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Hindgut fermentation in pigs induced by diets with different sources or starch

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Abstract

A proportion of dietary starch reaches the hindgut, being fermented there. The characteristics of *in vitro* caecal fermentation and microbial community in pigs given different sources of starch were studied. Twenty-four Duroc × (Landrace × Large White) gilts given diets based on barley (B), broken rice (R), maize (M) or peas (P) (n = 6) for five weeks were slaughtered with 93.6 ± 6.41 kg. No differences ($p > 0.10$) were recorded in caecal pH, total short chain fatty acid (SCFA) and total bacterial concentration, nor in *in vitro* gas production from caecal contents, indicating the lack of a quantitative dietary effect on caecal environment. This could be partly due to the length of fasting time before slaughter (around 10 h). Molar SCFA proportions did not differ among diets; however, relative proportion of *Lactobacillus sobrius/amylovorus* as the species-type in starch digestion in hindgut of pigs, was highest with P diet ($p = 0.010$), and gas production from potato starch as substrate with P diet was highest at 2 h incubation ($p = 0.012$), and higher than B and R diets at 4 ($p = 0.055$) and 6 ($p = 0.10$) h incubation. Caecal bacterial biodiversity was higher for M and R diets than for P and B diets (Shannon index, $p = 0.003$). Sources of resistant or slowly digestible starch such as peas promote a microbial community with a different profile and higher capacity to ferment the starch arriving to the organ than other sources which are mostly digested in the small gut.

Additional key words: gilts; caecal fermentation; starch; *in vitro;* bacterial diversity.

Introduction

Conventional diets for growing pigs are largely based on starchy ingredients as source of energy. Most dietary starch is digested in the small intestine, but a proportion depending on starch composition may escape and reach the hindgut, being fermented by microbial population. With diets differing in proportions of rapidly digestible (RDS), slowly digestible (SDS) and resistant (RS) starch, Regmi *et al*. (2011) recorded ileal starch flows ranging from 1.3 to 211 mg g^{-1} dry matter fed. However, starch output in faeces was in all cases below 25 mg g^{-1} dry matter fed, highlighting that starch arriving to the hindgut is totally fermented by microbial community. Thus, ileal starch digestibility in diets based on wheat, barley or rice may be over 0.95 (Canibe & Bach Knudsen, 2001; Sun *et al*., 2006), whereas it is reduced below 0.80 in sources with a high proportion of RS such as potato (Martin *et al*., 1998) or peas (Sun *et al*., 2006). In fact, RS is considered to behave physiologically as soluble dietary fibre in the hindgut (Haralampu, 2000). Comparison of *in vitro* digestibility of diets based on 0.60 of rice, barley or maize indicates a hindgut contribution to overall organic matter digestibility of 0.09, 0.07 and 0.15, respectively (Pérez-Vendrell & Torrallardona, 2010).

In the hindgut, microbial fermentation of starch renders short chain fatty acids (SCFA) that are partly absorbed and contribute to overall energy input. Thus, Bach Knudsen (2011) summarises that 4% of energy

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Abbreviations used: B (barley); Ct (cycle threshold); DGGE (denaturing gradient gel electrophoresis); M (maize); P (peas); R (broken rice); qPCR (quantitative Polymerase Chain Reaction); RDS (rapidly digestible starch); RS (resistant starch); SCFA (short chain fatty acids); SDS (slowly digestible starch); SEM (standard error of the mean); SI (similarity index).

comes from SCFA in a maize-based diet, whereas 7% and 44% may come from peas or potato starch diets, respectively. Not only the total concentration, but also the profile of SCFA is affected by the nature of dietary starch. Hedemann & Bach Knudsen (2007) reported that acetate proportion decreases, and that of butyrate increases, when the proportion of non-resistant starch (in the form of maize) was substituted by RS (as potato starch). Different symbiotic bacteria are able to digest starch, mostly *Bifidobacterium* and *Lactobacillus* spp. (Bird *et al*., 2007; Regmi *et al*., 2011). Recently, *Lactobacillus sobrius/amylovorus* has been described as a major amylolytic species for the pig digestive microbiota (Konstantinov *et al*., 2006; Marti *et al*., 2010).

It has been hypothesised that diets with starch sources differing in starch composition may promote different nutrient levels reaching the hindgut of growing pigs, thus affecting environment and microbial fermentation. Therefore, an experiment was designed with barley, broken rice, maize and peas as starch sources for growing pigs to study their effect on the characteristics of caecal fermentation and microbial community, either quantitatively or qualitatively.

