, soft faeces or hard faeces) from 3 rabbits.

[2]Raw materials defined in Tables 1 and 3.

SEM: standard error of mean, V_f: asymptotic gas production (mL), k: fractional gas production rate (%/h), L: initial delay in the onset of gas production (h), μ_m: maximum gas production rate (mL/h), t_i: time when μ_m is reached (h).

reported in previous studies. The intestinal microbiota in rabbits is mainly developed in the caecum, with reported concentrations of 10^{10}-10^{12} bacteria/g of caecal digesta, whereas bacterial concentrations in the ileum are smaller (10^{4}-10^{9} bacteria/g ileal digesta; Gouet and Fonty, 1979; Penney *et al.,* 1986; Padilha *et al.,* 1995). Differences in gas production values between the 2 *inocula* were more pronounced for the highly fermentable substrates (SBP and pectin) than for that with low degradability (wheat straw). However, there were no differences between ileal and caecal *inocula* in DMD and OMD, which might be explained by the hydrolysis and/or solubilisation of components of the substrate that were not fermented, but were lost in the filtration process and therefore not retained in the undegraded residue.

The results of Experiment 1 indicated that an adequate dose of inoculum for *in vitro* ileal fermentation might be between 1 and 2 g fresh digesta/g substrate DM. Therefore, a second experiment was conducted to test 2 doses of ileal inoculum (1.0 and 1.5 g fresh digesta/g substrate DM). The tested doses had a minor influence on the kinetics of gas production (Figure 2), and the accumulated gas production of both doses evolved in parallel. Whereas the 1.5 dose resulted in greater rates of gas production than the 1.0 dose for starch and pectin, the opposite was observed for wheat straw (Table 3). An excess of endogenous digesta makes the inoculum more heterogeneous and difficult to dose (Omed *et al.,* 2000; Bovera *et al.,* 2006), and this might have a more pronounced effect on low-fermentable substrates such as wheat straw than on high-fermentable substrates. It seems that the dose of 1.0 g fresh ileal digesta/g substrate DM might be preferable when studying ileal fermentation of different substrates. In addition, this would reduce the amount of ileal digesta required, which is an important point considering the small amount of ileal contents that can be obtained per rabbit.

Table 5: Effect of different *inocula* (ileal, caecal, soft faeces and hard faeces) from rabbits and substrate on dry matter (DMD) and organic matter disappearance (OMD) in 144 h incubations (Experiment 2)[1].

	DMD (g/g)	OMD (g/g)
Inoculum		
Ileum	0.424	0.444[a]
Caecum	0.498	0.542[b]
Soft faeces	0.478	0.511[b]
Hard faeces	0.436	0.453[a]
SEM	0.021	0.022
Substrate[2]		
Starch	—	—
Pectin	—	—
Sugar beet pulp	0.775	0.814
Wheat straw	0.143	0.161
SEM	0.015	0.015
P-value		
Type of inoculum	0.076	0.014
Substrate	<0.001	<0.001
Type of inoculum×Substrate	0.32	0.20

[a,b]Within each parameter, inoculum means with different superscripts differ ($P<0.05$).

[1]n=3; Each inoculum was a pooled content (ileal, caecal, soft faeces or hard faeces) from 3 rabbits.

[2]Raw materials defined in Table 1 and Table 3. Starch and pectin showed DMD and OMD values of 1.0 g/g for all *inocula* and these values were not included in the statistical analysis.

Although similar rates of gas production were observed for SBP and wheat straw in Experiment 2 (Table 4), the maximum gas production rate and the asymptotic gas production were greater for the SBP than for the wheat straw, which may be related to the lower lignification of the SBP cell wall compared with that of wheat straw (2.5 and 6.2 g lignin/100 g aNDFom-cp, respectively). These results are in agreement with the lower final pH and greater values of DMD and OMD observed in our study for SBP compared with wheat straw, and with the greater amount of degradable insoluble fibre reported in previous studies for low-lignified sources of fibre (García *et al.,* 2002; Trocino *et al.*, 2013). The high values (1.0 g/g) of DMD and OMD obtained for starch and pectin with the different *inocula* (Table 5) might be explained by either a complete fermentation of substrates after 144 h of incubation or a lack of retention of the potential remaining fermentation residues in the crucibles after filtering (Tagliapietra *et al.,* 2003).

