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IN VITRO CAECAL FERMENTATION OF CARBOHYDRATE-RICH FEEDSTUFFS IN RABBITS AS AFFECTED BY SUBSTRATE PRE-DIGESTION AND DONORS' DIET

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Abstract: The influence of substrate pre-digestion and donors' diet on *in vitro* caecal fermentation of different substrates in rabbits was investigated. Eight crossbreed rabbits were fed 2 experimental diets containing either low (LSF; 84.0 g/kg dry matter [DM]) or high soluble fibre (HSF; 130 g/kg DM) levels. *In vitro* incubations were conducted using batch cultures with soft faeces as inoculum and four fibrous or fibre-derived, low-starch and low-protein substrates: D-cellobiose (CEL), sugar beet pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS). Substrates in half of the cultures were subjected to a 2-step pepsin/pancreatin *in vitro* digestion without filtration, and the whole residue (soluble, insoluble and added enzymes) was incubated at 39°C. Gas production was measured until 144 h, and volatile fatty acid (VFA) production at 24 h incubation was determined. Cultures without substrate (blanks) were included to correct gas production values for gas released from endogenous substrates and added enzymes. Pre-digestion had no influence on *in vitro* gas production kinetic of WS, and only reduced the time before gas production begins (lag time; by 31%; P=0.042) for SBP, but for both substrates the pre-digestion decreased the molar proportion of acetate (by 9%; $P\leq0.003$) and increased those of propionate and butyrate ($P\leq0.014$). For CEL, the pre-digestion increased the gas and total VFA production (by 30 and 114%), shortened the lag time (by 32%), and only when it was combined with LSF inoculum 38 percentage units of acetate were replaced by butyrate ($P\leq0.039$). Treatments had a minor influence on in vitro fermentation traits of SBP pectin. The results showed that the pre-digestion process influenced the *in vitro* caecal fermentation in rabbits, but the effects were influenced by donors' diet and the incubated substrate. Pre-digestion of substrate is recommended before conducting *in vitro* caecal fermentations. The level of soluble fibre in the donors' diet also influenced the *in vitro* caecal fermentation, but its effect depended on the type of substrate.

Key Words: gas production, processing procedure, donors' diet, rabbits, caecal fermentation, volatile fatty acids.

INTRODUCTION

The inclusion of moderate levels of soluble fibre (mainly provided by sugar beet pulp) in rabbit diets has been reported to improve the gut barrier function in the small intestine (Gómez-Conde *et al.,* 2007) and the ileal and faecal digestibility of all fibre fractions (Abad-Guamán *et al.,* 2015). These effects might be partly mediated by the microbial end-products, such as volatile fatty acids (VFA). However, the quantification of VFA concentration in the digesta is not a good estimator of VFA absorption (Van der Klis and Jansman, 2002). The use of *in vitro* methodologies to study the microbial fermentation of carbohydrate-rich substrates in rabbits may be of interest, but a standardised *in vitro* methodology is not yet available. Some studies have used the *in vitro* gas production technique (Menke *et al.,* 1979) to investigate caecal fermentation in rabbits, and in most of them (Bovera *et al.,* 2006, 2009; Lavrenčič, 2007; Calabrò *et al.,* 2010; Yang *et al.,* 2010) substrates were just ground, but in others (Bindelle *et al.,* 2007a; Bindelle

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et al., 2007b; Rodríguez-Romero *et al.,* 2011) the insoluble residue obtained after the pepsin-pancreatin *in vitro* pre-digestion was used as substrate. Whereas this latter procedure might seem more appropriate (Coles *et al.,* 2005), it is also questionable due to the loss of soluble fibre during the filtration/centrifugation process. This problem could be solved by avoiding the filtration/centrifugation step and using the whole material (solid and liquid) obtained after the pre-digestion procedure as substrate for *in vitro* caecal incubations. However, using this alternative, the enzymes added in the pre-digestion procedure could be fermented and contribute to gas and VFA production (Carro *et al.,* 2005). To our knowledge, the influence of a pre-digestion procedure on the *in vitro* caecal fermentation in rabbits has not yet being investigated. Additionally, the lack of a filtration/centrifugation step would allow that glucose and amino acids potentially released from substrate starch and protein could be finally fermented, which would be an unwanted effect. However, the use of this procedure with substrates with low or even no content of starch and protein could limit this effect. In addition, donors' diet has been shown to influence the *in vitro* gas production and substrate degradability in pigs and ruminants (Bindelle *et al.,* 2007a,b; Mateos *et al.,* 2013; Sappok *et al.,* 2013), but information on the effects of donors' diet on *in vitro* caecal fermentation in rabbits is very limited (Rodríguez-Romero *et al.,* 2011). The aim of this study was to assess the *in vitro* caecal fermentation of four carbohydrate-rich substrates as influenced by the pre-digestion process and donors' diet.

Table 1: Ingredients and chemical composition of the experimental diets fed to donor rabbits.