Material and methods

Experimental design and sampling

All procedures were carried out under the Project License PI03/10 approved by the *Comisión Ética Asesora en Experimentación Animal* from the University of Zaragoza, Spain. Care and use of animals were performed according to the Spanish Policy for Animal Protection 32/2007, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

This experiment was carried out at the facilities of the *Servicio de Experimentación Animal* (University of Zaragoza), simultaneously with a larger production trial. On it, a total of 72 Duroc × (Landrace × Large White) crossbred gilts, with 116 ± 3 d of age and weighing 63.0 ± 4.55 kg of body weight were allocated by weight in groups of three animals to six blocks of increasing weight and distributed within each block to the experimental treatments in a randomised block design, resulting in six replicates of three gilts each per treatment. Animals were housed in 100% slotted floor pens (2.0 m × 2.0 m) provided with a grow feeder and an automatic drinking device, in a temperature-controlled barn (23-25°C).

Animals were given four experimental diets, based on different sources of starch: barley, maize, broken rice and peas. Diets were formulated to include 450 g starch kg^{-1}, provided by barley (B), barley and broken rice (R), barley and maize (M) or barley and peas (P) combinations. At least 0.40 of total starch from diets R, M and P was provided by rice, maize or peas, considering B (only barley as source of starch) as a control. In diet P, peas also substituted part of soybean meal as protein source. In diet R, a small proportion of soybean hulls was included to maintain a similar fibre level than the other diets. Diets were formulated to be isonutritive and to meet or exceed the levels recommended by FEDNA (2006) for growing pigs. The ingredient and nutrient composition of the experimental diets is shown in Table 1. Diets were given *ad libitum* on a meal form, and gilts had free access to fresh water throughout the experiment. The feeds were sampled weekly and pooled for subsequent chemical analyses.

After 5 weeks of feeding, one gilt per pen was randomly chosen and slaughtered in a commercial abbatoir, resulting 6 gilts per treatment (93.6 ± 6.41 kg of body weight). Animals were kept in lairage for 10 h, with full access to water but not to feed, before being stunned and exsanguinated according to standard commercial procedures. Immediately after slaughter, total digestive tract was obtained and the caecum excised. The pH of caecal contents was measured directly inside the organ with a glass electrode pH-meter (Crison 507, Crison, Barcelona, Spain). Approximately 20 g of contents were sampled into 22 × 70 mm polypropylene screw cap tubes and immediately frozen in liquid nitrogen for fermentation studies (Prates *et al*., 2010). Another sample of caecal contents (1 g) from each gilt was immediately taken, frozen in liquid nitrogen and stored at –80°C for subsequent microbiological analyses.

Caecal fermentation study

Samples of caecal contents were thawed at 38ºC for 1-2 min, and 1 g was added with 1 mL distilled water and 0.5 mL of a 0.5 M H_3PO_4 plus 0.05 M 4-metilvalerate solution, that were stored at –20°C for determination of SCFA concentration. For the study of *in vitro* caecal microbial fermentation, 10 g caecal contents were taken and diluted under a CO_2 stream in 40 mL of incubation solution, without resazurin and micromineral solution, using HCl-cysteine as the re-

Table 1. Ingredient and chemical composition of the experimental diets (g kg^{-1}, as-fed basis)

	Diet[1]			
	B	**R**	**M**	**P**
Ingredients				
Barley grain	780	490	490	490
Broken rice	—	230	—	—
Maize grain	—	—	270	—
Peas	—	—	—	400
Soybean meal (440 g crude protein kg^{-1})	172	198	198	60
Blended fat	20	19	10	19
Soybean hulls	—	33.5	3	4.3
Calcium carbonate	10	10	10	10
Dicalcium phosphate	8	10.5	9	8
Sodium chloride	4	4	4	4
L-Lysine	1.5	0.8	1.5	—
DL-Methionine	0.5	0.2	0.5	0.7
Vitamin and mineral premix[2]	4	4	4	4
Analysed composition				
Dry matter	902	897	901	900
Organic matter	934	940	940	938
Crude protein	173	167	172	180
Neutral detergent fibre	158	151	150	151
Ether extract	39	36	39	38
Total starch	425	454	455	425
Rapidly digestible starch (g kg^{-1} starch)	229	322	225	194
Slowly digestible starch (g kg^{-1} starch)	552	562	514	460
Resistant starch (g kg^{-1} starch)	218	116	260	347
Calculated composition[3]				
Metabolisable energy (kcal kg^{-1})	3,093	3,141	3,118	3,111
Lysine	9.0	9.4	9.2	9.6
Calcium	6.7	7.5	6.9	6.7
Digestible phosphorous	2.4	2.5	2.4	2.6

