Study of the effect of slurry dilution, structural carbohydrates and exogenous archaeasupply on in vitro anaerobe fermentation and methanogens population of swine slurry

Short title: Methanogens population and pig slurry fermentation

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Abstract:
Artificial slurry (4% DM) was prepared using fresh faeces and urine obtained from four pregnant sows fed a commercial diet (DE: 3,124 Mcal / kg; CP: 14%) using freeze-dried dairy cattle feces (external archaea) as co-inoculum (Co-i) and structural carbohydrates (CHO; AP, apple pulp, SBP, sugar beet pulp and WS, wheat straw) as substrate. Pig slurry and buffer (40:60 ratio of slurry: buffer) together with three amount of Co-i (0, 5 and 10 % of the bottles dry matter) and 600 mg of each CHO were incubated in quadruplicate sets in a 3 x 4 x 3 factorial design. Bottles were incubated (39±1°C for 56 days) and gas production (Gp) was measured (mbar) and converted to the volume. A gas sample (0.1ml) was collected manually and analyzed for CH₄ concentration using a GC-chromatography. Incubation bottles were opened on days 0, 25 and 56 to determine concentrations of total bacteria, total and hydrogenotrophic methanogens archaea (HMA) by quantitative PCR and population biodiversity by denaturing gradient gel electrophoresis (DGGE). Gels were scanned and analyzed for the presence or absence of bands. Incubation time reduced the titers of total bacteria and archaea (P<0.01) but did not modify HMA population. Doses of Co-i showed a positive correlation with HMA titres although interacted with incubation period (P<0.001), at 5 % Co-inoculation total bacteria decreased
significantly (0 to 25 days) but remained steady until day 56 (P>0.05) whereas at 10% Co-
inoculation titters decreased constantly. Most of the archaeal DGGE bands were observed in all
samples, suggesting a common microbial population origin but Co-i supply altered the DGGE-
structure of archaea populations.

**Keywords:** Archaea, Biodiversity, DGGE, Methane, Pig slurry

**Introduction:**
The emission of CH$_4$ and other greenhouse gases by livestock, its implications and mitigation
technologies have been the subject of many studies in recent years (i.e. Steinfeld et al., 2006).
Livestock emit CH$_4$ due to anaerobic organic matter (OM) fermentation processes occurring,
mostly in the rumen, but also in post-gastric compartments such as colon (horses, pigs) or
caecum (rabbit). Moreover, as anaerobe fermentation continues, CH$_4$ synthesis may persist during
manure storage (> 30 days; Jarret et al., 2011), especially relevant in the pit’s slurry located
under swine pens (Alvarez-Rodriguez et al., 2013). Methane emission from pigs is lower than
ruminants (Monteny et al., 2001) and a significant fraction (around 50% of the enteric synthesis)
(Van Amstel et al., 1993) comes from slurry storage.
Methane emission from slurry should be considered with a double perspective, firstly
environmental pollution of the emission from pits, which is a process highly dependent on
storage conditions [time, temperature, dilution, pH (Monteny et al., 2001)]. Secondly,
"methanization" of the slurry-OM through its co-digestion, which can be treated as a potential
renewable energy source [CH$_4$] for industrial purposes (Bonmati, 2001; Flotats et al., 2009).
Methane synthesis involves a synchronic OM fermentation by a consortium of microbial species.
Initially, organic substrate is degraded to monomers that are fermented (by acidogenic bacteria)
to volatile fatty acids (VFA) and ketones among others. Through acetogenesis, products are
transformed into hydrogen and acetic acid (Ostrem, 2004) and then hydrogenotrophic and
methylotrophic methanogens transform them into methane (Verma, 2002). Both, methylotrophic
and hydrogenotrophic methanogens are essential in the whole process, but the knowledge of their
roles over the storing process of slurry is rather limited (Demirel and Scherer, 2008). Also,
enhancing the microbial activity by inoculating the slurry with external source of microbes and
supplying extra fermentable organic matter have been suggested as feasible strategies
This work aimed to study the abundance and biodiversity of methanogens during “in vitro” anaerobic slurry-OM conversion to CH₄ and the alteration induced by dry matter dilution and the supply of agro industrial by-products and cattle’s faeces as external source of structural carbohydrates and microbial inoculants, respectively.

**Material and Methods**

1. Materials

   **Inoculums.** Slurry was artificially prepared from fresh faeces and urine to obtain a 10% dry matter (DM) concentration. Excreta were obtained from four pregnant sows (Centre d'Estudis Porcins Torrelameu, Lleida, Spain) fed a commercial diet (DE: 3,124 Mcal / kg; CP: 14%), and faeces collected by rectal extraction and urine by vulva massage. Faeces and urine were homogenized, sampled (10%, for DM analysis) and retained (for 24 hours at 4°C) until use.

   **Co-inoculum (Co-i).** Fresh faeces from dairy cattle (as source of external methanogens) were used. They were collected fresh, lyophilized, grounded and stored in sealed jars until use.

   **Substrates (CHO).** Commercial agricultural by-products (SBP, sugar beet pulp; AP, apple pulp; and WS, Wheat straw) were chosen as substrates. By-products samples were dried (60°C, 48 h), grounded with a hammer mill (1 mm) and stored in sealed jars until use. The proximate chemical analysis of inoculums and substrates is shown in Table 1.