	Experimental diets	
	Low soluble fibre	High soluble fibre
Ingredients (g/kg as fed)		
Dehydrated alfalfa	150.0	150.0
Soybean meal	80.0	80.0
Wheat	227.0	217.0
Wheat bran	280.0	129.7
Wheat straw	100.0	50.0
Beet pulp	-	180.0
Sunflower meal	99.7	130.0
High oleic sunflower oil	8.5	8.5
Sunflower oil	21.5	21.5
L-Lysine HCl	4.4	4.4
DL-Methionine	0.8	0.6
L-Threonine	3.1	3.2
Calcium carbonate	12.0	7.0
Sodium chloride	3.0	3.1
Calcium phosphate	5.0	10.0
Vitamin/mineral premix[1]	5.0	5.0
Chemical composition (g/kg DM)		
Dry matter	908	908
Ash	70.8	67.5
Crude protein	169	168
Total dietary fibre[2]	391	442
Soluble fibre[3]	84.0	130
Neutral detergent fibre[2]	307	312
Acid detergent fibre[4]	165	185
Acid detergent lignin[4]	31.0	33.0
Starch	226	182
Ether extract	53.8	48.7

[1] Provided by Trouw Nutrition (Madrid, Spain). Mineral and vitamin composition (per kg of complete diet): 20 mg of Mn as MnO, 59.2 mg of Zn as ZnO; 10 mg of Cu as $CuSO_4\,5H_2O$; 1.25 mg of I as KI; 0.495 mg of Co as $CoCO_3\,H_2O$; 76 mg of Fe as $FeCO_3$; 8375 UI of vitamin A; 750 UI of vitamin D_3, 20 UI of vitamin E as DL-α-tocopherol acetate, 1.0 mg of vitamin K; 1.0 mg of vitamin B1; 2 mg of vitamin B2; 1 mg of vitamin B6; 20 mg of Niacin; 54.1 mg of Betaine; 137.5 mg of Choline chloride; 66 mg of robenidine; 50 mg of ethoxyquin.

[2] Values were corrected for ash and crude protein.

[3] Calculated as total dietary fibre minus neutral detergent fibre.

[4] Values corrected for ash.

MATERIALS AND METHODS

All procedures involving animals were carried out in accordance with the Spanish guidelines on experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes) after being approved by the Directorate General of Livestock and Agriculture of the Community of Madrid.

Animals and diets

Eight hybrid rabbits (New Zealand White×Californian) were weaned at 34 d of age and assigned randomly to each of the 2 experimental diets (4 rabbits/diet). The experimental diets were formulated to differ in their content of soluble fibre mainly provided by sugar beet pulp (84.0 and 130 g/kg for the low soluble fibre [LSF] and high soluble fibre [HSF] diets, respectively; dry matter [DM] basis) and starch (226 and 182 g/kg DM for LSF and HSF, respectively). Ingredients and chemical composition of diets are given in Table 1. Rabbits had *ad libitum* access to feed and fresh water over the trial and received no antibiotic. At 41 d of age (1.13±0.01 kg body weight) rabbits were fitted with plastic collars from 9:00 to 10:00 h (maximum) to collect the soft faeces to be used as inoculum for the *in vitro*

incubations. A soon as they were produced, the soft faeces were wrapped in aluminium foil to reduce air contact and thermal shock, and were immediately transported to the laboratory in thermal flasks.

Substrates and pre-digestion procedure

Four fibrous or fibre-derived, low-starch, and low-protein ingredients having a wide range of fermentation rate and extent were used as substrates for the *in vitro* incubations: D-cellobiose (CEL; NPC Cello-Oligo, Nippon Paper Industries Co., Tokyo, Japan), sugar beet pulp (SBP; Fipec, Nordic Sugar, Copenhagen, Denmark), sugar beet pulp pectin (PEC; Betapec RU 301, Herbstreith & Fox, Neuenbürg, Germany) and wheat straw (WS; Pagran, PITE S.A., Tordesillas, Spain). Most of the chemical constituents in all ingredients selected for this study cannot be hydrolysed by endogenous enzymes of adult rabbits. According to the manufacturer, CEL contained 96.6% cellobiose β1-4, 1.9% cello-oligosaccharide, 1.5% glucose, and no nitrogen content. The SBP had 646 g total dietary fibre (TDF), 369 g neutral detergent fibre (NDF), 86.3 g crude protein (CP), and 39.4 CP-TDF/kg DM, PEC had 934 g TDF, 6.4 g NDF, 53.1 g CP and 16.2 g CP-TDF/kg DM, and WS contained 785 g TDF, 748 g NDF, 28.0 g CP and 27.9 g CP-TDF/kg DM. All TDF and NDF values were corrected for their ash and CP content.