[1] Diets based on barley (B), broken rice (R), maize (M) and peas (P). [2] Providing (per kg of diet): vitamin A: 6,500 IU; vitamin D_3: 2,000 IU; vitamin E: 6 IU; vitamin B_2: 4 g; vitamin B_6: 1.5 g; vitamin B_{12}: 16 g; niacin: 18 g; calcium pantothenate: 9 g; choline chloride: 75 g; Zn: 110 g; Mn: 16.6 g; Fe: 99.9 g; Cu: 12 g; Co: 0.48 g; Se: 0.21 g; I: 0.99 g; 4920 6-Phytase: 499.8 FTU; E 4818 Endo-1.4 β xylanase: 10 IU. [3] According to FEDNA (2010).

ducing agent (Mould *et al*., 2005). Bicarbonate concentration in the buffer solution was adjusted to maintain an incubation pH of 6.5 (Kohn & Dunlap, 1998). Aliquots (10 mL) were taken and inoculated into duplicate bottles (32 mL total volume) with 170 mg potato starch (Panreac 121096, Barcelona, Spain) as substrate, according to the method proposed by Theodorou *et al*. (1994). Two additional bottles without substrate were also prepared as blanks for each inoculum, in order to adjust for the potential contribution of other solubles in the extracts on overall gas production (expressed per unit of inoculum dry matter) and correct to readings of substrate-including bottles from self-fermentation of inocula. Bottles were added with another 10 mL of incubation solution, capped with a rubber septum and an aluminium seal, and incubated for 24 h in a water bath at 38°C. Four incubation runs with two samples per treatment on each run, were carried out. The remaining sample was used for dry and organic matter determination.

Internal pressure of bottles was recorded at 2, 4, 6, 8, 10, 12, 15 and 24 h with an HD2124.2 manometer with a TP704 pressure gauge (Delta OHM, Caselle di Selvazzano, Italy). Pressure readings (Pres) were converted into volume (vol) by using a pre-established linear regression between pressure recorded in the

same type of bottles and known inoculated air volumes (Pres = 1.301 + 75.10 vol; $n = 35$; $R^2 = 0.997$). The gas volume for each incubation time was corrected for the inoculum organic matter, and when substrate was added it was expressed per unit of incubated substrate, once discounted the contribution of the inoculum itself.

Microbiological analyses

Samples for microbiology analysis were treated with bead beater (60 s at 80 rpm) and then DNA was extracted from frozen samples using a QIAamp DNA Stool Mini Kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions, except that the samples were heated at 95°C for 5 min to lyse bacterial cells. DNA concentrations were measured at 260 nm with a NanoDrop (3.0.1, ND-1000, Willington, USA) and purity was accessed by measuring the A_{260}/A_{280}.

For denaturing gradient gel electrophoresis (DGGE) analysis of bacterial caecal community, fragments of V3 region of the 16S rRNA genes were amplified (200 pb) from the extracted DNA by PCR using the bacterial universals primers (*E. coli* positions 341-534) 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGGCCTACGGGAGGCAGCAG-3' and 5'-ATTACCGCGGCTGCTGG-3' (Muyzer *et al*., 1993). PCR amplification conditions used were 94°C for 4 min; 32 cycles (94°C 1 min, 55°C 1 min, 72°C 1 min) and 72°C for 4 min. PCR reactions used 50 ng DNA, in 50 µL reaction mix containing 1 mM of buffer, 1.25 mM of each primer, 0.8 mM of dNTPs mix, 2.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase, and were carried out in a DNA engine gradient cycler (Bio-Rad®) thermocycler. The resulting amplicons were analysed on a 1.5% (w/v) TAE agarose gel to check for PCR products of approximately the predicted size (200 bp).