2. Experimental development.

   **In vitro** anaerobic co-digestion of slurry was conducted following Theodorou et al. (1994) procedure and modified by Fondevila and Pérez-Espés (2008). A total of 108 bottles were assigned to a 3 x 3 x 3 factorial arrangement involving 3 slurry dilutions (2, 4 and 6% DM), three Co-i concentrations (Co-i: 0, 5 and 10%) and three CHO (SBP, AP, WS). Briefly, three mixtures were prepared to obtain 20:80, 40:60 and 60:40 slurry: buffer concentrations, (2, 4 and 6% of DM concentration, respectively). Three levels of Co-i: 0, 5 and 10%, respectively (based on the slurry DM) were added to the artificial slurry together with 600 mg DM of the different types of CHO (SBP, AP and WS). In addition, the corresponding blanks combinations were tested:

   i) buffer + slurry; ii) buffer + slurry + Co-i (at 5 and 10% DM, respectively).
The bottles containing the media were incubated (39±1°C) in quadruplicate for 56 days and the measurements were repeated in two incubation consecutive batches.

**Media preparations.** Bottles were filled with the corresponding substrate and Co-i doses the day before the experiment began. Incubation solution was prepared following the initial protocol (Theodorou et al., 1994). Then, bottles were sealed with butyl rubber stopper and aluminium crimps seal, in the presence of constant CO₂ stream. The pressure was released using hypodermic needle through the septum, after shaking the bottles and the incubation started in an air oven at 39±1°C for 56 days.

3. **Sampling protocol.**

Gas production was measured from headspace pressure (mbar) (DELTA OHM, Caselle di Selvazzano, Italy) equipped with a probe (TP704). Gas measurements were performed daily (day 1 to 7), twice a week (day 8 to 21) and once a week (day 22 to 56). In order to prevent slight differences in total volume among bottles, pressure headings were converted to volume by a linear regression established between the pressure recorded in each bottle and known inoculated air volumes at the same incubation temperature (Fondevila and Pérez-Espés, 2008). Gas volume at each incubation time was expressed per unit of incubated organic matter (OM). After gas pressure measurements, a sample (0.1 ml) from headspace gas was collected manually (Hamilton syringe, Gustight® 1001SL1.0 ml SYR 22 / 2 "/ 2 L, Hamilton Company, Reno, Nevada, USA) and immediately analysed for CH₄ concentration.

Incubation bottles were opened on days 0, 25 and the two remaining on day 56, pH was immediately determined (pH meter 2000 Crucible, Crucible Instruments. Barcelona, Spain) and 12 ml of the liquid media were weighed, frozen in liquid N and stored at -80°C to use to determine total bacteria and total and hydrogenotrophic archaea titters and biodiversity were determined by real time PCR (qPCR) and DGGE (Denaturing gradient gel electrophoresis), respectively. The remaining contents was filtered through a metal sieve (1 mm Ø) and two samples were taken for NH₄⁺-N (2 ml were acidified with 0.8 ml HCl 0.5 N) and volatile fatty acids (VFA) determination (4 ml were mixed with preservative solution [1 ml, 2 g / l mercuric chloride, 20 ml / l ortho-phosphoric acid and 2 g / l 4-methylvaleric acid in distilled water]). Samples were immediately frozen (-20°C) until further analyses.

4. **Chemical analyses.**
DM content was determined in an oven at 105°C until a constant sample weight. Ash content was determined sample incineration on muffle furnace (550°C for 4 h). Crude protein (CP), crude fibre (CF), ether extracts (EE) and ammonium-N were analyzed according to AOAC (1999). NDF and ADF were analyzed according to Van Soest et al. (1991) procedures. VFA concentrations was determined by gas chromatography (GC) based on the technique proposed by Jouany (1982) using a gas chromatograph (Agilent Technologies 7890A, Net Work GC System, Beijing Elmer, Boston, USA), equipped with a flame ionization detector (FID) and a capillary column (BP21 30m x 0.25 mm ID x 0.25 m). Methane was analyzed using the same GC equipment, equipped with a different column(113-4332, GS- Gaspro capillary 30m x 0.32 mm ID), operating at 70°C for the column, 150°C in the injector and 200°C in the detector. The carrier gas was helium (99.999% purity [C50], Carburos Metalicos, Spain) and the total injection time was 1.8 minutes. Methane concentration was calculated from the peak concentration: area ratio using as a reference peak area generated from standard gas (CH₄; 99.995% purity[C45], Carburos Metalicos, Spain). Then different headspace volumes of standard mixture [0.1, 0.3, 0.5, 0.7 and 0.9 ml] were manually injected to the gas chromatograph to obtain a standard curve.

5. DNA extraction, DGGE, and real time-PCR analyses

The DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer’s instructions. For DGGE analysis, specific Archaea region of the 16S rRNA gene was amplified by PCR using the primers described by Cheng et al. (2009). PCR amplification conditions used were as follows: 1 cycle (94 ºC for 4 min); 30 cycles (94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min); 1 cycle (72 ºC for 7 min). PCR reactions used 50 ng DNA, in a 50 mL reaction mix containing 1 mM buffer, 1.25 mM of each primer, 0.8 mM of dNTPs, 2.5 mM MgCl₂ and 2.5U of Taq DNA polymerase in 10mM Tris-HCl (pH 9.0). The resulting amplicons were visualized on a 2% (w/v) TBE (89 mM Tris, 89 mM Boric acid, 2 mM Na₂EDTA, pH 8.3) agarose gel to check PCR products within the predicted size range. DGGE was performed using a BDH DGGE V20-HCDC Unit from VWR international Ltd (UK), following the manufacturer’s guidelines. PCR products (10 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 30–65% 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 60ºC for 16 h. DNA was then visualized by silver-stained using a Bio-Rad Silver Staining kit following the manufacturer’s instructions.