Samples (250 mg DM) of each substrate were carefully weighed into 115-mL glass vials. In half of the vials, the substrates were subjected to a 2-step pepsin-pancreatin *in vitro* digestion (pre-digestion) according to Ramos *et al.* (1992), with the exception that the contents of the vials were not filtrated at the end of the procedure. All vials were stored at 4°C overnight (to stop the digestion) and placed in an incubator at 40°C for 1 h before starting the *in vitro* incubations. A total of 64 vials with substrate (2 pre-digestion×2 donors' diet×4 substrates×4 rabbits/diet) were incubated. In addition, 16 vials without substrate (blanks; 2 per rabbit) were prepared and half of them underwent the pre-digestion procedure.

In vitro incubations

Soft faeces from each rabbit were mixed with the culture medium described by Goering and Van Soest (1970) and homogenised with a blender for 2 min. The soft faeces/medium ratio (720 mg/100 mL) was selected from previous studies by our group (it was selected from concentrations ranging from 415 to 2000 mg/100 mL (unpublished results). Vials were filled up with 25 mL of the mixture using a Watson-Marlow 520UIP31 peristaltic pump (Watson-Marlow Fluid Technology Group, Cornwall, United Kingdom), sealed with rubber stoppers, and incubated at 40°C for 144 h. This temperature (40°C) was set up in the incubator to reach 39°C in the content of the glass vials used for the incubations, consistently with the internal body temperature of rabbits. This long incubation time was selected to reach the potential degradation in all samples. Preparation of the medium, its mixture with the soft faeces and filling of vials were carried out under continuous CO_2 flushing at 40°C. Gas production was measured at 4, 6, 9, 12, 20, 24, 30, 35, 48, 58, 72, 96, 120 and 144 h using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe, and the gas produced at each measurement time was released. Immediately after measuring the gas production at 24 h, 1 mL of each vial content was taken using an insulin syringe, mixed with 20 µL of H_2SO_4 (10% vol/vol) and stored at –20°C for VFA analysis.

Chemical Analyses

The AOAC procedures (2000) were used to determine DM (method 934.01), ash (method 942.05), crude protein (method 968.06), ether extract (method 920.39), starch (amyloglucosidase-α-amylase method; method 996.11), total dietary fibre (method 985.29) and acid detergent fibre (method 973.187) content in the experimental diets. Dietary NDF and acid detergent lignin were determined according to Mertens (2002) and Goering and Van Soest (1970), respectively.

Samples for VFA analysis were thawed, centrifuged (13 000 ×*g*, 15 min, 4°C), and 0.8 mL of supernatant were mixed with 0.5 mL of deproteinising solution (0.06% of crotonic acid and 2% of metaphosphoric; in volume) and stored overnight at 4°C. Samples were centrifuged again (13 000 ×*g*, 15 min, 4°C) and the supernatant was transferred to chromatography vials and stored at –20°C until analysis by gas chromatography (Carro *et al.,* 1992) using a Pelkin Elmer Autosystem XL gas chromatograph (Perkin Elmer Inc., Shelton, CT, USA) equipped with an automatic injector, detector flame ionisation and a semi-capillary column TR-FFAP 30 m×0.53 mm×1 µm (Supelco, Barcelona, Spain).

Calculations and statistical analysis

The values of gas produced at each measurement time and those of VFA at 24 h were corrected for the amount of gas and VFA, respectively, produced in the corresponding blanks. Gas production data were fitted to the logistic model described by Schofield *et al.* (1994):

Gas production=Vf/[1+e $^{[2-4\ k\ (t\text{-lag time})]}$], where Vf is the final asymptotic gas production, k is the fractional rate of gas production, lag time is the initial delay in the onset of gas production (lag time) and t is the time of gas measurement. The Vf, k and lag time parameters were estimated by an iterative least squares procedure (Marquardt algorithm) using the NLIN procedure of SAS (version 9.2; SAS Inst. Inc., Cary, NC, USA). 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 μ_m=k×Vf and T_i=lag time+(Vf/[2 × μ_m]).

Data on gas production parameters and VFA production were analysed as a mixed model including the substrate processing (with or without pre-digestion procedure), the diet of donor rabbits (LSF and HSF), the type of substrate and their interactions as fixed effects, and donor rabbits (inoculum) as a random effect. When a significant effect of substrate was detected (P<0.05), the Tukey test was used for mean comparisons. Significance was declared at P<0.05, whereas P<0.10 values were considered to be a trend.

RESULTS

The solubility of substrates during pre-digestion was as expected, but PEC flocculated under the acidic conditions of the first step of the pre-digestion. Table 2 shows the effects of pre-digestion and donors' diet on gas production kinetics and VFA production in the cultures without substrate (blanks). Pre-digested blanks had greater (P=0.015) values of Vf (about three-fold increase), μ_m and T_i, total VFA production (six-fold increase) and butyrate and isovalerate proportions, as well as lower (P=0.004) k values and acetate proportions, than those untreated. Donors' diet affected

Table 2: Effect of pre-digestion and type of diet fed to donor rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) on gas production kinetics and volatile fatty acid (VFA) production and molar proportions in blanks (cultures without substrate) (n=4 rabbits/diet)[1].