The DGGE was performed using the model DGGE-1001 from CBS Scientific Company, following the manufacturer's guidelines. PCR products (16 µL) were loaded onto 8% (w/v) TAE polyacrylamide gels (40 mM of Tris base, 20 mM of glacial acetic acid and 1 mM of EDTA, pH 8.3), which contained a 40-60% denaturant gradient (100% denaturant, 7 M of urea and 40% (v/v) deionised formamide). Electrophoresis was performed at a constant voltage of 80 V and at a constant temperature of 60 °C for 16 h. Bacterial DNA was then visualised by silver-stained using the kit DNA Silver Staining (Amershan Biosciences, Uppsala, Sweden) following the manufacturer's instructions. The DGGE was scanned and the image was analyzed with Quantity One Software (BIO-RAD Laboratories, Ltd, Hemel Hempsteads, Hearts, UK) by scoring for the presence or absence of bands at different positions in each line. DGGE banding profiles were analyzed using Dice Coefficient and Unweighted Pair Group Method with Arithmetic Mean clustering algorithm, and were shown graphically as a dendrogram. The percentage of similarity was indicated by the similarity coefficient bar located above each dendrogram. Each band position present in the gel was binary coded for its presence or absence within a lane and each lane was compared by using a similarity matrix. The binary data generated were used to calculate the number of bands, *i.e.* the richness index. The Shannon index was used as a diversity index (Buckland *et al*., 2005) and was calculated as follows: $H = -\Sigma(pi)(\ln pi)$, where *pi* is the proportion of bands *i* in the sample.

Real time PCR (qPCR) was performed using an ABI PRISM® 7000, Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Concentration of total bacterial DNA was measured using the primers (50 mM) to target 16S rDNA BAC338F (5'-ACTCCTACGGGAGGCAG-3') and BAC805R (5'-GACTACCAGGGTATCTAATCC-3') (Yu *et al*., 2005). Concentration of *L. sobrius/amylovorus*, as type species implicated in starch digestion in the hindgut of pig were measured with the primers forward 5'-TTCTGCCTTTTTG GGATCAA-3' and reverse 5'-CCTTGTTTATTCAAGTGGGTGA-3' (Konstantinov *et al*., 2005). DNA (2 µL) was added to amplification reaction (25 µL total volume) containing 0.25 µL of each primer, 12.5 µL of Master Mix Power Sybr® Green qPCR of Applied Biosystems. Three replicates of each DNA samples were used, and non-template (sterile distilled water) negative control was loaded on each plate to screen for possible contamination and dimmer formation. The plate was briefly centrifuged and placed in the termocycler for amplification. The calibration curves were made with a DNA mixture of bacterial extracts of caecal contents from two pigs, diluted from 10^{-1} to 10^{-5}. Concentration was expressed as ng DNA μL^{-1} extracted DNA. Cycling condition for absolute quantification of total bacteria consisted of an initial hold to 95ºC for 10 min followed by 40 cycles of 95°C for 15 s and 61°C for 1 min, and 72°C for 30 s. For the relative quantification of *L. sobrius/amylovorus*, cycling conditions were: 95°C for 10 min and 45 cycles of 95°C for 15 s, 62.5°C for 45 s and 72°C for 30 s. Fluorescence readings were taken after each

extension step and a final melting analysis was made by slow heating with fluorescence measurements at 0.1°C intervals to determine the PCR specificity. The bacterial concentration in each sample was measured as $\log_{10}$ copy number by the interpolation of the cycle threshold (Ct) values obtained by the samples and the standard calibration curves. The relative quantification of specific bacteria was expressed as a proportion of total caecal bacterial *16S* rRNA (pg ng^{-1} total DNA) according to Denman & McSweeney (2005). Efficiency of the amplification was calculated using serial dilutions, and results were accepted only when efficiency was over 90%. Considering that some qPCR data will not meet the ANOVA requirements for normality, total microbial concentration was transformed on a logarithmic basis for the statistical analysis.