The gel was scanned and the image was analyzed with molecular analysis fingerprinting software (Quantity One - BIO-RAD Lab, Inc.) by scoring for the presence or absence of bands at different positions in each line. DGGE banding profiles were compared using Dice coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm and shown graphically as a dendogram. The level of similarity was indicated by the percentage similarity coefficient bar located above dendogram. Richness was estimated by counting the number of detected band in each DGGE profile. The Shannon index was used as a diversity index, as described in the following equation (Cox, 1979): $H = -\Sigma(p_i)(\ln p_i)$, where $p_i$ is the ratio of one specific group of bacteria to the total microorganisms in the samples, and $i$ is the total number of microbial species in the samples.

Real time PCR was used to quantify the numbers of total bacteria (Maeda et al., 2003) and archaea (Matarazzo et al., 2011) as described previously. Hydrogenotrophic methanogens were quantified as described by Denman et al. (2007). Analyses were performed on iQ5 multicolor Real-Time PCR Detection System (BioRad, Laboratories Inc., Hercules, CA, USA).

6. Calculations and statistical analysis.

Pressure values (mbar) were transformed to the volume gas unit by building a standard curve using generated pressure against the known air volume, final equation was $(y = 11.46^{-1}x - 0.289; R^2 = 0.9984)$ being $(x, \text{mbar})$ and $(y, \text{mL})$ pressure and gas volume, respectively.

Gas, methane production, pH were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA), considering as fixed effects the replicate (1, 2), slurry DM concentration (2, 4 and 6%), type of substrate (blank, WS, AP and SBP), Co-i level (0, 5 and 10%), incubation period (day 7, 14, 21, 28, 35, 42, 49 and 56), and their second-degree interactions; and bottle unit as a random effect.

VFA and N-NH$_4^+$ and qPCR analyses were carried out only in a single replication and hence this fixed effect was not accounted in the statistical model concerning these variables. The copy numbers of total bacteria, total archaea and hydrogenotrophic methanogens were transformed to their logarithm [log 10] to perform the statistical analyses. The relative quantification of methanogens was carried out using $\Delta C_t$ ($\Delta C_t = C_t \text{ total bacteria} - C_t \text{ MA}$). The mean separation
between treatments was performed using the Tukey test and the differences were considered significant at P<0.05.

**Results**

To study the evolution of the population of bacertia and archaea trough 56days of *in vitro* incubation of swine slurry, their activity [CH$_4$ production], changes in the incubation media [pH, ammonia, VFA concentration], titres of total and hydrogenotrophic methanogens and the archaeas biodiversity were analysed.

1. Methane production.

Figure 1 shows the accumulated CH$_4$ production through the incubation period [from 0 to 56 days] analysing the effect of slurry dilutions and substrate supply into the incubation media (Figure 1a and 1b, respectively). CH$_4$ production was modified by slurry concentration (P<0.001), although the effect was not linear, levelling off at 4% concentration when slurry was incubated alone (53.42, 72.92 and 52.72 ml/g OM SE =6.53) or supplemented (50.30, 70.21 and 55.84 ml/g OM SE = 8.27 for slurry concentrations at 2, 4 and 6% of DM, respectively). Addition of wheat straw (WS) and apple pulp (AP) improved total CH$_4$ production (296 and 361 ml CH$_4$/bottle; P<0.05) and production efficiency (132.2 and 138.6 ml CH$_4$/ OM; P<0.05) in relation to blanks (251 ml CH$_4$/bottle and 124.18 ml CH$_4$/mg OM). However, addition of sugar beet pulp (SBP) depressed both total (211 ml CH$_4$/bottles) and production efficiency (88.48 ml CH$_4$/mg OM) as compared to blanks. Co-inoculation with cows faeces from did not alter CH$_4$ production.

2. Changes in pH, ammonia and volatile fatty acids in the media along the incubation period.

2.1 Media acidity.

Along the incubation period, pH showed the pattern described in Figure 2, which decreased at 25- days and increasing at 56-days period (P<0.001), although such it was more pronounced in supplemented than in non-supplemented media (blanks) in which pH reduction at 25-days period was negligible (Interaction Days x Substrate: P<0.01). A differential evolution among substrates was also observed with slurry dilution, as pH decreased in SBP-supplemented bottles with slurry concentration (6.9, 6.79 and 6.67). The opposite was observed in non-supplemented bottles
(blanks: 7.07, 7.27 and 7.33 for 2, 4 and 6% slurry DM concentration), whereas no significant variations were detected in those bottles supplemented with straw or AP (Interaction Slurry dilution x Substrate: P<0.01). Type of substrate altered average media acidity (P<0.01) and pH was lower in pulps (pH =7.02 and 6.8 for AP and SBP) than in blanks bottles (7.23; SE: 0.04).