Pre-digestion	No		Yes		SEM[2]		*P*-value		
Donors' diet	LSF	HSF	LSF	HSF	Pre-digestion/ donors' diet[2]	Pre-digestion ×donors` diet	Pre-digestion	Donors' diet	Pre-digestion ×donors' diet
Gas production kinetics[3]									
Vf (mL/culture)	4.63	4.26	13.8	15.7	0.435	0.615	<0.001	0.240	0.092
k (%/h)	0.179	0.127	0.098	0.082	0.0086	0.0122	<0.001	0.017	0.159
Lag time (h)	0.000	0.272	0.000	0.000	0.0963	0.1363	0.337	0.337	0.337
μ_m (mL/h)	0.861	0.539	1.35	1.28	0.0893	0.1264	<0.001	0.148	0.334
T_i (h)	2.91	4.26	5.25	6.15	0.237	0.335	<0.001	0.006	0.512
VFA production									
Total VFA (mmol/L)	1.13^a	0.757^a	6.53^c	5.31^b	0.0976	0.1380	<0.001	<0.001	0.010
Molar VFA proportions (mol/100 mol)									
Acetate	100	100	91.1	97.5	1.13	1.60	0.004	0.069	0.069
Propionate	0.00	0.00	1.05	0.66	0.0439	0.621	0.193	0.760	0.760
Butyrate	0.00	0.00	3.39	0.99	0.224	0.775	0.015	0.148	0.148
Isobutyrate	0.00	0.00	0.64	0.32	0.549	0.317	0.337	0.337	0.337
Isovalerate	0.00^a	0.00^a	3.82^b	0.86^a	0.313	0.443	<0.001	0.006	0.006

[1] *In vitro* incubations for determining gas production parameters and VFA production lasted for 144 and 24 h, respectively. [2] SEM of the mean values for pre-digestion and donors' diet were the same for all variables. [3] V_f: asymptotic gas production; k: fractional rate of gas production; Lag time: initial delay in the onset of gas production; μ_m: maximum gas production rate; T_i: time when μ_m is reached.
[a, b, c] Means with different superscripts in the same row differ (P<0.05).

Table 3: Effect of pre-digestion, type of diet fed to donor rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) and incubated substrate on gas production kinetics in 144 h *in vitro* incubations (n=4 rabbits/diet)[1].

	Vf (mL/g DM)	k (%/h)	Lag time (h)	μ_m (mL/h)	T_i (h)
Pre-digestion					
No	170	0.032	15.8	5.96	37.2
Yes	198	0.042	13.1	9.79	30.4
SEM	4.68	0.0030	0.98	0.771	1.37
Donors' diet					
Low soluble fibre	187	0.042	13.6	9.58	31.9
High soluble fibre	180	0.032	15.3	6.17	35.7
SEM	4.68	0.0030	0.98	0.771	1.37
Substrate					
Cellobiose	295^{d}	0.069^{b}	25.3^{d}	21.0^{c}	34.4^{b}
Pectin	261^{c}	0.024^{a}	11.3^{b}	6.25^{ab}	36.3^{b}
Sugar beet pulp	142^{b}	0.021^{a}	16.7^{c}	3.05^{a}	41.7^{c}
Wheat straw	36.8^{a}	0.035^{a}	4.52^{a}	1.20^{a}	22.7^{a}
SEM	6.62	0.0042	1.388	1.089	1.94
P-value[2]					
Pre-digestion	<0.001	0.029	0.065	0.001	0.001
Donors` diet	0.292	0.028	0.214	0.003	0.056
Substrate	<0.001	<0.001	<0.001	<0.001	<0.001
Pre-digestion × donors' diet	0.886	0.132	0.786	0.017	0.601
Pre-digestion × substrate	0.001	0.517	0.003	0.003	0.535
Donors' diet × substrate	0.009	0.006	0.014	<0.001	0.021
Pre-digestion × donors' diet × substrate	0.116	0.006	0.471	<0.001	0.432

[a-d] For each item, means for substrates in the same column with different letters differ significantly (*P*<0.05).
[1] V_f: asymptotic gas production; k: fractional rate of gas production; Lag time: initial delay in the onset of gas production; μ_m: maximum gas production rate; T_i: time when μ_m is reached.
[2] Interactions are shown in Figure 2.

k and T_i values, with the LSF-inoculated blanks having greater k (*P*=0.017) and lower T_i values (*P*=0.006) than those HSF-inoculated. There were no pre-digestion×donors' diet interactions (*P*>0.05) for any gas parameter in the blanks with the exception of total VFA production and isovalerate molar proportion (*P*=0.010), and a trend (*P*=0.092) was detected for Vf and acetate proportion.