Chemical analyses

Pooled samples of feeds were ground to a 1 mm maximum size with a Wiley mill and analysed in duplicates following the procedures of AOAC (2005). Dry matter was determined by oven drying at 105°C to constant weight (ref. 934.01), organic matter and total ash by muffle furnace (ref. 942.05), crude protein by the Kjeldahl method (ref. 976.05) and ether extract by Soxhlet analysis (ref. 2003.05). The content of neutral detergent fibre was determined according to Van Soest *et al*. (1991) using an Ankom 220 Fibre Analyser equipment (Ankom Technol., NY). The neutral detergent fibre is expressed exclusive of residual ashes, and α-amylase was used in the analysis. Sodium sulphite was not used. Total starch content was determined enzymatically from samples ground to 0.5 mm, using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). The RDS and SDS were determined after 20 and 240 min enzyme hydrolysis (Van Kempen *et al*., 2010), and the RS was estimated by difference with total starch. Analysis of SCFA was carried out by gas-liquid chromatography in an AGILENT 6890 apparatus equipped with a capillary column (Tecknokroma WAW80/100, 10% SP-1,200 + 1% H_3PO_4), using helium as carrier gas.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (1990). The model included diet as the main effect, and the animal donor of caecal content was considered as the experimental unit (n = 6). For *in vitro* microbial fermentation, the incubation run was taken into account as a block. A p-value < 0.05 was considered as a significant difference between treatments, and a p-value between 0.05 and 0.10 was considered as a trend for differences. The least significant difference test was used to compare means when the variance analysis indicated a significant effect.

Results

Caecal environmental traits at slaughter, in terms of pH, total SCFA concentration and molar SCFA proportions (Table 2) were not affected by the experimental treatments ($p > 0.10$). Average caecal pH varied between 6.26 for P to 6.34 for M diet.

Total bacterial concentration in the caecum of gilts was not affected ($p = 0.42$) by the dietary treatments (Table 3); however, the proportion of *L. sobrius/amylovorus* was highest with P diet ($p = 0.010$). Bacterial

Table 2. Caecal pH, total short chain fatty acid (SCFA) concentration (mM) and molar proportions of the main SCFA in caecal contents of gilts fed on diets with barley (B), broken rice (R), maize (M) and peas (P) as sources of starch

	B	R	M	P	SE[1]	*p*-value
pH	6.30	6.29	6.34	6.26	0.081	0.91
SCFA	70.6	66.9	65.3	78.9	4.64	0.22
Acetate	0.591	0.608	0.584	0.594	0.0125	0.59
Propionate	0.244	0.242	0.256	0.246	0.0094	0.70
Butyrate	0.121	0.107	0.110	0.116	0.0070	0.48
Valerate	0.020	0.017	0.021	0.018	0.0021	0.65
Isobutyrate	0.012	0.013	0.014	0.013	0.0013	0.81
Isovalerate	0.013	0.014	0.016	0.014	0.0017	0.57

[1] SE: standard error of mean (n = 6).

Table 3. Total caecal bacterial concentration (log_{10} nº copies g^{-1}) and relative concentration (%) of *Lactobacillus sobrius/amylovorus,* together with richness and Shannon diversity indexes of bacterial community determined by DGGE, in caecal contents of gilts fed on diets with barley (B), broken rice (R), maize (M) and peas (P) as sources of starch

	B	R	M	P	SE[1]	*p*-value[2]
Bacterial concentration	10.06	10.07	10.04	9.97	0.045	0.42
L. sobrius/amylovorus	0.305[b]	0.234[b]	0.160[b]	0.661[a]	0.0975	0.010
Richness index	36.0[b]	46.7[a]	51.8[a]	37.3[b]	2.744	0.001
Shannon index	3.56[b]	3.83[a]	3.94[a]	3.62[b]	0.069	0.003

[1] SE: standard error of mean (n = 6). [2] Within a row, means with different superscript letter differ ($p < 0.05$).

biodiversity indexes are also showed in Table 3. The number of bands detected in DGGE was higher for M and R diets than for P and B diets ($p = 0.001$), and Shannon index responded consequently ($p = 0.003$). Bacterial caecal profile in the DGGE gel is presented in Fig. 1. Samples of caecal contents of gilts given M and R clustered together, with a similarity index (SI) of 0.61. Besides, each of these treatments showed a high internal homogeneity (SI of 0.68 for either M or R diets). In contrast, caecal communities of B and P diets are not clearly separated according to diet, but in any case are grouped in a different cluster that M and R diets, with an average SI of 0.55 among them.