2.2 Ammonium concentration

Ammonia-N concentration in the incubation media varied between 1.18 and 3.02 g/l and were significantly modified by experimental treatment. ammonia concentration increased along the incubation period at the highest level of slurry concentration (2.57, 2.72 and 3.02 g/l in 6% at 0, 25 and 56 days and 1.58, 1.92 and 2.11 g/l in 4% slurry DM concentration, respectively). However no changes were observed at the lowest concentration level of slurry (1.56, 1.18 and 1.22 in 2% slurry DM at 0, 25 and 56-d incubation period, respectively; Interaction Slurry concentration x Days: P<0.01). The effect of slurry concentration on ammonia was also modulated by type of substrate, thus for SBP-supplemented bottles and for blanks bottles ammonia concentration increased proportionally with DM concentration but in those bottles supplemented with straw or AP, the rise was only observed when slurry concentration increased from 4 to 6% of DM.

2.3 Volatile fatty acids (VFA) evolution.

Average VFA concentration (mM) in the original media (12.7), raised (28.8) at day 25 and decreased (6.1) at day 56 of the incubation period (Table 3). Differences among slurries in the time evolution of VFA concentration did reach statistical significance (Interaction Slurry concentration x Days: P<0.01). The highest production (from day 0 to 25) was registered in media with highest DM concentration (6%) whereas the highest disappearance rate (from day 25 to 56) was observed with the medium slurry concentration (4% DM). Changes in molar proportion of main individual VFA (i.e: acetic, propionic and butyric) are shown in Figure 3. In general, at different rates, acetate proportion increased, to the detriment of the rest, although such transformation process was modulated for both, slurry concentration and type of substrate. During the incubation period, the average concentration of acetate was greater whereas the concentration of propionate was lower in blank and WS than in AP and SBP within the most diluted slurry (2% DM) (P<0.05). This response was not observed in 4 and 6% slurry DM, which showed similar average concentration of acetate and propionate among substrates (P>0.05). As shown in Figure 3, the relative production of propionate was nearly negligible at day 56 of
incubation in all the substrates added to 4 and 6% slurry DM but it was noticeable in AP and SBP from the 2% slurry DM.

The concentration of valerate and branched-chain fatty acids (iso-butryate plus iso-valerate acids) was affected by the interaction Slurry dilution x Incubation time (P<0.01; Table 3). Valerate proportion peaked at day 25 in 4 and 6% DM (2.55 and 2.02±0.16%, respectively), whereas this increase was not observed in 2% slurry DM (1.51±0.16%, P<0.05). Likewise, the proportion of branched-chain fatty acids peaked at 25 days of incubation period in 4 and 6% slurry DM (6.62 and 6.31±0.56%, respectively), but not in 2% DM (3.40±0.56%, P<0.05). In addition, valerate and branched-chain fatty acids concentrations were negligible at 56 days of incubation.

3. Changes in total bacteria and archaea titres along incubation time.

Figure 3 evidenced that the intermediate slurry concentration (4% of DM) incubated at 56-day period provided the most favourable fermentation conditions to allow the complete OM fermentation. Most of the substrate was fermented to CO$_2$ and CH$_4$, at this point the complete transformation or “methanization” of the fermentable substrate can be assumed. This process neither was reached at highest nor lowest slurry concentrations media. For these reasons the profile and evolution of total bacteria and archaea populations were only analysed in 4%-slurry concentration bottles and corresponding data are presented in Table 4.

The addition of different types of substrate did not alter total bacteria concentration in the bottles, neither comparing blanks titres against those derived from supplemented media nor comparing among substrates. However, along the incubation period total bacterial biomass decreased significantly (from 15.1 to 3.1 log gene copy numbers 16SrRNA/g FM, Table 4). Co-i addition level interacted with incubation day with regard to total bacteria gene copy numbers (P<0.001).

In 5% Co-i level, total bacteria decreased significantly from day 0 to 25 (14.8 vs. 4.9, P<0.05) but remained steady until 56 incubation days (3.9, P>0.05), whereas in 10% Co-i level the early evolution was similar (9.4 vs. 5.1, P<0.05) but the final concentration was the lowest (3.4, P<0.05).

The concentration of total archaea was also depressed along the incubation time (P<0.01), independently of substrate and Co-i supply. Contrarily to the pattern observed in total archaea population, hydrogenotrophic methanogens population showed an increase in the relative abundance at day 25 and then declined from day 25 to 56. Also their titres increased
proportionally with Co-i presence in the media (Table 4, P<0.05). Moreover, hydrogenotrophic methanogens titres were depressed in those bottles supplemented with WS (P<0.05).

4. Biodiversity, DGGE analyses

The structure of the archaeal community in samples from swine faeces (inoculums), and after anaerobic fermentation including different substrates and cows faeces (Co-i) were studied by DGGE (Figure 4). The amplification of DNA using the set of archaeal primers (Table 2) generated 38 major different bands, 25 in the original faeces (B0). Overall, samples shared 59% similarity in the banding profile and clear clustering pattern by incubation time: samples from day 56 clustered separately from those at days 0 and 25. Within another cluster, samples from days 0 and 25 also resulted in a different profile. Moreover, at long time incubation period (56 days) the differences induced by Co-i addition were reflected in their community structure and those samples supplemented either 5 or 10% of Co-i DM, clustered separately. In the latter case (addition of 10% of Co-i DM) the number of bands increased which was reflected in higher diversity. Indeed, in the original samples without (B0) or with Co-i (B5%=25 and B10%=25 bands) at 0 day incubation period, the number of bands increased with Co-i addition (addition of 10% of Co-i DM) although differences were more pronounced at 25 days of incubation period (25, 25.5 and 29.5 bands) than at 56 days [25, 25.5 and 27.5 band corresponding to Co-i addition of 0, 5 and 10% of DM, respectively; Interaction Day x Co-i reached statistical significance (P<0.05)]. Shannon index showed a similar pattern as band numbers.