Multiple interactions among the studied factors (pre-digestion, donors' diet and substrate) were observed for gas production parameters in the cultures with substrate (Table 3 and Figure 1). Pre-digestion×substrate and donors' diet×substrate interactions were detected for Vf, lag time and µm values (*P*=0.014). As shown in Figure 2, for CEL substrate Vf values were greater (*P*=0.033) and lag time values were lower (*P*=0.039) in the pre-digested cultures than in those untreated (333 *vs.* 256 mL/g DM, and 20.5 *vs.* 29.9 h, respectively), and in the LSF-inoculated cultures compared with those HSF-inoculated (318 *vs.* 271 mL/g DM, and 20.3 *vs.* 30.1 h, respectively). In contrast, Vf values were unaffected (*P*=0.174) by any studied factor for the other substrates.

Similarly to that observed for CEL, the pre-digestion shortened lag time values for SBP (13.6 *vs.* 19.7 h; *P*=0.042), but enlarged them for PEC (19.8 *vs.* 8.6 h; *P*=0.044) and had no effect for WS (4.4 *vs.* 4.7 h; *P*=0.804). No influence (*P*=0.136) of donors' diet on lag time values was observed for SBP, PEC and WS. The LSF-inoculum decreased T_i values for CEL (27.8 *vs.* 40.9 h for LSF and HSF inoculum, respectively; *P*=0.021), but had no effect for the other substrates, thus resulting in a donors' diet×substrate interaction for this parameter. Pre-digestion×donors' diet×substrate interactions were detected for k and μ_m (*P*=0.006). These triple interactions were explained by the increase of k and μ_m values when the LSF-inoculum was used to ferment the pre-digested CEL compared with the HSF-inoculum (0.111 *vs.* 0.042%/h, and 40.8 *vs.* 12.2 mL/h, respectively), whereas no effect was detected for SBP and WS substrates. For PEC, the pre-digestion increased k and μ_m values only with the HSF-inoculum (0.035 *vs.* 0.013%/h and 10.1 *vs.* 3.3 mL/h, respectively).

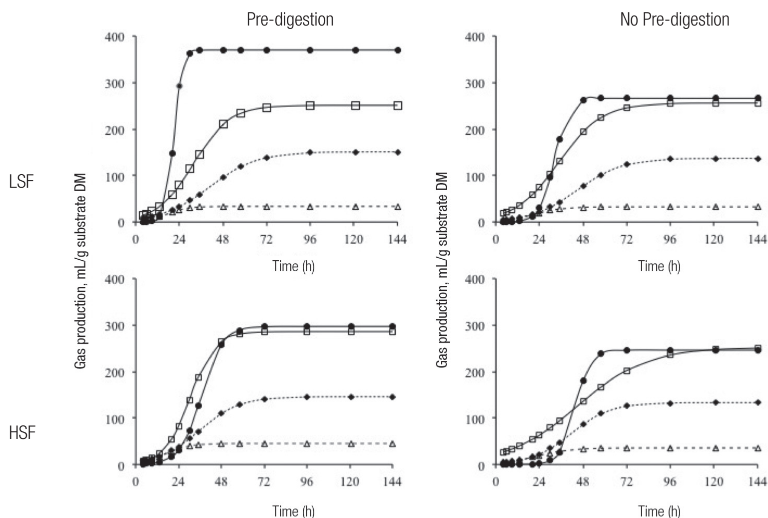

Figure 1: Effect of pre-digestion, type of diet fed to donor rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) and incubated substrate on gas production kinetics in 144 h of *in vitro* incubations (n=4 rabbits/diet. Standard error=9.7 mL/g DM substrate). —●— : CEL; —□—: PEC; ···◆···: SBP; --△---: WS.

Pre-digestion×substrate and donors' diet×substrate interactions were also observed for total VFA production (P=0.049; Table 4 and Figure 3). Whereas the pre-digestion increased total VFA production for CEL (13.0 *vs.* 6.1 mmol/L; P=0.036), the opposite was observed for WS (6.6 *vs.* 8.2 mmol/L; P=0.022), and no effect was detected for SBP and WS (P=0.502). Furthermore, the LSF-inoculum increased total VFA production for CEL (14.4 *vs.* 5.4 mmol/L;