The final pH after 24 h *in vitro* fermentation trial from caecal contents from gilts given B, R, M and P were 6.56 ± 0.20, 6.50 ± 0.13, 6.52 ± 0.21 and 6.52 ± 0.078, respectively when incubated without substrate, and 5.84 ± 1.64, 6.00 ± 1.67, 6.08 ± 1.69 and 5.94 ± 0.19 when incubated with potato starch. The pattern of *in vitro* microbial fermentation of caecal contents (without substrate) is shown in Fig. 2. There were no significant differences among treatments, but from 6 to 12 h, incubation of R diet rendered numerically lower gas volume than those of B and M diets ($p = 0.11$ to 0.13) and it tended to be lowest with R diet after 24 h ($p = 0.091$). When potato starch as a source of RS was

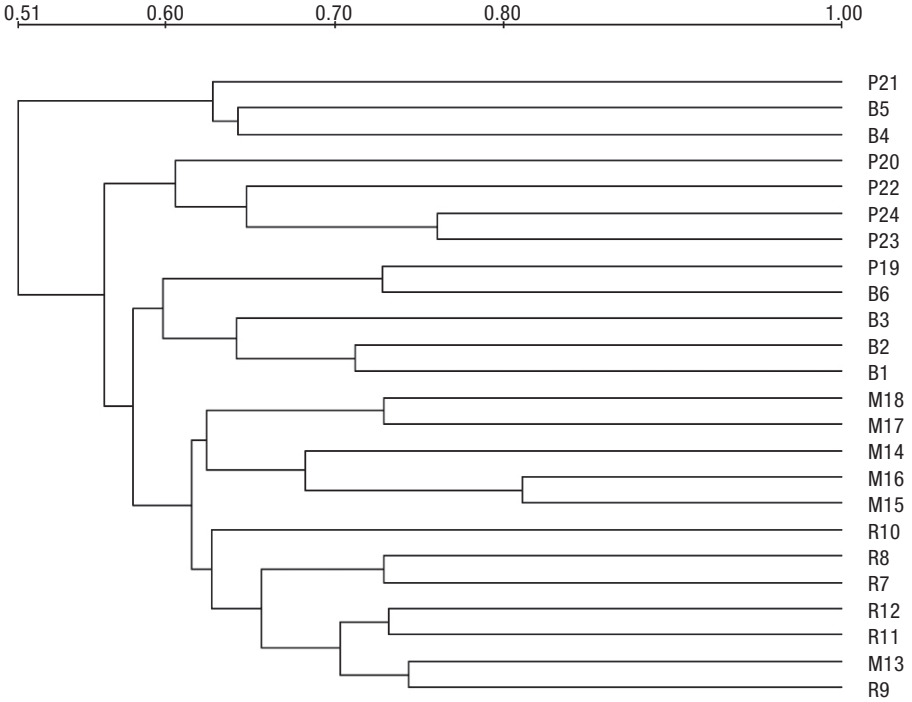

Figure 1. Dendrogram of caecal bacterial diversity from DGGE gel in gilts fed on diets with barley (B1 to B6), broken rice (R7 to R12), maize (M13 to M18) and peas (P19 to P24) as sources of starch.

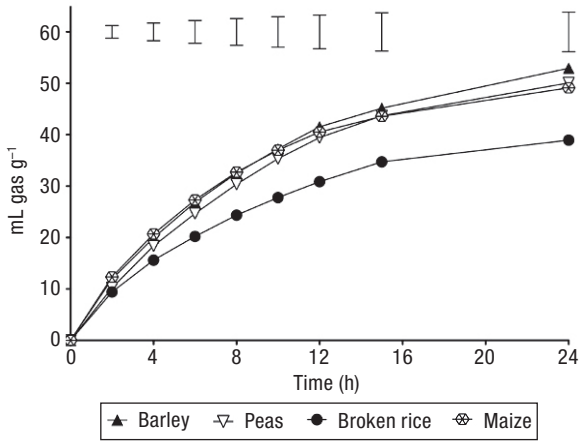

Figure 2. Pattern of cumulative gas production (mL g^{-1} of inoculum organic matter) from caecal contents of gilts fed on diets with barley, broken rice, maize and peas as sources of starch.

included (Fig. 3), gas production with inoculum from gilts given P diet was highest at 2 h incubation ($p = 0.012$), and it tended to be higher than that of B and R diets at 4 ($p = 0.055$) and 6 ($p = 0.10$) h incubation. After, differences became non-significant, probably because of the magnitude of the error term (variation coefficient from 0.40 to 0.55), despite from 8 to 15 h gas with P diet inoculum was 1.6 to 1.7-fold higher than with R diet.