Discussion

1. Methodological approach.

Pig slurry is a mixture of excreta, including faeces and urine together with water and some food residues (Babot et al., 2011) stored in the animal’s pit and bulked temporally in the pool. Slurry hydration comes mostly from urine but also, and in different proportions, from water refusals, animal’s cleaning or drinking losses. Moreover slurry storage periods are tagged to farm structure, management and legal conditions imposed for their application on the fields. Indeed it results in a wide heterogeneity of slurry compositions and characteristics for instant, slurry DM content (g DM/100 ml) along the Spanish commercial pig farms ranges from 1 to 12. Thus in the present work we restricted the study of evolution of archaea titres along the anaerobic OM conversion to methane to specific slurry dilution rank varying from 2 to 6% of
DM. Fresh pig’s faeces were diluted with fresh urine up to 10% to simulate the normal faeces/urine ratio and further dilutions (to 6 and 2% of DM) were obtained using tap water. To compensate the low C/N ratio of the slurry and thus to improve bacterial growth, media were supplemented with crop or agro-industrial by-products characterised by high proportion of different type of structural carbohydrates assuming that starch and other non-structural sources was previously digested in the pig’s intestine. The anaerobic digestion system employed was the original methodology proposed by Theodorou et al. (1994) designed to optimize anaerobic degradation of substrate working at 40°C, by buffering the pH variation and using small amounts of substrate. Under such experimental approach the present paper has been built and discussed.

2. Methane production and incubation media characteristics.

Methane production at 56-days (ml/bottle) was directly related to the amount of available OM, but differences among treatments in relation to the production efficiency (ml/g OM fermented) were relevant. Methane production (ml/g OM) was within the range reported for those authors working in similar conditions of both, slurry concentration (4.3 to 13.6% DM) and incubation periods (up to 56 days) (75, 75 and 82 days; Campos et al., 1999, 2000 and 2002), although higher production has been also reported in the existing literature (290-490 ml/g OM; Jarret et al., 2011). Differences in efficiency production must be interpreted in terms of variation in the fermentation conditions and initial substrate (Vedrenne et al., 2008).

CH$_4$ production levelled off at 4% slurry concentration and then there was not a direct relationship between OM concentration in the media and CH$_4$ production efficiency, which agrees with previous reports (Massé et al., 2003; Dinuccio et al., 2008). In this sense, it has been hypothesised that the absence of a direct relationship could be derived either from the presence of toxic substances in the slurry (i.e. lignin and its derivatives: Kaparaju and Rintala, 2005; Chen et al., 2008) or because the incubation media became inappropriate or toxic, for the acidity induced by VFA excess (Kaparaju and Rintala, 2005) or contrarily alkalinity under the ammonia load (Chen et al., 2008).

The high buffer capability of the slurry was able to compensate acidity induced by increases in VFA production in the case of blanks and bottles supplemented with wheat straw, since pH level was kept within the appropriate range reported for a correct anaerobic digestion of the OM (6.6-7.6; Carrillo, 2003). However, pH was below this threshold level and reached a critical value in those media supplemented with pulps, which was able to explain the inhibition of the
fermentation processes and the depression registered in methane production using pulps (Hansen et al., 1998). Sugar beet pulp is quickly fermented in the initial digestion process (day 1 to 10; Ward et al., 2008) due to its high proportion of high digestible hemicelluloses and a low proportion of lignin and cellulose (Alkaya and Demirer, 2011), which yields a high production/accumulation of VFA (Hansen et al., 1998) and the acidification of the media may depress temporally microbial fermentation (Jain et al., 1982; Fang et al., 2011). Few results are available in relation to apple pulp digestion, but values registered with pear pulp (Campos, 2001) were similar to those in the present study.

In any case, the changes in the pH cannot justify the depression in methane production registered at the highest level of slurry concentration. Slurry as substrate for microbial growth has two mains constraints: the low availability of OM together with a high concentration of N mostly as ammonium form. Rank of N-NH$_4^+$ concentration in the blanks fits well with the normal values determined in the slurries from commercial farms (Babot et al., 2011; Alvarez-Rodriguez et al., 2013). Ammonium nitrogen comes from urea hydrolysis (carried out for ubiquitous microbial urease (Cortus et al., 2008) and it may explain the variations in N-NH$_4^+$ concentration according to dilution of the original slurry (blanks). The evolution of N-NH$_4^+$ concentration along the incubation period was altered by the experimental treatment and it may reflect the equilibrium between microbial protein degradation and synthesis (Hristov et al., 2011). Those media with the lowest slurry concentration, synthesis may prevail over degradation processes and N-NH$_4^+$ concentration decreased whereas protein degradation may predominate as slurry concentration increased. The N-NH$_4^+$ un-balance increased with carbohydrate supplementation, basically with pulps. If N-NH$_4^+$ values reached, or not, toxic values for microbial growth is open to the discussion. Hansen et al. (1998) reported that N-NH$_4^+$ would exert inhibition when values reach 3.3 g/l whereas Chen et al. (2008) increased such threshold level up to 5 g N-NH$_4^+$ /l if there was a previous media adaptation to the substrate. More specifically Van Velsen (1979) and Hashimoto (1986) described a depression in titres of methanogen archaia when N-NH$_4^+$ reached values up to a range in between 1.5-2.5 g/l. That was our case and a negative effect of N-NH$_4^+$ level on microbial growth with the highest slurry concentration could be not discarded.