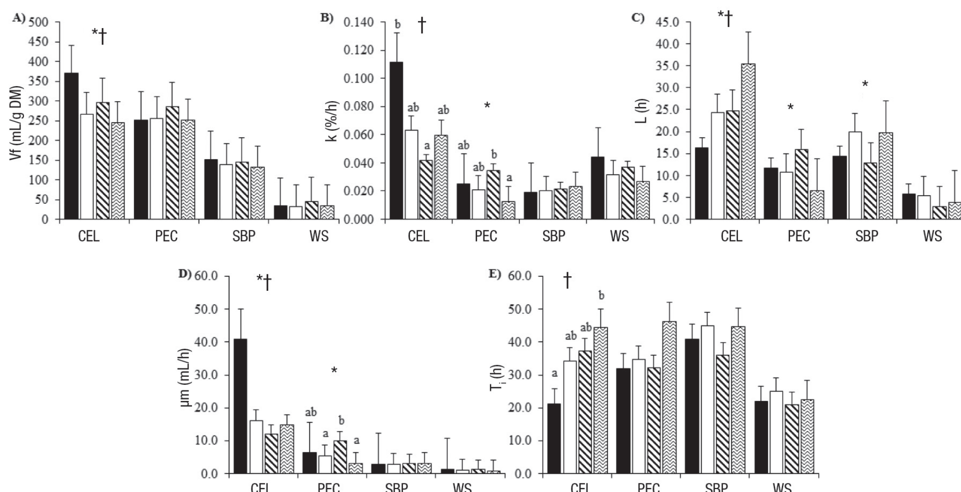

Figure 2: Effect of pre-digestion (Yes *vs.* No), type of diet fed to donor rabbits of soft faeces (low soluble fibre [LSF ■] or high soluble fibre [HSF ▨]) and incubated substrate (D-cellobiose (CEL), sugar beet pulp pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS)) on gas production kinetics in 144 h *in vitro* incubations (n=4 rabbits/diet). A) V_f: asymptotic gas production; B) k: fractional rate of gas production; C) L (lag time): initial delay in the onset of gas production; D) μ_m: maximum gas production rate; E) T_i: time when μ_m is reached. a, b: Within each substrate, columns with different letters differ (P<0.05). Bars indicate the standard error. Within each substrate, * and † indicate an effect (P<0.05) of pre-digestion and donors' diet, respectively. ■: yes LSF; □: no LSF; ▨: yes HSF; ▩: no HSF.

Table 4: Effect of pre-digestion, type of diet fed to donor rabbits of soft faeces (low soluble fibre [LSF] or high soluble fibre [HSF]) and incubated substrate on gas production, total volatile fatty acid (VFA) production and individual VFA profile after 24 h of *in vitro* fermentation (n=4 rabbits/diet).

	Gas production (mL/g DM)	Total VFA (mmol/L)	Molar proportions (mol/100 mol)				
			Acetate	Propionate	Butyrate	Isobutyrate	Isovalerate
Pre-digestion							
Yes	77.7	10.2	87.1	2.78	8.32	0.66	1.18
No	30.5	8.99	98.8	0.00	1.17	0.00	0.00
SEM	5.273	0.854	1.17	0.408	0.964	0.160	0.213
Donors’ diet							
LSF	69.6	11.1	92.4	0.86	6.14	0.25	0.32
HSF	38.5	8.03	93.5	1.93	3.35	0.40	0.87
SEM	5.273	0.854	1.17	0.408	0.964	0.160	0.213
Substrate							
Cellobiose	94.4[b]	9.61[a]	85.0[a]	0.47	13.5[b]	0.36	0.67
Pectin	78.4[b]	14.2[b]	96.1[b]	1.69	1.36[a]	0.31	0.50
Sugar beet pulp	28.5[a]	7.14[a]	96.7[b]	1.11	1.55[a]	0.20	0.48
Wheat straw	15.0[a]	7.40[a]	94.0[b]	2.30	2.58[a]	0.45	0.72
SEM	7.457	1.207	1.65	0.578	1.363	0.226	0.301
P-value[1]							
Pre-digestion	<0.001	0.338	<0.001	<0.001	<0.001	0.006	<0.001
Donors` diet	<0.001	0.013	0.541	0.070	0.046	0.510	0.073
Substrate	<0.001	<0.001	<0.001	0.151	<0.001	0.887	0.925
Pre-digestion×donors’ diet	0.006	0.638	0.193	0.070	0.006	0.510	0.073
Pre-digestion×substrate	<0.001	0.049	0.003	0.151	<0.001	0.887	0.925
Donors’ diet×substrate	<0.001	0.010	0.005	0.583	0.001	0.780	0.419
Pre-digestion×donors’ diet×substrate	<0.001	0.327	0.001	0.583	<0.001	0.780	0.419

[a-b] For each item, means for substrates in the same column with different letters differ significantly (P<0.05).
[1] Interactions are shown in Figure 3.

P=0.036), but decreased it for WS (6.3 *vs.* 8.5 mmol/L; P=0.022). No effects of either the pre-digestion or the donors’ diet on total VFA production were observed for PEC and SBP.

A pre-digestion×donors’ diet×substrate interaction (P=0.001) was observed for molar proportions of acetate and butyrate. The pre-digestion of CEL caused a sharp decrease in the proportion of acetate and an increase in that of butyrate (by 37 and 36 percentage units, respectively) when it was fermented with the LSF-inoculum, but these effects were not observed with the HSF-inoculum. In contrast, similar qualitative changes in VFA profile (decrease in acetate and increase in butyrate by 9 and 4 percentage units, respectively) were observed by pre-digesting WS with the HSF-inoculum. For SBP, pre-digestion caused similar changes in VFA profile with both inocula. Finally, the pre-digestion of all substrates resulted in greater (P=0.006) proportions of propionate, isobutyrate and isovalerate than untreated ones. No valerate was detected in any sample.