Discussion

Engylst *et al*. (1992) first characterised starch according to its rate of digestion in the human small gut, as RDS (digested *in vitro* in the first 20 min), SDS (from 20 to 120 min digestion) and RS (undigested after 120 min). However, van Kempen *et al*. (2010) suggested that RS should be estimated at a larger time in pigs, since a considerable starch digestion (up to 45%) has been noticed from 120 to 400 min. Consequently, assuming that the mean retention time of digesta through the small gut of pigs with conventional diets is around 4 h (Wilfart *et al*., 2007), a time of 240 min has been considered for the estimation of potential amylolytic activity, and SDS and RS have been calculated accordingly. Thus, our results for RDS, SDS and RS agreed well with those expected from results of Sun *et al*. (2006) and Van Kempen *et al*. (2010), showing that P and R diets promote the highest and lowest proportion of potentially fermentable starch to the hindgut, respectively.

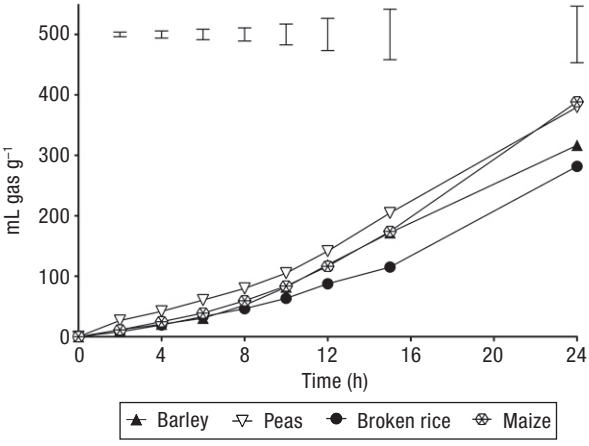

Figure 3. Pattern of cumulative gas production (mL g^{-1} organic matter) from potato starch of caecal contents of gilts fed on diets with barley, broken rice, maize and peas as sources of starch.

It is assumed that total tract digestibility of starch is almost complete (Bach Knudsen, 2011). However, a certain fraction of starch (RS and some proportion of SDS) resists digestion in the small intestine and reaches the caecum, being fermented by the microbial population. Therefore, starch sources with high proportion of RS and SDS would lead to a higher extent of fermentation, affecting hindgut environment. An enhanced gas production was recorded (Fondevila *et al*., 2002) when ileal contents from pigs given maize or acorn-shorghum diets that ensured three-fold differences in starch flow to the hindgut (Morales *et al*., 2002) were incubated.

For a closer approach to caecal conditions, *in vitro* incubation pH was adjusted to 6.5, as it has been achieved in previous incubation studies with rumen contents (Fondevila & Pérez-Espés, 2008; Bertipaglia *et al*., 2010). Despite reduction in bicarbonate concentration in the medium may limit the buffering capacity of the solution, pH after 24 h incubation when starch was added was in the expectable range (present study; Canibe & Jensen, 2003) and far from the level considering as harmful for amylolytic bacteria (Russell, 1992). In the present experiment, *in vitro* incubation of caecal contents without added substrate did not show differences among diets, except for being numerically lower ($p < 0.13$) with R from 6 h onwards. This might indicate that a low amount of starch reached the caecum with diets based in conventional, low RS sources such as barley, maize and peas, and therefore its effect should be minor and can be masked by the presence of other fermentable components (De Schrijver *et al*., 1999), such as fibre. It has to be considered that positive response in hindgut fermentation were ge-

nerally observed when using sources rich in RS such as potato or processed starches (Topping *et al*., 1997; Martin *et al*., 1998). With rice as starch source, the level of both RS and SDS were smaller than in the other treatments (Table 1), and thus its fibre content was not enough to buffer the extent of fermentation. In any case, starch digestion in the small intestine increases with age (Pluske *et al*., 1997), and therefore the magnitude of the response in fermentation diminishes in growing compared with weaned pigs, thus reducing the amount of starch entering the caecum. Besides, caecal contents were sampled after 10 h fasting, and this might also affect substrate concentration in the organ, minimising treatment differences since transit time of digesta through the upper gastrointestinal tract lasts from 5 to 11 h (Wilfart *et al*., 2007; Solá-Oriol *et al*., 2010).

The lack of a significant response in either total SCFA concentration or molar SCFA proportions is in agreement with the lack of response in gas production from self-fermentation of caecal contents, and could be also explained because of both the scarce amount of fermentable substrate at the time of sampling and a masking of the effect of starch reaching the caecum by the fibrous component of diet. An increased starch concentration in the proximal colon with slowly-digestible (high amylose) starch resulted in higher faecal concentration of total SCFA and proportion of propionate, whereas that of acetate decreased (Topping *et al*., 1997). Hedemann & Bach Knudsen (2007) recorded a higher SCFA concentration in the caecum given resistant potato starch, with an increased proportion of butyrate at the expense of acetate, and similar results were reported by Le Blay *et al*. (1999) in rats. However, no differences in total concentration or molar proportions of SCFA in the caecum were observed by Morales *et al*. (2002), even with 3-fold differences in starch flowing to the hindgut, in agreement with results of De Schrijver *et al*. (1999) and Martínez-Puig *et al*. (2003). In any case, it has to be borne in mind that SCFA concentration is a relative, but not a direct indicator of microbial fermentation, since they are absorbed to a great extent through the organ wall, at different rates of each individual acid (Argenzio & Southworth, 1975; De Schrijver *et al*., 1999).

In the same trend as gas production from self-fermentation of caecal contents and total SCFA concentration, no dietary differences were detected on caecal total bacterial concentration. In agreement with our results, there is no evidence of a clear effect of a high input of starch to the caecum on total bacterial concentration in this organ. Morales *et al*. (2002) and Martínez-Puig *et al*. (2003), using purine bases as microbial marker, did not either find any effect on caecal bacterial concentration of diets with different levels of starch reaching this organ nor Le Blay *et al.* (1999) using culture techniques and Regmi *et al*. (2011) using molecular techniques.

Despite the lack of response of *in vitro* gas production by self-fermentation of caecal contents, diet P promoted a microbial community more prone to digest RS and caecal contents from gilts given diet R were the least able to ferment the substrate, as it was showed during the initial 6 h of incubation. The flux of starch to the caecum, even at low concentration, may induce the ability of the microbial community to rapidly degrade a RS source. *In vitro* results from Fondevila *et al*. (2002) with Landrace pigs also showed that ileal contents richer in starch (from an acorn/sorghum based diet) as substrate produced more gas in the first 8 h when incubated with caecal inoculum of pigs given such diet compared to others given a maize diet that ensures less starch reaching the hindgut.

This would be supported by the higher proportion of *L. sobrius/amylovorus* observed in P diet. The *L. sobrius/amylovorus* is a bacterial species capable to degrade starch, that has been observed in abundant concentration in the intestinal tract or faeces of piglets and adult pigs, in a range between 5 and 9 $\log_{10}$ cells per g (Konstantinov *et al*., 2006; Marti *et al*., 2010), constituting up to 80% of total lactobacilli. A typical feature of this species is its ability to utilize starch, producing lactic acid (Du Toit *et al*., 2001), and therefore it was chosen as a target amylolytic species to compare changes in the caecal ecosystem induced by the experimental diets. In our study, proportion of this species was the highest with diet including peas, probably in response to a higher proportion of starch, both SDS and RS, arriving to the hindgut. Similarly, increased concentrations of starch-degrading *Bifidobacterium* and *Lactobacillus* spp. in the proximal colon, and that of *Bifidobacterium* spp. in pig faeces, were reported by Bird *et al*. (2007) and Regmi *et al*. (2011) as resistant starch increased.

From results of similarity in Fig. 3, it is clear that R and M diets promoted specific caecal communities, whereas those from gilts given B or P diets are not well differentiated, but far from those with the former starch sources. Biodiversity indexes (Table 3) show that diets P and B are associated to a less diverse population,

which could be related with a higher specialization. This is supported by the faster increase in gas production when potato starch was incubated with caecal contents from animals given P (Fig. 2), although in the case of barley its starch composition (Table 1) suggests that a minimum amount of starch should reach the hindgut. In contrast, the higher diversity of bacterial communities promoted by diets M and R could be associated to a low availability of nutrients in the caecal ecosystem. In this regard, Durmic *et al*. (2000) obtained a two-fold bacterial isolates in the colonic digesta of pigs when starch flux to the hindgut was reduced from 0.134 to 0.006 RS by extruding the cereal source (wheat).

Under our experimental conditions, it is concluded that the dietary ingredients such as peas, which ensures an input of starch either as resistant or slowly digestible starch into the hindgut, compared with the other starch sources promoted a microbial community with a different profile. Such community is characterised by a higher proportion of starch-degrading bacteria such as *L. sobrius/amylovorus*, which seems more prone to ferment the starch that may arrive to the organ. However, the present experimental conditions do not allow detecting quantitative differences in terms of fermentation of caecal contents and SCFA and total bacterial concentration.

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