The anaerobic decomposition of biowaste occurs in a continuous process, although specific phases could be distinguished (Ostrem, 2004), in our case bottles were sampled at discrete timing, at 0-days, VFA concentration reflect those values from the original slurry.
In the adult pig’s intestine, starch and other non-structural carbohydrates are digested and absorbed, so those remaining in the faeces belong mostly to structural group (cellulose and other fibrous products) and thus VFA profile was mostly acetic in agreement with previous papers reporting VFA profile in the fresh slurry (Møller et al., 2004; Peu et al., 2006). At 25-day sampling period a significant increase in VFA concentration was observed. Media reflected the initial stage of the syntrophic degradation characterized by products hydrolysis and VFA synthesis (Jain et al., 1982). Finally at 56-day sampling, syntrophic degradation was ending with a significant disappearance of VFA with acetic acid prevalence. It would reflect the transformation of short fatty acid and other product to acetic acid by acetogenic bacteria and further degradation to methane by methanogens (Ostrem, 2004).

Volatile fatty acids concentration and profile were modified by the experimental treatment. Slurry dilution and pulp addition increased propionic acid prevalence at 56-d sampling. We hypothesised that non-acetic VFA prevalence may be explained by two reasons, an excess of substrate which delayed the syntrophic degradation process or an increase in propionate proportion derived from pulp supply (Canh et al., 1997; Gerrits and Verstegen, 2006), which promoted the concentration of sulfate-reducing population (Chen et al., 2008) and they may compete against methanogens for substrate (Barredo and Evison, 1991). The greatest branched-chain VFA concentration at 25 days of sampling in the most concentrated slurries (4 and 6% DM) was in line with the increasing N-NH₄⁺ concentration derived from protein degradation and subsequent availability of branched-chain amino acids, which are necessary for iso-fatty acids synthesis (Cunningham and Klein, 2007). However, the accumulation of branched-chain VFA was not sustained at day 56-days of incubation even though N-NH₄⁺ remained increasing.

3. Evolution of methanogens titres and biodiversity

Methanogens are sensitive to changes in their niche, as their habitat is limited to strict conditions and their substrates are generally limited (Kaspar and Wuhrmann, 1987). The physicochemical conditions, bacterial community composition and organic matter are the key environmental factors that allow methanogens to survive in both “in vitro” anaerobic digestion and natural ecosystems (i.e., marshes, riverbed sediments, and lake sediments; Calli et al., 2005). In fresh slurries several archaea communities were described (Dabert et al., 2008), being such communities dominated by hydrogenotrophic and methylotrophic microorganisms that are able to adapt and survive in “in vitro” conditions. However, ammonia (Massé et al., 2003) and a
excess of organic load (González-Fernández and García-Encina, 2009) were identified as the common limiting factors. For these reasons and for the low methane production efficiency registered at the lowest and highest concentrated slurry, the effort was focused on 4% concentration slurry in which complete substrate degradation was almost reached. In the present work, the quantitation of total archaea population (by targeting the 16sRNA gene) and the hydrogenotrophic group (by targeting the methyl-CoM reductase mcrA gene), allowed us to monitor the patterns of the two main methanogenic groups described in this environments. This was done assuming that the difference between total and hydrogenotrophic would correspond to acetotrophic methanogens.

It is true that conditions imposed by an “in vitro” system are more uniform than natural ecosystems (Angenent et al., 2002), however “in vitro” cultivation and/or slurry maturation reduced both, total bacterial and archaeal biomass.

Agreeing with previous reports, hydrogenotrophic methanogens constituted a minor proportion within the archaea group in the slurry, which suggests that the major group corresponded to methylotrophic methanogens (Jeon et al., 2009). In our work, as the in vitro incubation progressed, titres showed a decline in the abundance of archaea and an increase followed by a decline of hydrogenotrophic group. In this sense (Dabert et al., 2008), reported significant changes in methanogens population through slurry maturation due to a differential capability and efficiency in the adaptation process. Addition of freeze dry cows faeces to the incubation media, improved titres of total archaea and hydrogenotrophic methanogens and throughout the incubation period also improved biodiversity. The predominance of hydrogenothopic methanogens in the ruminants digestive system (Kim et al., 2011) may explain the amelioration in the hydrogenotrophic population titres into the cultivation media and their slow adaptation capability to the new “in vitro” environment (Snell-Castro et al., 2005). The fact that biodiversity index (band numbers) was only increased at longest time incubation period. The improvement in hydrogenotrophic titres did not improve total methane production but increases in the production efficiency were relevant. The optimization of the use of other sources of methanogens appears as an interesting area of work.