DISCUSSION

The greater gas production in the pre-digested blanks than in those untreated was attributed to fermentation of the added enzymes. Carro *et al.* (2005) analysed the effects of treating several substrates with fibrolytic enzymes on *in vitro* ruminal fermentation, and also observed greater gas production in the blanks including the fibrolytic enzymes. These authors also noticed that using different blanks (with or without enzymes added) for correcting gas production values altered the differences between experimental treatments and changed the interpretation of results. The increased total VFA production and the shifts in VFA profile observed in our study when the blanks were pre-digested also indicate a fermentation of enzymes, as the amino acids resulting from proteolysis can be deaminated

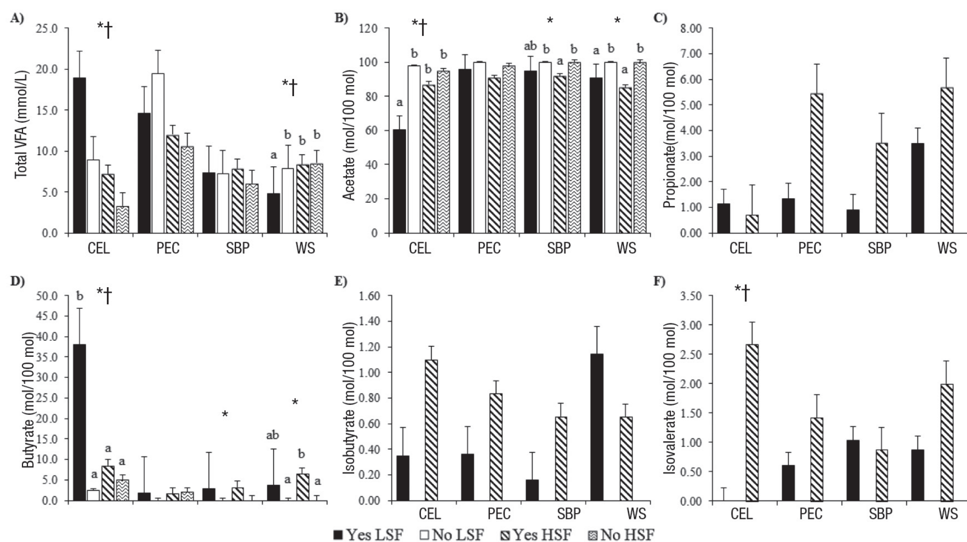

Figure 3: Effect of pre-digestion (Yes *vs.* No), type of diet fed to donor rabbits of soft faeces (low soluble fibre [LSF ■] or high soluble fibre [HSF ▧]) and incubated substrate (D-cellobiose (CEL), sugar beet pulp pectin (PEC), sugar beet pulp (SBP) and wheat straw (WS)) on total volatile fatty (VFA) production and molar proportions of individual VFA after 24 h of *vitro* incubation (n=4 rabbits/diet). a, b: Within each substrate, columns with different letters differ (P<0.05). Bars indicate the standard error. Within each substrate, * and † indicate an effect (P<0.05) of pre-digestion and donors' diet, respectively. ■: yes LSF; □: no LSF; ▧: yes HSF; ▩: no HSF.

and the generated carbon skeletons be fermented to VFA (Van Soest, 1994; Vanegas *et al.,* 2017). The greater isovalerate proportions detected in the pre-digested blanks are consistent with this hypothesis, as these VFA generally arise from the fermentation of leucine (Wallace and Cotta, 1988). Therefore, both gas and VFA production values in our study were corrected for the amount of gas and VFA, respectively, produced in the corresponding blanks. The inclusion of enzymes also increased the proportion of butyrate, which is quantitatively the second VFA in the rabbit caecum (García *et al.,* 2002).

The effect of both the pre-digestion and donors' diet on gas and VFA production depended on the incubated substrate (Figures 2 and 3). The pre-digestion had no influence on the *in vitro* gas production kinetics of WS and had only a moderate effect on that of SBP (reduction of lag time), although in both cases the pre-digestion decreased the molar proportion of acetate and, in combination with the donors' diet, modified total VFA production for WS. The similarity in the results observed for SBP and WS contrasts with the different proportion of solubilised carbohydrate fraction obtained after the pre-digestion procedure (40 and 22%, respectively) previously reported by Abad *et al.* (2013) for the same substrates. The lack of a stronger influence of the pre-digestion on the fermentation of these substrates might be explained by a quick solubilisation of this carbohydrate fraction when the untreated WS and SBP were mixed with the inoculum. Using a different experimental approach (pig faeces as inoculum) and the insoluble residue of the pepsin-pancreatin pre-digestion of SBP (and other carbohydrate-rich substrates) as substrate, Bauer *et al.* (2003) reported lower *in vitro* gas production for pre-digested than for untreated substrates. However, it should be noted that these authors obtained the residue of the pre-digestion by centrifugation, and therefore the soluble potentially-fermented compounds were lost, which might partly account for the different results obtained. In our study, the substrate was not centrifuged/filtered after the pre-digestion, and thus contained all generated compounds (mainly soluble fibre, but also protein, especially in SBP), that could be further fermented by the caecal microbiota. However, no increase in either gas or VFA production was observed by the pre-digestion of SBP and WS. Contrary to the results of Bauer *et al.* (2003), the use of the pre-digested insoluble residue (excluding by filtration the soluble fraction) of SBP did not modify the *in vitro* gas production compared with the untreated one, using rabbit caecal digesta (Kermauner

and Lavrenčič, 2013). These results might indicate a minor influence of the solubilised fraction in pre-digestion on the *in vitro* fermentation kinetics. Differences either in the *in vitro* solubility of SBP or in the caecal inoculum can also help explain the controversial results observed in different studies.