The kinetics of gas production of the ileal inoculum differed from the other *inocula* (Figure 3), especially for highly-fermentable substrates (starch and pectin). The greater gas production rate of starch observed for the ileal inoculum is in agreement with the 2.7 and 2.4 times higher amylase and maltase activity reported by Marounek *et al.* (1995) in the ileal digesta of rabbits compared with the caecum, although enzymatic activities may depend on the type of diet (Falcão-e-Cunha *et al.,* 2004). The use of the ileal inoculum resulted in greater differences among substrates

than using the other *inocula*. The results also confirm a relevant fermentative potential at the ileum, which would be limited mainly by the lower microbial concentration and the short mean retention time of digesta in this portion of the digestive tract (Gidenne, 1994; García *et al.,* 1999).

To our best knowledge, no previous study has compared the ileal fermentation with that produced in other sections of the rabbit digestive system. In pigs, Wang *et al.* (2013) observed that ileal inoculum produced a lower cumulative amount of gas, but a higher maximum fermentation rate than the faecal inoculum when using corn starch as substrate. Similarly, Tagliapietra *et al.* (2003) reported that the ileal inoculum of suckling piglets produced a lower accumulated gas production than the faecal inoculum when using starch as substrate, but no differences were found in the maximum fermentation rate or in the volatile fatty acids production; in contrast, there was no difference between ileal and faecal *inocula* when SBP was used as substrate. The results of different studies must be considered with caution, as the comparison of different *inocula* is affected by many factors such as the diet of donor animals, dilution rate of the inoculum, inoculum/substrate ratio and the type of substrate, among others (Mould *et al.,* 2005).

There was no difference in the gas production kinetics between the caecal and faecal inoculum, which might be consistent with the lack of effects on the volatile fatty acid production (but with different molar proportions) when dehydrated alfalfa meal, sugar beet pulp and barley grain were incubated with these 2 *inocula* (Bovera *et al.,* 2006). These results would indicate that faeces (either soft or hard) might be used as *inocula* to assess caecal fermentation in rabbits. In contrast, other studies by the same authors revealed differences between the caecal and faecal inoculum with significant type of inoculum×substrate interactions (Bovera *et al.,* 2008, 2009). The higher caecal (caecal digesta or soft faeces) OM degradability of the sugar beet pulp and wheat straw compared to the faecal inoculum is consistent with the *in vitro* digestibility using caecal and faecal *inocula* of a mixture of corn, alfalfa hay and wheat middlings (Pascual *et al.,* 2000). However, these authors found no differences between caecal and faecal *inocula* for 4 other feeds, and caecal inoculum produced lower digestibility values than those of faecal inoculum for sugar beet pulp. These results highlight again the existence of interactions between the type of substrate and type of inoculum used.

CONCLUSIONS

A dose of 1.0 g fresh digesta/g substrate DM was adequate for both ileal and caecal *inocula* for *in vitro* incubations with rabbits digesta. The kinetics of gas production was different among the ileal, caecal, soft faeces and hard faeces inoculum, especially for highly fermentable substrates (starch and pectin). The ileal inoculum demonstrated a relevant fermentative potential, but it was lower compared with the other *inocula*. Gas production parameters for ileal inoculum were more similar to those for hard faeces than to those determined for caecal inoculum. Caecal, soft faeces and hard faeces inoculum resulted in similar gas production parameters. The results indicate that soft or hard faeces could substitute the caecal inoculum in future *in vitro* studies, thus avoiding the slaughter of rabbits.

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