Conclusions
Slurry dilutions affected, non-proportionally, methane production. An excessive slurry dilution (2 % DM) or substrate load (6 % DM) depressed OM methanization whereas no amelioration was detected in methane production (ml/g OM) with structural carbohydrates supply. Archaeas population constituted a minor fraction of total bacteria in swine slurry (0.3-0.4%), they were unaltered by structural carbohydrate supplementation and decreased along the incubation period. Hydrogenotrophic methanogens concentrations were depressed by the presence of straw in the media and improved significantly by addition of cow’s faeces as exogenous archaeas source. Titters of hydrogenotrophic species did not improve total methane production (ml/g OM) but they were reflected in its production rate (ml/g OM/day).

The dilution of the slurry (DM content) is the major factor affecting methane production in vitro, while the addition of the three sources of external fermentable carbohydrates used here (wheat straw and apple and sugar beet pulp) or cows faeces do not seem to improve overall methanization. The archaeal community structure adapts along the incubation time and is highly influenced by the addition of cows faeces, which results in higher methane productions rates. The study of the use of microbial inoculum to optimize methane production deserves further research.

**Table 1.** Chemical composition of the inocula (swine and cattle faeces) and substrates (sugar beet pulp: SBP; apple pulp: AP; wheat straw: WS) (g/100 g DM)

<table>
<thead>
<tr>
<th></th>
<th>Inoculum</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Swine</td>
<td>Cattle</td>
</tr>
<tr>
<td>Organic matter (OM)</td>
<td>77.7</td>
<td>90.1</td>
</tr>
<tr>
<td>Ether extract (EE)</td>
<td>2.22</td>
<td>2.27</td>
</tr>
<tr>
<td>Crude protein (CP)</td>
<td>15.5</td>
<td>17.8</td>
</tr>
<tr>
<td>Crude fibre (CF)</td>
<td>20.2</td>
<td>29.2</td>
</tr>
<tr>
<td>Neutral-detergent fibre (NDF)</td>
<td>45.1</td>
<td>46.4</td>
</tr>
<tr>
<td>Acid-detergent fibre (ADF)</td>
<td>21.2</td>
<td>26.2</td>
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**Table 2.** Authors and sequences of primers used in the present experiment.
<table>
<thead>
<tr>
<th>Target</th>
<th>Author</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Forward</td>
</tr>
<tr>
<td>DGGE Archaeae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bacteria</td>
<td>(Maeda et al., 2003) Maeda et al. 2003</td>
<td>5´-gtgStgcYgYggYtgtcgtca-3´</td>
</tr>
<tr>
<td>Total Archaeae</td>
<td>Denman et al. 2007</td>
<td>5´-tccggtgggcagrgc</td>
</tr>
</tbody>
</table>
Table 3. Media fermentation parameters (pH, NH$_4^+$ and VFA) as affected by slurry concentration (2, 4 and 6 g DM/100 ml), incubation time (0, 25 and 56 days) and substrate supplementation (600 mg DM/bottle; Wheat straw (WS) Apple pulp (AP) or Sugar beet pulp (SBP)).

<table>
<thead>
<tr>
<th>Item</th>
<th>Slurry DM concentration (%)</th>
<th>SEM</th>
<th>Substrate</th>
<th>SEM</th>
<th>Days</th>
<th>SEM</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
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<tr>
<td>pH</td>
<td>7.0</td>
<td>7.2</td>
<td>7.3</td>
<td>0.04</td>
<td>7.3</td>
<td>7.2</td>
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<tr>
<td>N-NH$_4^+$, g/l</td>
<td>1.6</td>
<td>2.3</td>
<td>3.4</td>
<td>0.07</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>VFA, mM</td>
<td>15.0</td>
<td>12.8</td>
<td>19.8</td>
<td>1.34</td>
<td>15.1</td>
<td>13.9</td>
</tr>
<tr>
<td>VFA, mol/100 mol</td>
<td>Acetate</td>
<td>63.1</td>
<td>73.8</td>
<td>72.9</td>
<td>2.53</td>
<td>76.9</td>
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<tr>
<td></td>
<td>Propionate</td>
<td>27.0</td>
<td>16.8</td>
<td>16.4</td>
<td>2.27</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>Butyrate</td>
<td>4.7</td>
<td>4.7</td>
<td>5.3</td>
<td>0.28</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Valerate</td>
<td>1.0</td>
<td>1.3</td>
<td>1.1</td>
<td>0.09</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>BCFA</td>
<td>4.2</td>
<td>3.5</td>
<td>4.3</td>
<td>0.37</td>
<td>3.7</td>
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<table>
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<th>Substrate</th>
<th>Day</th>
<th>Slurry x Substrate</th>
<th>Slurry x Day</th>
<th>Substrate x Day</th>
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<tbody>
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<td>**</td>
<td>**</td>
<td>ns</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>N-NH$_4^+$, g/l</td>
<td>**</td>
<td>ns</td>
<td>*</td>
<td>**</td>
<td>ns</td>
<td>**</td>
</tr>
<tr>
<td>VFA, mM</td>
<td>**</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
</tr>
<tr>
<td>VFA, mol/100 mol</td>
<td>Acetate</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Propionate</td>
<td>**</td>
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<td>ns</td>
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<td></td>
<td>Valerate</td>
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<td>ns</td>
<td>**</td>
<td>ns</td>
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<td></td>
<td>BCFA</td>
<td>ns</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
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ns=P>0.05, *=P<0.05, **=P<0.01
**Table 4.** Effect of substrate supplementation (600 mg DM/bottle; wheat straw (WS), apple pulp (AP) or sugar beet pulp (SBP)) incubation time (0, 25 and 56 days) and Co-inoculum addition (0, 5 or 10% of bottle DM) on methane production (ml/bottle/day) and concentration in the medium of total bacteria (gene molecules 16S-rRNA/g FM), methanogenic archaeas [% of total bacteria; ΔC(t)] and hydrogenotrophic methanogens [% of total methanogens; ΔC(t)] in 4% DM slurry.