The CEL was the substrate most affected by both the pre-digestion and the donors' diet (Figures 2 and 3). The results indicate that the combination of pre-digestion and LSF-inoculum caused an increase in the amount of CEL fermented, as cumulative gas production is directly related to the organic matter fermented (Menke *et al.,* 1979); however, this effect was not observed for the other substrates. The increased total VFA production observed when CEL was pre-digested (mainly with the LSF inoculum) helps strengthen this hypothesis. These results might be explained by an eventual modification of CEL structure due to the pre-digestion procedure and/or a potential synergy between the added enzymes and CEL, combined with a specific interaction with the microbiota present in the LSF-inoculum. There is some evidence for the potential modification of CEL by the pre-digestion procedure, as CEL can be hydrolysed at low pH values (2.0-3.0) such as that used in the first step of the pre-digestion procedure (Bobleter *et al.,* 1986). Moreover, CEL can be extensively digested in the small intestine of rats by a β-galactosidase present in the mucosal membrane (Morita *et al.,* 2008), and the presence of cellobiosidase in the intestine of herbivores has been described previously (Nakamura, 2005). However, it is unlikely that the pancreatin used in our study had these enzymatic activities, although this point would require further confirmation. The combination of added enzymes and CEL might have interacted specifically with the microbiota of the LSF-inoculum, as suggested by the marked shifts observed in the proportions of acetate and butyrate. In agreement with our results, Falcão-e-Cunha *et al.* (2004) found that caecal fibrolytic activity in rabbits depended on the type of dietary fibre. Others have also reported that a reduction in the dietary level of soluble fibre reduced the ileal and faecal digestibility of different fibrous fractions (Abad-Guamán *et al.,* 2015) and modified the caecal microbiota of rabbits (Gómez-Conde *et al.,* 2009; Gómez-Conde *et al.,* 2007).The microbial communities in the LSF-inoculum might have also interacted with the pre-digested CEL, causing changes in VFA profile. A change of butyrate for acetate also resulted from the caecal *in vitro* fermentation of untreated CEL using rabbit caecal contents obtained from donor rabbits fed with a low soluble fibre diet (Yang *et al.,* 2010). All these results could indicate that the amount of fermentable substrate available for the microbiota would have been lower for the LSF compared with the HSF diet, which in turn might have influenced the activity and/or adaptability of the microbiota. An adaptation of the microbiota to CEL seems to be required according to the large lag time values observed for this substrate (25.3 h), although the time for maximal rate of gas production (T_i) was similar to that for PEC and lower than that for SBP (Table 3). It seems that a synergy occurred when the pre-digested CEL was incubated with the LSF-inoculum, which might be related to the potentially high demand of amino acids at initial stages of fermentation, thus avoiding the use of amino acids as energy source. The lack of isovalerate production detected for the pre-digested CEL with the LSF-inoculum (although not different from the other treatments) would support this hypothesis.

Contrary to that observed for CEL, k and μ_m values increased when pre-digested PEC was fermented by the HSF-inoculum compared with the untreated PEC, but there was no effect with LSF-inoculum. This might be related to a better adaptation of the microbiota from the HSF-inoculum to the pre-digested PEC and/or a potential modification of PEC by the pre-digestion, as suggested by the PEC flocculation observed under the acidic conditions of the first step of the pre-digestion. These results are in agreement with the changes previously observed by Tran *et al.* (2016) in the gas production, VFA molar proportions and microbiota profile when mucins (another fraction of the endogenous substances) were added to *in vitro* batch fermentations, although only minor interactions were detected between mucins and the incubated substrates (cellulose and inulin). Further studies would be required to assess whether supplementation of the substrate with other quantitatively relevant endogenous substances (like mucins) should be implemented, as previous in vivo and *in vitro* studies have reported a high fermentability of mucins (Abad-Guamán *et al.,* 2015; Marounek *et al.,* 2000).

In conclusion, pre-digestion of the substrate before *in vitro* caecal fermentation is recommended in order to exert potential changes in the substrate similar to those occurring during digestion in the stomach and small intestine, and to provide some endogenous substances for caecal fermentation. The level of soluble fibre in the donors' diet also influence the *in vitro* caecal fermentation, but its effect was heavily dependent on the type of substrate.

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