<table>
<thead>
<tr>
<th>Item</th>
<th>Substrate</th>
<th>SEM</th>
<th>Days</th>
<th>SEM</th>
<th>Co-inoculum</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methane Production (ml/ day)</td>
<td>Blank</td>
<td>48.8</td>
<td>WS</td>
<td>53.8</td>
<td>AP</td>
<td>47.2</td>
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<tr>
<td></td>
<td>0</td>
<td>48.7</td>
<td>25</td>
<td>46.5</td>
<td>56</td>
<td>102.4</td>
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<tr>
<td></td>
<td>0%</td>
<td>32.6</td>
<td>5%</td>
<td>55.9</td>
<td>10%</td>
<td>60.4</td>
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<tr>
<td>Total bacteria</td>
<td>7.8</td>
<td>15.1</td>
<td>3.1</td>
<td>0.38</td>
<td>9.0</td>
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<tr>
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<td>7.7</td>
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<td>0.05</td>
<td>0.2</td>
<td>0.4</td>
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<tr>
<td></td>
<td>7.5</td>
<td>3.1</td>
<td>0.3</td>
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<td>0.4</td>
<td>0.43</td>
</tr>
<tr>
<td>Total archaeas</td>
<td>0.4</td>
<td>0.5</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.05</td>
<td>0.1</td>
<td>0.4</td>
<td>0.43</td>
</tr>
<tr>
<td>Hydrogenotrophic methanogens</td>
<td>0.5</td>
<td>0.1</td>
<td>0.6</td>
<td>0.16</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.6</td>
<td>0.3</td>
<td>0.17</td>
<td>0.7</td>
<td>0.16</td>
</tr>
</tbody>
</table>

**Biodiversity of total Archaea**

| Bands number | 25.3 | 25.5 | 24.2 | 25.0 | 0.50 | 22.7 | 26.5 | 25.8 | 0.53 | 24.7 | 24.3 | 26.0 | 0.48 |
| Shannon Index | 3.2  | 3.2  | 3.2  | 3.2  | 0.02 | 3.1  | 3.3  | 3.3  | 0.02 | 3.2  | 3.2  | 3.2  | 0.02 |
| Evenness      | 0.8  | 0.8  | 0.8  | 0.8  | 0.005| 0.8  | 0.8  | 0.8  | 0.005| 0.8  | 0.8  | 0.8  | 0.004|

**P Value**

<table>
<thead>
<tr>
<th>Item</th>
<th>Substrate</th>
<th>Days</th>
<th>Co-inoculum</th>
<th>Co-inoculum x Day</th>
<th>Substrate x Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methane Production</td>
<td>ns</td>
<td>***</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>ns</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>ns</td>
</tr>
<tr>
<td>Total Archaea</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Hydrogenotrophic methanogens</td>
<td>*</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Biodiversity**

| Bands number | ns   | **  | **  | **  | ns              |
| Shannon Index | ns   | **  | *   | **  | ns              |
| Evenness      | ns   | *** | *   | **  | ns              |

ns=P>0.05, *=P<0.05, **=P<0.01, ***=P<0.001
Figure 1. Methane production (ml/g OM) along the incubation period considering the effect of slurry concentration (a) or substrate inclusion (b).
Figure 2. Average pH value registered for the different supplements (Blanks; WS: Wheat straw; AP: Apple pulp; SBP: Sugar beet pulp) along the incubation times at 0, 25 and 56 incubation days.

Different superscripts \(^{(a,b,c)}\) denote statistical differences (\(P<0.05\)) among slurry DM concentrations (2, 4, and 6\% DM) within the incubation period whereas \(^{(x,y)}\) denote statistical differences (\(P<0.05\)) within each slurry DM concentration throughout the incubation period.
Figure 3. Concentration (mmol/l) of acetic acid, propionic and butyric acid in bottles (media) containing as inoculum (Pig slurry at 2, 4 or 6 gDM/100g FM) at different in incubation period (0, 25 and 56 days) supplemented with different levels of co-inoculum (0, 5 and 10 g/100 g DM lyophilized cows faeces) and crop by-products as substrates (B, Blank; WS: Straw; AP: Apple pulp and SBP: Sugar beet pulp) supplementation.
**Figure 4.** DGGE derived dendrograms illustrating the effect of incubation period (0, 25 and 56 days), co-inoculum (0, 5 and 10% lyophilized cows faeces additions) and substrate (B, Blank; WS: Wheat straw; AP: Apple pup and SBP: Sugar beet pulp) supplementation. The scale bars show Hamming distances.
References:


