The impact of reducing dietary crude protein and increasing total dietary fiber on hindgut fermentation, the methanogen community and gas emission in growing pigs

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ABSTRACT

Sixty-four cross bred 6 week-old intact male pigs (initial BW = 13.8 ± 2.3 kg) were randomly distributed to 4 separated modules using a three-phase feeding program in which two dietary crude protein (CP) and total dietary fiber (TDF) levels were tested in a 2 × 2 factorial arrangement under a commercial-like production system. The room air was sampled and analyzed for NH3 and CH4 while the slurry pit air was sampled and analyzed for CH4 content during the early growing (phase I, 13.8–38.6 kg of body-weight), growing (phase II, 38.6–72.8 kg of body-weight) and finishing periods (phase III, 72.8–108.7 kg of body-weight); at the end of the finishing phase, 16 random pigs were sacrificed and cecum and colon contents were sampled to determine fer- mentation and microbial parameters. The pH and ammonium content increased with digesta transit being lower in cecum (6.0 and 69.7 mg/L) than in colon (6.3 and 156.3 mg/L) whereas the opposite trend was seen for total VFA and acetate (175.2 mM and 62.6 mol/100 mol vs. 141.1 mM and 57.2 mol/100 mol, respectively; $P < 0.05$). Low protein (LP) and high fiber (HF) diets showed a higher NH3 concentration in the colon but not in cecum samples. Dietary fiber also altered intestinal VFA concentration where animals fed Low fiber (LF) diet showed high VFA's concentrations and such effect was more pronounced in colon samples. Total NH3 (1.8, 4.8 and 8.5 g/day) and methane (2.5, 3.5 and 7.5 g/day for Phase I, II and III, respectively) emissions increased consistently with age ($P < 0.05$), dietary CP level increased NH3 volatilization (6.3 vs. 3.8 g/d for high protein (HP) and LP diets respectively; $P < 0.01$) and fiber tended to increase methane emission (5.0 vs. 4.0 for HF and LF diets, respectively $P < 0.1$). The methane pro- duction measured at slurry pit contributed significantly to total CH4 emission (3.26, 9.02 and 16.91% in the phases I, II and III respectively). Dietary CP increased total bacteria (TB; 9.7 vs. 9.5; $P < 0.03$) and total methanogenic archaea (TMA; 7.2 vs. 6.4; $P < 0.01$) abundances in the intestinal as well as the slurry (6.8 vs. 6.3 Log n° copy/ g fresh matter (FM); $P < 0.01$) samples whereas TDF did not alter microbial titers. Differences in CH4 emission did not reflect the TMA concentration in hindgut contents.

Keywords: fermentation parameters, greenhouse gas emission, microbiota, pig production

Introduction

The environmental impact of the intensified pig production in Europe is significant. Pig manure is a source of greenhouse gases (GHG) like methane (CH_4) and other harmful gases such as ammonia (NH_3). Increased public concern on the livestock environmental footprint made EU legislation to regulate the potential quota of atmospheric pollution (IPPC Directive; Directive EU 2016/2284 on the reduction of national emissions of certain atmospheric pollutants) where animal nutrition is considered as a key strategy. Under intensive production, ≈ 20% dietary N is retained in the animal's body (Canh et al., 1997). Irreversible NH_3-losses (through the urine and feces) may approach 50% of N intake (Ryden et al., 1987; Hartung and Phillips, 1994) due to the excess or unbalanced dietary protein together with manure-handling strategies. Methane is identified as a main contributor to global warming (Johnson et al., 2002) and represents an irreversible energy loss. Methane production in pigs has been commonly linked to dietary gross energy intake (1.2% of DE intake; Christensen, 1987), and a positive correlation between fiber intake and methanogens diversity (Cao et al., 2012) or abundance (Liu et al., 2012) has been reported. Methanogens have been identified in hindgut digesta and pig feces, being the genus *Methanobrevibacter* (Steinberg and Regan, 2009; Luo et al., 2012) the most abundant. CH_4 production is the main disposal sink for reducing equivalents (H^2), and a competition between H^2-consuming organisms (hydrogenotrophic) and sulphate reducing bacteria (SRB) has been reported in the human colon (Bernalier et al., 1996) and rabbit caecum (Belenguer et al., 2011) but factors that determine the end-routes of reducing equivalents (H^2) are not well understood.

This study was designed to determine the impact of dietary factors on emissions of harmful gases in pigs under commercial-like conditions and to analyze the ecology of lower gut methanogens to prevent the negative impact of CH_4 production on energy utilization and the environment.

Materials and methods

All procedures were carried out under Project License CEEA 03/01-10 and approved by the in-house Ethics Committee for Animal Experiments at the University of Lleida. The care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental purposes.

Installations, animals, diets and experimental design

The study was conducted at the Swine Research Center of Catalonia, located in Torrelameu (CEP; Lleida, Spain); the center comprised a growing-finishing facility with 4 separated modules that were

connected to each other by isolation doors. The space inside each module was divided into four pens (2 pens at each side of the module) of 4.2 m^2 (2.1 m × 2 m) and keeping 0.55 of its area covered by concrete slatted floor, and thus facilitating the access to the slurry pit beneath. Each two lateral pens drained into a single slurry pit (145 cm × 150 cm × 40 cm) and each one was equipped with a single space self-feeder in the concrete floor area and a square nipple drinker in the slatted floor area. The modules were sealed allowing a single air inlet (34 cm × 106 cm), at 208 cm from the ground level along with a single exhaust outlet (40 cm Ø) located at 185 cm of height and ventilated (24 h continuous air flow) using an air extractor (Fan coil, SP 400, Hept, SODECA, Barcelona, Spain), regulated by a flow controller (Ambitrol 100 Environmental Controller, Model 103, Electronic Systems Progress SA, Bellpuig, Spain).The air outflow was determined twice a day (0800 and 1700 h) during gas collection period by an anemometer (Testo 425, telescopic tube speed / temperature, temperature sensor NTC, Postfach 1140. Lenzkirch 79849, Germany). Temperature and relative humidity were controlled inside each module through a data logger (Testo 174 H; Testo AG, Lenzkirch, Germany) suspended at 185 cm of height at a distance of 150 cm from the aperture of the exhaust outlet.

The experiment was performed during spring time March-June (105 days) in 3 feeding phases. The manure collection system and sampling protocol was described by Morazán et al. (2015a), briefly, the slurry was maintained into the pits for 15 days period (maximum depth = 0.5 m each) and at the end of each period, the slurry of 4 pits of a single module was homogenized, sampled (≈ 1 kg) and immediately frozen at -20 °C then drained into a lagoon. Slurry production was calculated considering the area of the pit and the depth of the slurry produced. The temperature and the relative humidity were 23.9 °C (SD 2.4) and 52.3% (SD 13.9), 21.7 °C (SD 2.5) and 67.5% (SD 10.3), 25.9 °C (SD 3.4) and 60.5% (SD 11.1) for phases 1, 2 and 3 of the experiment, respectively.

Sixty four entire male pigs (the progeny of Large-White sires × Landrace dams) were purchased at 6 weeks of age from Nucleus S.A.S., Le Rheu, France. Piglets were weighed (mean initial BW = 13.8 ± 2.3 kg) before entering the fattening installation and were randomly assigned to four modules of 16 individuals based on minimum BW variation. Piglets inside each module were accom- modated in 4 pens (4 pigs/pen) once again based on minimum BW variation in order to comply with homogeneity and avoid any competition between the animals to procure the feed.

To assess the impact of dietary changing on the emission of gases as well as evaluate the hindgut fermentation, two crude protein (CP; High *vs.* Low) and total dietary fiber (TDF; Low *vs.* High) concentrations were compared in a 2 × 2 factorial arrangement

considering 4 replicates (pen) per each treatment through a three-phase feeding program: phase I (from 6 to 11), II (from 12 to 16), and III (from 17 to 21 wk of age). The diets were formulated to be iso-energetic and to meet or exceed the CP (*i.e.* SID lysine) and TDF levels recommended by the NRC (2012) and Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA, 2006) for pigs of that BW interval.

The diets (ground using 6 mm screen which resulted in 1–2 mm feed meal) were composed primarily of cereals plus soybean meal/rapeseed meal as the CP source and/or sugar-beet pulp as an extra fiber source.

Diet description is provided in Morazán et al. (2015a) and briefly described in Table 1.

Gas collection

Gas emission (CH_4 plus NH_3) was measured at the end of each experimental period (during 48 h continuously) when the slurry stored into the pit reached the maximum level (end of every 15 days slurry collection period), in the case of CH_4, slurry from total emissions were considered separately. For total gas emission, a representative air sample was obtained from (i) outside of each experimental module and (ii) the midpoint of the exhaust air outlet in each module following the procedure suggested by (AMCA, 2009). Continuous samples of both (air flow rate of 1 l/h), outside and exhaust air from each module were continuously pumped (Gilson, Mini pulse 3, Le Bel Villiers, France) through a Teflon tube (0.1 mm Ø) toward a separate inert gas collection bag (10 L volume).

To collect the air sample from the slurry pits, portables flux chamber (PFC, dome-made using PVC with a coverage area of
283.5 cm^2 [20 cm Ø with 40 cm length]) were devised and placed in duplicate inside each pit under the concrete slatted floor (Fig. 1). The lateral trunk of the flux chambers located below the slurry surface was pierced to allow the dynamic efflux of the slurry in order to maintain homogeneous of the material in-out of the PFC. During the gas collection period, the head space area at the top of the PFC was being washed out by the fresh outside inlet air (reaching the PFC through Teflon tube) and the contaminated air was being extracted through a second Teflon tube located at counterpart of the outside inlet air aperture. The air sample was being collected in a separate inert gas collection bag. Air flow was also adjusted to 1 L / h using a digital flow check (Alltech, IL, USA).

Slaughtering procedure and collection of intestinal digesta

At the end of the experiment, 16 pigs (1 pig per pen chosen at random) were selected and sacrificed approximately 18 h post feeding after captive bolt stunning. Immediately after sacrifice, the ventral side was opened and the whole gastrointestinal tract was ligated and excised. The cecum and colon segments were

mid-incised and the pH of their contents was recorded using a portable pH meter equipped with a Crison 507 penetrating electrode (Crison Instruments S.A., Barcelona, Spain). Thereafter, subsamples (15 g) of digesta were collected from cecum and colon in falcon tubes, immediately frozen in liquid nitrogen tank, then transported to the laboratory and kept frozen at −80 °C until further analyses. Four grams of the same gut sections were also sampled into tubes containing 1 mL of an extraction solution (20 mL/ ortophosphoric acid and 2 mL/L of 4-methylvaleric acid as internal marker) until further analysis for volatile fatty acids (VFA). Samples were also taken to determine the ammonia nitrogen (2 g over 0.8 mL of 0.5 *N* HCl). Both types of samples were stored at −20 °C.

Chemical analyses

The air from each storage bag was sampled with intervals of 3 h (from 0900 to 2100 h) and 12 h (from 2100 to 0900 h of the day after) during 48 h using 100-mL plastic syringes adapted with a valve (Maris et al., 2016). The air sample was held inside the syringe by closing the valve and immediately analyzed for NH_3 and CH_4 concentration using the photoacoustic technique (Innova 1312 Photoacoustic Multigas Monitor, Denmark).

Digesta samples collected from the cecum, and colon were thawed at 4 °C overnight, dried in an oven at 60 °C for 48 h for determination of their DM content and the dried samples were used to measure the crude protein (CP) content using Kjeldahl method (ref. 976.05) and the proportion of neutral detergent fiber (aNDFom) according to Van Soest et al. (1991) procedures, using α- amylase but not sulphites, and subtracting ash from the residue. The fermentable fiber (was estimated as sum of total soluble fiber and hemicellulose components. The total dietary fiber (TDF) was determined using a gravimetric-enzymatic procedure (AOAC, 2000; method 991.43) with α-amylase, protease, and amyloglucosidase treatments (Megazyme Int. Ireland Ltd., Wicklow, Ireland). Total soluble fiber was estimated by subtracting the aNDFom content from the total dietary fiber (TDF) whereas hemicellulose content was estimated as the difference between aNDFom and ADFom contents (NRC, 2012). VFA concentrations were determined by gas chromatography (Jouany, 1982) using a capillary column (BP21 30 m × 0.25 mm i.d. × 0.25 μm, Australia). Ammonia-N concentration was determined (Chaney and Marbach, 1962) after sample centrifugation (25,000 *g*, 20 min).

DNA extraction and quantification of microbial communities

The cecum and colon samples kept at −80 °C were freeze dried and the DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions. The yield and purity of extracted DNA was assessed using a Nanodrop™, by measuring the absorbance intensity at 260 nm and the absorbance ratio 260/280, respectively. Quantification of microbial

communities was performed by quantitative PCR (qPCR) using a CFX-96 Touch Real-Time PCR Detection System. PCR amplification conditions used were as follows: 1 cycle (95 °C for 10 min); 40 cycles (95 °C for 15 s, 60 °C for 10 s, 72 °C for 55 s); amplicon specificity was assessed using melting curve analyses of the PCR end products by increasing the temperature from 55 °C to 95 °C at a rate of 0.5 °C/30 s. Reactions were run in triplicate in 96-well plates which contained 0.5 µL of each forward and reverse primer, 12.5 µL of 1X Bio-Rad SYBR Premix Ex Taq, and 2 µL of DNA template. MiliQ water was added to reactions to complete a final volume of 25 µL. Abundance of Total Bacteria (TB), Total Archaea (TA) and Total Methanogenic Archaea (TMA) were assessed using absolute quantification with a standard curve. Technical triplicates were averaged while checking overlaying of amplification plots at threshold cycle (Ct) value. Absolute quantification of TB, TA and TMA were expressed as log10 rrs or mcr*A* gene copies number/g FM, respectively.

The primer sets and amplicon sizes are described in Table 2.

Calculation for gases [i.e.NH3 and CH4] production

The airflow rate of the outlet air (V_{St}) was calculated by multiplying air velocity at exhaust (m/s) by the exhaust outlet area (m^2) and was corrected to standard temperature (T_{St}) and pressure (P_{St}) as: [$V_{St} = P_0V_0T_{St}/P_{St}T_0$] where P_0 is the atmospheric pressure and V_0 is the gas volume under the experimental condition. Then the gas emission production (difference between outlet and inlet) was calculated as [M_{gas} (g) = V_{gas} ρ_{gas}], where V_{gas} = V_{St} × gas ratio in air sample and ρ_{gas} (g/L) is the density of gas at standard temperature (Cao et al., 2013).

Statistical analyses

Data of fermentation parameters (pH, NH_3-N, concentration and proportion of VFA) together with microbiota quantification were tested using a repeated measures ANOVA (PROC MIXED, SAS Institute Inc., Cary, NC, USA) using the following model:

$$Y_{ijklm} = \mu + S_i + Aj + CP_k + TDF_l + (S_i \times CPj) + (S_i \times TDF_l) + (CP_k \times TDF_l) + \varepsilon_{ijklm}$$

Being: Y_{ijklm}, dependent variable, µ overall mean; Si: Intestinal section within each individual (i = cecum and colon); Aj, animal (random effect) j; CP_k, dietary CP level effect (k = HP and LP); TDF_l, dietary TDF level effect (l = HF and LF); and ε_{ijklm}, residual error term.

The same statistical model but including three experimental phases; Ph_i (i = 6–11 weeks, 12-16weeks

and 17-21weeks of age) instead of the intestinal section (cecum *vs* colon) was used to analyze statistical significances for gases emission and slurry com- position. The Tukey multiple comparison procedure was applied and significant differences and tendencies were declared at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

Results

The experiment was carried out using a three phase feeding system, in which the amino acid supply was adjusted for each production stage to match nutrient intake with requirements. Intake, digestibility, growth and carcass characteristics were fully addressed and discussed previously in Morazán et al. (2015a).

Fermentation traits

The effect of sample collection site (cecum *vs.* colon) and dietary CP and TDF content as well their interactions on pH value, ammonia (mg/L) and total VFA (m*M*) concentrations, and their proportion (as percentage) are shown in Table 3. Data obtained from slurry are also presented.

Sampling site (cecum *vs*. colon) affects pH values, pH being lower in the cecum than in the colon (6.0 *vs.* 6.3 SEM 0.06;

$P < 0.01$). Neither CP nor TDF altered pH values, although TDF level interacted with sampling site, pigs fed HF diets showed greater pH than those fed LF but only in the colon (6.4 *vs.* 6.1 SEM 0.08; $P = 0.03$). Samples in the slurry pits were apparently more alkalinized than digesta and slurry from animals fed HP diets showed higher pH value than those fed LP (7.5 *vs.* 7.0; $P = 0.04$).

Similar to pH, NH_3 concentration was higher in the colon than in the cecum (156.3 *vs.* 69.7 mg/L SEM 8.38; $P < 0.01$) and sampling site interacts with both dietary treatments. In colon samples, LP diets caused higher NH_3 concentration (182.1 *vs.* 130.5 mg/ L SEM 11.85; $P = 0.02$ for HP and LP diets respectively) as HF diets (166.7 *vs.* 145.9 mg/L for HF and LF SEM $P = 0.05$, respectively). High concentrations of ammonia were found in the slurry (up to 6 g/L) where the ammonia concentrations in pits of animals fed HP were greater than LP ($P < 0.01$). No differences related to fiber composition in the diet were detected.

Total VFA's concentrations and the proportion of acetic acid were higher in cecum samples, whereas butyric and branched chain fatty acids (BCFAs; iso-butyric and iso-valeric acids) showed the opposite tendency being higher in colon samples. No significant changes were detected in propionic and valeric acids ($P > 0.1$). Dietary CP tended to increase valerate concentration.

Dietary fiber altered intestinal VFA concentration and its profile; animals fed LF diets showed high VFA's concentrations being this effect explained mostly by differences registered in the colon compartment (Interaction intestinal segment × TDF, $P < 0.04$). HF diets induced higher acetate and lower butyrate

concentrations than LF diets whereas in the slurry samples, HF diets numerically increased total VFA concentrations and LF diets tended to reduce the proportion of propionate.

Gut microbiota

Changes throughout the hindgut together with the effect of dietary CP and TDF content and their interactions on absolute abundances of Bacteria (TB), Archaea (TA) and Methanogenic Archaea (TMA) are shown in Table 4. Abundances of TB, TA and TMA were greater in colon than in cecum samples. Dietary CP increased TB ($P = 0.03$) and TMA ($P < 0.01$) abundances in the intestine as well as in the slurry ($P < 0.01$) samples whereas TDF did not alter TB, TA and TMA abundances. However, slurry from animals fed HF presented abundances (Log N° copy/g FM) of TB (9.7 *vs.* 9.5), TA (9.2 *vs.* 8.8) and TMA (6.6 *vs.* 6.4) higher than those receiving LF diets.

Interaction detected between main effects (CP × TDF) in slurry microbiota is shown in Fig. 2. The abundances of TB in the LP diets were not modified by level of TDF although in HP diets those animals fed HF showed higher abundances of TB. Moreover, TMA abundances in pigs fed LP-LF diet showed a significant reduction in TMA titers comparing with animals receiving LP-HF diets. Animals fed HP diets did not show changes in TMA abundance related to TDF supply.

Gas emissions

Environmental (directly produced by animal plus slurry pit g/an/day) emissions of NH_3 and CH_4 together with CH_4 emission from slurry pit (expressed as g CH_4/an/day and g CH_4/m^3 of slurry) during the three experimental phases at the experimental modules are presented independently in Tables 5 and 6, respectively. The interaction between dietary CP and NDF did not alter gas emission throughout the experimental period ($P > 0.05$), therefore results are described as independent effects.

Total (environmental) NH_3 volatilization increased with the age of the animals, reaching the maximum at 21st week (8.5 g per animal and day; $P < 0.01$) of age. Dietary CP content had a significant impact on this parameter. Animals fed HP showed higher NH_3 volatilization than those fed LP diets ($P < 0.01$) where the differences were mostly explained by the volatilization values recorded in phase 3 (Interaction CP level × Phase $P < 0.02$). No effect of TDF concentration on NH_3 volatilization was observed.

Methane emission increased with the age ($P < 0.01$), the last phase (108.7 kg LW; phase III) marked the highest methane emission values whereas no statistical differences in CH_4 emission between the initial (38.6 kg LW; phase I) and the middle (72.8 kg LW; phase II) phases were observed. In addition, emission

per unit of DMI also increased, mostly at the end of the fattening period (1.8, 1.6 and 2.8 g/kg DMI for Phase I, II and III respectively, SEM 0.875; $P < 0.05$). Neither CP nor TDF affected the emission of methane, although those pigs fed HF diets tended ($P < 0.1$) to increase CH_4 emission in relation to those fed LF diets.

In order to distinguish CH_4 origin emission values corresponding to slurry stored in pits are presented in Table 6.

Methane emission from the slurry pits were 2.5, 3.5 and 7.6 g/m^3 slurry, for I, II and III feeding phase respectively ($P < 0.01$) and also the proportion of CH_4 in total emissions increased with age (3.26%, 9.02% and 16.9%, for the phases I, II and III, respectively). No effect of dietary CP level was detected but TDF altered CH_4 emission although the effect only reached statistical significance in the Phase III (Interaction TDF × phase; $P < 0.01$)

Slurry composition

Chemical composition of the slurry stored into the pits is presented in Table 7; samples were taken at the end of each experimental phase when the 15 days slurry storage period was ended. The interaction between dietary CP and TDF did not affect pH and OM, N (total or ammonia-N), P and K concentrations throughout the experimental period ($P > 0.05$); therefore, they are described as independent effects. In case of dry matter concentration, CP and TDF interacted and values of Table 7 are completed with Fig. 3. Data on dry matter production (kg/animal/day), conductivity and density were detailed in Morazán et al. (2015a).

Mean values of pH registered in the slurry were closed to neutrality (7.3 SEM 0.11) and were not altered by growing phase nor dietary TDF content, however the slurry of those pigs fed LP diets were slightly acidic ($P < 0.04$) than those fed HP diets. The water content of the slurry was high (Average dry matter content was 7.6% SEM 1.43), and was independent of the phase of study although an interaction was observed between CP and TDF contents of the diets as the main effects. Among those animals fed HP diets those received HF diets showed the highest concentration of dry matter whereas the opposite was true in those pigs fed LP diets and the highest DM concentration was observed in those animals fed LF diets (Fig. 3). Total N concentration in the slurry averaged 78.2 g/kg DM (SEM 7.07), which mostly was as ammonia-N form (45.6 g/kg DM SEM 8.18). No significant effect of the experimental treatment was found in total N content or ammonia-N. The average (expressed as g/kg DM) of Phosphorous (42.6 SEM 2.95) and Potassium (59.7 SEM 10.01) contents of the slurries were not affected by the experimental treatments.

Discussion

Experimental approach

In the present approach CH_4 emission and NH_3 volatilization were analyzed simultaneously in an open-circuit system while trying to maintain the "standard" commercial housing conditions and minimizing the impacts of the experimental handling over the ani- mals.
Total emissions included gases originating from the digestive tract and also those originated from the slurry storage; in this sense, authors are aware of a wide variation existing in both housing conditions (*i.e.* aeration level, slats/concrete ratio, water supply, *etc*) but mainly in the features in which manure is stored into the pits (*i.e.* DM composition, temperature, period, depth and aeration rate).

In such scenario it is important to relate slurry gas emission to specific storage conditions, i.e: storage period was fifteen days and gas emission was measured in the last 48 h, no intervention into the manure ecosystem and a constant aeration rate of the experimental plot surface was assumed.

Gas emission from the slurry is commonly estimated by "simulation" approaches (*i.e.* Jarret et al., 2012; Morazán et al., 2015b) due to the experimental difficulties to obtain consistent values of the real, "*in situ*" situation (Le et al., 2008). In the present approach, gas emission from slurry surface was obtained using PFC and data has been treated with caution due to the particular condition in which gas samples were harvested.

Ammonia volatilization

Ammonia volatilization falls within the limits proposed by Best Available Techniques (Commission Implementing Decision (EU) 2017/302 of 15 February 2017); however, due to a high variability in pig housing conditions, several authors have already reported lower (Osada et al., 1998; Philippe et al., 2007) and higher values (Fernández et al., 1999).Moreover, NH_3-volatilization increased with dietary CP supply and growth phase (Table 5), even when volatilization was expressed by unit of metabolic body weight (127, 209 and 262 mg NH_3/kg $BW^{0.75}$, for Phase I, II and III respectively, SEM 16.5; $P < 0.05$).

For instance, an association between N excretion, mostly urine, and NH_3 volatilization does exist and reductions in N excretion and NH_3-volatilization per decrease in dietary CP supply have already been proposed (Canh et al., 1998; Zervas and Zijlstra, 2002; Le et al., 2008; Sajeev et al., 2018). Thus, NH_3 volatilization was proportionally reduced by 8.6% per each percentage unit (%) of CP reduction when comparing LP and HP diets in the last phase of the study. Similar responses in NH_3-volatilization (from 3.2–

12.5%) have been reported in studies with an excess of dietary CP (Latimier and Dourmad, 1993; Kay and Lee, 1997; Canh et al., 1998).

It is important to mention that lower NH_3 volatilization might be a consequence of lower NH_3 content in the slurry, considering fresh basis (see important differences in Table 3), presumably originated from a lower urine N excretion of low CP diets. Differences in slurry DM contents can also be an important factor which may be affecting the emissions. Besides, previously it was mentioned that the slurry of those pigs fed LP diets were slightly acidic than those fed HP diets and the slurry pH also affects NH_3 emissions. Therefore, reducing CP might have a combined effect reducing NH_3 emissions by reducing the amount of total ammonia nitrogen on the one hand and reducing pH on the other hand.

Following the conventional rationing protocol, dietary CP was reduced (2.5, 2.2 and 5% for phase I, II and III, respectively). This practice has been demonstrated to be effective at substantially reducing N wastage (Rademacher, 2000; Sajeev et al., 2018); however, NH_3-volatilization still increased with animal maturity, which suggests that CP could be further reduced in the employed rations if such reduction is combined with the adequate supply of essential amino acids (Dourmad et al., 1993).

Increasing total dietary fiber improves microbial hindgut fermentation and allows the redirection of plasma N toward the hindgut and thus constitutes a strategy to reduce N wastage through urine excretion (Kreutzer and Machmüller, 1993; Canh et al., 1997). In our case, increasing TDF did neither reduce NH_3 volatilization nor alter microbial fermentation conditions in the colon, (Tables 4 and 5); in fact, animals fed HF diets showed lower colonic concentrations of VFAs and NH_3, and higher pH values than LF ones (Table 3), such discrepancy with the existing studies (Kreutzer and Machmüller, 1993; Canh et al., 1997) could be due to low inclusion level of SBP.

Methane emissions

Methane emission (g/animal per day) varied between 2.23 and 8.83 g per animal per day (between 189.2 and 283.4 mg/kg $BW^{0.75}$). Values fall within the range reported in the existing literature (Christensen, 1987; Osada et al., 1998; Jørgensen, 2007; Hansen et al., 2014), and lower than those reported by Atakora et al. (2011). Portables flux chambers allow measuring emission of CH_4 from the surface of the slurry (Table 5) and therefore the values can be distinguished from the total emission ones (Table 6). Enteric origin constituted the main source for methane emission, although a relevant fraction (averaging 9.7%) did come from the slurry pit, this contribution reached 17.8% at the end of the fattening period. Since slurry was removed every 15 days, a lower proportion of CH_4 emission from the slurry was expected, considering that IPCC (2006) proposes important differences in emission factors from stored slurry depending on the slurry storage time (less or more than 1 month). Methane emission from the slurry

pits (between 0.07 and 15.2 g/ m^3) placed within the range reported using both, "*in situ*" (from 0.3 to 30 g CH_4/m^3; Zhang et al., 2007) or '*in vitro*' (from 3.4 g to 41.9 g CH_4/m^3 ; Amon et al., 2007; Moset, 2009; Moset et al., 2010) procedures.

In agreement with previous results of Ji et al. (2011), the current data suggest that maturation of the fermentation compartment does occur probably through an increase in the symbiotic gastrointestinal population, among them methanogenic microorganisms (Haeussermann et al., 2006). In relation to dietary effects, CP supply did not alter CH_4 emissions, in line with other studies (Le et al., 2009; Atakora et al., 2011; Osada et al., 2011), however increases in TDF intake trended to increase CH_4 emissions. However, we could not detect an equivalent improvement in microbial activity (by increasing VFA concentration and pH reduction) in the colon of those pigs that received HF diets and trended to emit more CH_4 (Table 5). It is known that microbial activity depends on the indigestible material that reaches the hindgut compartment (Morales et al., 2002). In our experiment SBP as an extra fiber source, has a high fermentability and most of its structures may be fermented earlier, in the terminal ileum (up to 50%, Gidenne and Jehl, 1996). In this scenario, an increase in CH_4 production would occur with little or no effect on colon fermentation.

Hindgut fermentation and Methanogen Community

Throughout the ceco-colonic tract, digesta showed relevant changes; pH and VFA concentration were reduced by fiber level, which may reflect either a reduction in microbial activity or a hypothetical increase in VFA absorption rather than production when fermentable substrate availability decreases. The former possibility seems to be unlikely because throughout the intestine, abundance of TB showed a low but significant increase ($P < 0.05$), assuming that TB in the pigs' hindgut includes all the predominant digesting bacteria (Varel and Yen, 1997).

Changes observed in fermentation conditions between the intestinal digesta and the slurry stored into the pits are shown in Table 3. Among other factors, significant alterations in bacterial fermentation end products (*i.e.* NH_3, VFA) would reflect changes in microbial population in such compartments; indeed, the decrease in TB (between 10^{11}-10^9 log gene copies/FM) probably can be explained by a reduction in substrate (*e.g.* TDF and CP) availability into the media.

Bearing in mind that data available in methanogens diversity and concentration in hindgut of pigs are scarce (Luo et al., 2012; Cao et al., 2016), density of methanogens (Log N° *mcrA* gene copies/ g FM) found in caecal (5.9 ± 0.19) and colonic digesta (7.7 ± 0.14) falls within the range described in existing literature using the conventional culture methods (6–8; Sorlini et al., 1988; Butine and Leedle, 1989) and it is slightly lower than those values proposed by Luo et al. (2012) using the same PCR based approach (8.80 or 8.23 Log N° *mcrA* gene copies/ g FM).

In agreement with previous results (Butine and Leedle, 1989) methanogen concentration increased throughout the cecum-colon tract (5.9 *vs*. 7.7 log N° copy/ g FM, $P < 0.01$), reflecting an improvement in the archaea fermentation conditions. Gut fermentation is a complex synchrony where archaea occur in the latter positions in the metabolic-ecological niche and therefore their increment may occur in latter and more favorable sections of the intestine, such as colon (Seradj et al., 2015).

Those pigs receiving high TDF tend to emit more CH_4, but no relevant changes in methanogen concentrations were detected in respect; effectively, the relationship between abundance of methanogens and CH_4 production in the hindgut ecosystem remains unclear and our results would confirm previous assays conducted by (Cao et al., 2013, 2016), where it was demonstrated that fiber availability improved the diversity of methanogens but not their abundance. NDF fraction in TDF stimulates microbial species within the complex cellulolytic-methanogens (Miller and Lin, 2002). The reductive activity (H^2) released during the degradation process is used by methanogens to reduce carbon dioxide to methane (Zhou et al., 2010; Seradj et al., 2014). However, such relationship is masked by the promotions of SRB in hindgut (Lin et al., 1997) that compete with methanogens for the substrate (H^2).

The extra methane production from aceticlastic archaea may also bias the relationship between fiber availability and CH_4 gen- eration. Presence of the aceticlastic methanogens has been previously evidenced (Smith and Ingram-Smith, 2007) and with the aim of clarifying the aceticlatic role within the hindgut ecosystem and using the specific 16S rRNA gene for *Methanosaeta ssp*., proposed originally by Rowe et al. (2008), we detected *Methanosaeta* ssp. in all the samples and its quantification, relative to total archaea was in general, high and varied from 5 to 50 ($2^{-\Delta ct}$; Livak and Schmittgen, 2001). Relative abundance was higher in colon than in cecum and relative concentration was neither altered, nor by the experimental treatment nor by acetate concentration (as unique substrate for *Methanosaeta ssp*) in hindgut compartments. The aceticlastic role in hindgut compartments has been recently evidenced but we decided to consider such values with caution due to the limited value of 16S rRNA gene identification when low number of copies is found (Fogel et al., 1999; Acinas et al., 2004; Fricke et al., 2006).

Animals fed HP diets showed greater abundances of methanogens than those fed LP ones. The authors are not aware of data describing a positive relationship between dietary CP and methanogen abundance although theoretically, the effect of dietary CP on methanogen abundances should be exerted through ammonia concentration as essential nutrient for microbial survival and growth (Jha and Berrocoso, 2016). We hypothesize that the differences might be due to two reasons, i) the low NH_3 concentration registered in the cecum [close to threshold level (50 mg/l; Satter and Slyter, 1974)] limits microbial and methanogen growth and, ii) the synchronic competition between methanogens and some NH_3 degradative species as *S. Ruminantium* (Saengkerdsub and Ricke, 2014). This competitive mechanism has already been described in human GI tract between *Mbb. Smithii* and *B. thetaiotamicron* (Samuel et al., 2007); S. *ruminantium* also

possesses NH_3 fixation pathways (Ricke and Schaefer, 1996) and may also explain the (numerical: $P > 0.05$) lower concentration of methanogens and NH_3 registered in the cecum compartment of animals fed HF diets, compared with those fed LF diets. In the colon the protein degradative activity or plasma recycling improves colonic NH_3 concentration but the ceca-carry over effect would maintain differences in the latter compartment.

Conclusions

In the present approach, in an open-circuit system designed to maintain commercial-like conditions NH_3-volatilization increased with dietary CP supply and growth phase and proportionally reduced as a whole, a mean values of 8.6% per each percentage unit (%) of CP reduction. Enteric origin constituted the main source for CH_4 emission, although a relevant fraction (averaging 9.7%) did come from the slurry pit. Pigs of HF diets trended to increase CH_4 emission. Methanogen concentration increased throughout the cecum-colon tract reflecting an improvement in the archaea fermentation conditions, but no changes between middle colon digesta and slurry may due to the ability of the methanogen archaea communities to adapt to the new environmental imposed into the pits. Differences in CH_4 emission did not reflect the TMA concentration whereas the aceticlastic populations were rather relevant in hindgut compartment and its role in CH_4 emission has to be further developed.

Declarations of interest

None.

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Table 1

Main ingredients (g/kg) of the three-phase experimental diets, differing in CP content (high, HP *vs*. low, LP) and/or TDF content (Low, LF *vs*. high, HF) from 6 to 21 weeks of age.

Item	Feeding phase											
	I (6–11 weeks of age)				II (12–16 weeks of age)				III (17–21 weeks of age)			
	HP		LP		HP		LP		HP		LP	
Ingredients	HF	LF	HF	LF	HF	LF	HF	LF	HF	LF	HF	LF
Barley	193	267	253	203	297	276	301	268	217	274	398	253
Soybean meal, 47% CP	266	265	199	217	186	246	102	156	114	226	16	90
Sorghum	52	260	59	151	–	205	–	227	–	200	–	101
Wheat	379	152	382	375	296	205	298	288	201	199	201	396
Rapeseed meal 00	–	–	–	–	70	–	78	–	100	–	81	–
Maize	–	–	–	–	16	–	95	–	173	60	152	101
Sunflower meal	–	–	–	–	–	–	–	–	80	–	36	–
Sugar beet pulp	53	–	50	–	53	–	50	–	50	–	50	–
Soybean oil	3.1	–	3.2	–	8.6	–	6.9	–	9.9	–	8.7	–
Animal-vegetable fat 3/5	30	31	31	31	40	31	40	30	40	23	40	31
Vitamin-mineral premix and additives[1]	4.1	4.1	4.1	4.1	4.4	4.4	4.4	4.4	4.5	4.5	4.5	4.5
Calcium carbonate	2.8	8.9	2	–	2.5	5.9	0.8	0.8	4.3	7	4.2	13.5
Monocalcium phosphate	9	5.5	8.1	9.1	6.1	6.3	7.1	7.5	3.4	4.2	5.5	6.3
Sepiolite	–	–	–	–	4.1	8.4	3.3	4.2	–	–	–	–
Sodium chloride	1.8	2.1	2.1	2.1	2	1.9	1.9	2.1	1.9	2.2	1.9	2.2
l-Lysine, CP 50%	2.51	3.57	1.63	3.66	–	–	0.9	1.4	–	–	1.4	1.53
dl-Methionine, 88%	1.6	0.5	1.73	–	–	–	–	–	–	–	–	–
l-Threonine	1.1	0.3	1.22	3.96	5.29	–	–	–	–	–	–	–
l-Tryptophan	1.08	–	1.21	–	–	–	0.82	–	0.5	–	–	–
Chemical Composition (g/kg as fed basis)												
DM[2]	891		887		883		883		890		887	
CP[1]	197.5		172		173		151.5		175		125.5	
aNDFom[1]	141	120	154	123	174	130	162	126	175	123	167	135
ADFom[2]	41	36	43	35	51	44	52	32	66	35	56	31
CF[2]	26	27	29	21	39	27	39	23	55	28	47	26
TDF[2]	195	162	182	165	196	178	223	169	228	167	225	138
Fermentable fiber	155	126	138	130	145	134	172	137	161	132	169	108
Starch	383	380	399	380	374	384	380	444	361	417	426	472
AEE[1]	50	49	48	48	60	49	64	45	67	42	60	46
Ash	56	66	51	48	44	67	48	46	48	47	46	63

[1] The vitamin and mineral premix compositions for pigs in the three phases were already described by Morazán et al. (2015a).

[2] DM, dry matter; CP, crude protein, aNDFom, neutral detergent fiber expressed exclusive of residual ash; ADFom, acid detergent fiber expressed exclusive of residual ash; CF, crude fiber; AEE = acid hydrolyzed ether extract; TDF = total dietary fiber.

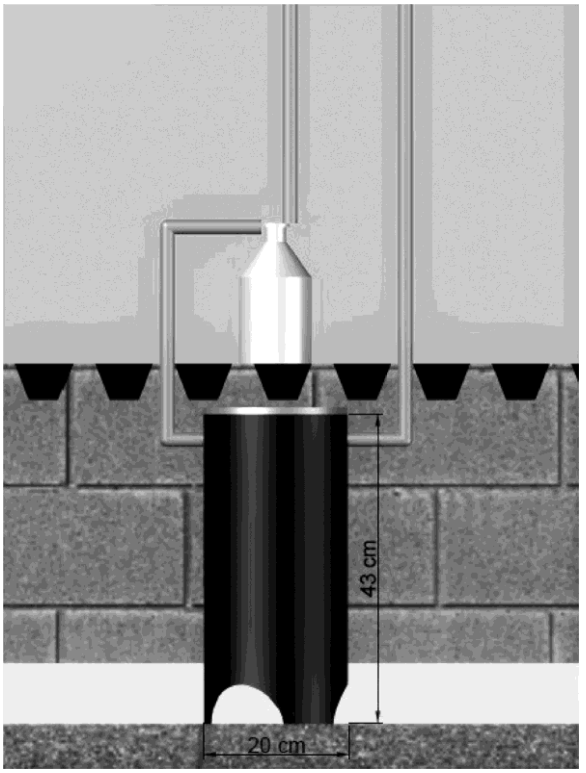

Fig. 1. Flux chamber (made of PVC) used to collect the air sample from the slurry pits, located under the concrete slatted floor.

Table 2

Specific primer sets for qPCR used in the experiment.

Target	Sequences	References
Total bacteria	F 5´-GTGSTGCAYGGYTGTCGTCA-3´ R 5´-ACGTCRTCCMCACCTTCCCC-3´	(Maeda et al., 2003)
Total Archaeae	F 5´- AGGAATTGGCGGGGGAGCA-3´ R 5´- BGGGTCTCGCTCGTTRCC-3´	(Einen et al., 2008)
Total Methanogens	F 5´-TTCGGTGGATCDCARAGRGC -3´ R 5´-GBARGTCGWAWCCGTAGAATCC-3´	(Denman et al., 2007)
Methanosaeta	F5´-GGGGTAGGGGTGAAATCTTGTAATCCT-3´ R5´-CG-GCGTTGAATCCAATTAAACC GCA-3´	(Rowe et al., 2008)

Table 3

The effect of crude dietary protein and total dietary fiber content as well as sample collection site (cecum, colon and slurry) and their interactions on pH value, ammonia concentration (mg/L), the absolute quantity (m*M*) of total volatile fatty acids and the proportion (in percentage) of each VFA.

		Segment	CP		TDF		mean	SEM	P values				
			HP	LP	HF	LF			Seg	CP	TDF	Seg × CP	Seg × TDF
pH		Cecum	5.9	6.1	5.8^{k}	6.1	6.0^{k}	0.08^{1}	<0.01	0.54	0.84	0.08	<0.01
		Colon	6.3	$6.4^{e,j}$		6.1^{f}	6.3^{j}	0.06^{2}					
		Slurry	7.5^{a}	7.0^{b}	7.2	7.2		0.12^{3}		0.04	0.94		
	NH3	mg/L Cecum	75.3^{k}	64.1^{k}	55.5^{k}	84.0^{k}	69.7^{k}	11.85	<0.01	0.10	0.75	0.01	0.05
		Colon	$130.5^{b,j}$	$182.1^{a,j}$	166.7^{j}	145.9^{j}	156.3^{j}	8.38					
		g/L Slurry	6.2^{a}	2.9^{b}	5.1	4.1		0.39		<0.01	ns		
	Total VFA	m*M* Cecum	169.6	180.8	175.6^{j}	174.8	175.2^{j}	13.92	0.02	0.57	0.04	0.82	0.04
		Colon	138.8	143.5	$110.7^{f,k}$	171.6^{e}	141.1^{k}	9.85					
		Slurry	219.3	162.7	176.7	205.3		33.70		ns	ns		
	Acetate	mol/100 mol TVFA Cecum	60.9	64.2	64.1	61.0	62.6^{j}	1.33	<0.01	0.25	0.04	0.19	0.85
		Colon	57.2	57.2	59.0	55.5	57.2^{k}	0.95					
		Slurry	66.7	64.4	65.8	65.4		1.02		ns	ns		
	Propionate	Cecum	24.3	23.8	24.5	23.5	24.0	0.87	0.20	0.41	0.66	0.76	0.12
		Colon	23.4	22.4	22.0	23.8	22.9	0.62					
		Slurry	14.2	17.8	17.9	17.1		0.26		t	t		
	Butyrate	Cecum	10.8	8.9^{k}	7.8	11.9	9.9^{k}	0.73	<0.01	0.73	<0.01	<0.01	0.09
		Colon	11.5	14.0^{j}	11.8	13.7	12.7^{j}	0.54					
		Slurry	11.6	10.3	11.4	10.5		2.56		ns	ns		
	Valorate	Cecum	1.7	1.1	1.2	1.6	1.4	0.30	0.06	0.07	0.59	0.82	0.60
		Colon	2.2	1.7	1.9	2.0	2.0	0.21					
		Slurry	1.4	1.7	1.6	1.5		0.18		ns	ns		
	BCFA	Cecum	2.2	1.9	2.3	1.9	2.1^{k}	0.68	<0.01	0.37	0.68	0.63	0.93
		Colon	5.6	4.7	5.3	5.0	5.2^{j}	0.48					
		Slurry	6.1	5.7	6.3	5.5		0.55		ns	ns		

[1]Standard error of the mean for comparisons between CP or TDF content within cecum and colon, [2]between segments and [3]between CP or TDF content within slurry. Different upper case superscripts (j, [k)] within columns denote differences between segments ($P < 0.05$). Different upper case superscripts [(a, b)] within rows denote differences among high/low CP and different upper case superscripts [(e, f)] within rows denote differences among high/low TDF content ($P < 0.05$).

Table 4

The impacts of crude protein and total dietary fiber content as sole and their interactions with the sample collection site (cecum, colon and slurry) on absolute counts of total bacteria and, total archaeas and total methanogen archaeas (TMA).

		SegCP		TDF			SEM	P values					
		HP	LP	HF	LF	mean		Seg	CP	TDF	CP × TDF	Seg × CP	Seg × TDF
Absolue quantification, Log N° copy/ g FM													
Total Bacteria	Cecum	11.6	11.5	11.6	11.6	11.6^{k}	0.11^{1}	0.01	0.03	0.31	0.06	0.14	0.16
	Colon	12.1	11.7	12.1	11.8	11.9^{j}	0.08^{2}						
	mean	11.9^{a}	11.6^{b}	11.8	11.7		0.08^{3}						
	Slurry	9.7^{a}	9.5^{b}	9.7^{e}	9.5^{f}		0.03^{4}		0.02	0.01	0.04		
Total Archaea	Cecum	9.3^{k}	9.8	9.5	9.5	9.5^{k}	0.15	0.03	0.43	0.32	0.42	<0.01	0.13
	Colon	9.9^{j}	9.7	10	9.6	9.8^{j}	0.11						
	mean	9.6	9.7	9.7	9.6		0.12						
	Slurry	9.1	9	9.2^{e}	8.8^{f}		0.04		0.27	<0.01	0.16		
TMA	Cecum	6.2	5.6	5.7	6	5.9^{k}	0.19	<0.01	<0.01	0.17	0.13	0.33	0.96
	Colon	8.2	7.2	7.6	7.9	7.7^{j}	0.14						
	mean	7.2^{a}	6.4^{b}	6.7	7		0.15						
	Slurry	6.8^{a}	6.3^{b}	6.6^{e}	6.4^{f}		0.03		<0.01	0.01	0.01		

Different upper case superscripts (j, $^{k)}$ within columns denote differences between segments ($P < 0.05$). Different upper case superscripts $^{(a, b)}$ within rows denote differences among high/low CP and different upper case superscripts $^{(e, f)}$ within rows denote differences among high/low TDF content ($P < 0.05$).

[1]Standard error of the mean for comparisons between CP or TDF content within cecum and colon, [2]between segments, [3]between CP or TDF content and [4]between CP or TDF content within slurry.

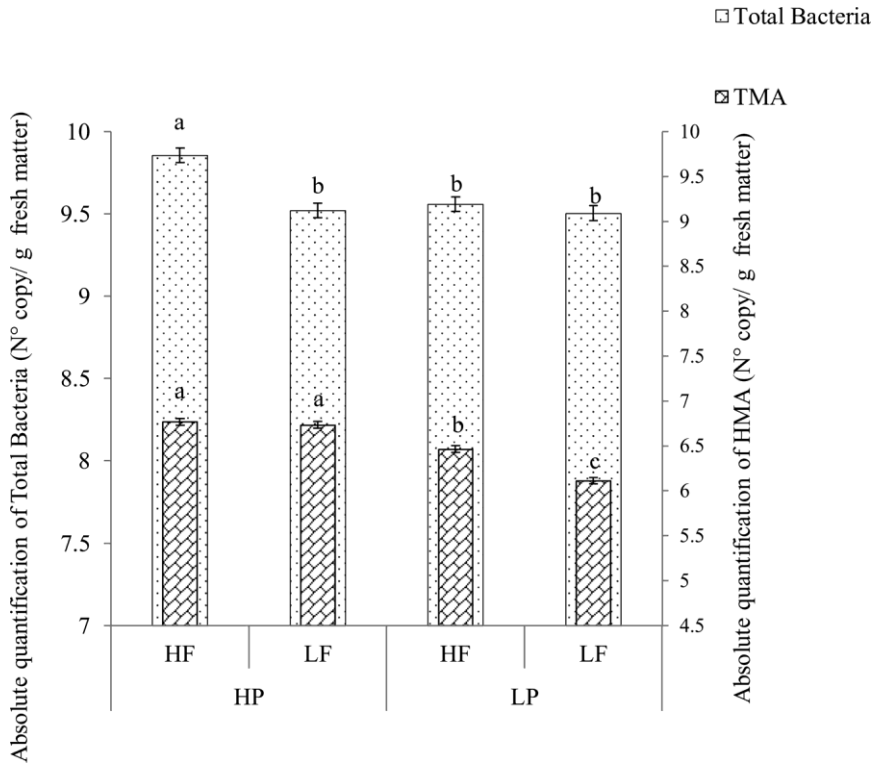

Fig. 2. The interactions between CP level (high or low) and TDF content (high or low) of the experimental diets on absolute abundance (N° copy/ g FM) of Total Bacteria and Total Methanogenic Archaea in the slurry.

Table 5

Values of methane and ammonia emitted at modules (environment).

Gas		Unit	Phase	CP		TDF	mean		SEM	P values				
				HP	LP	HF	LF			Ph	CP	TDF	Ph × CP	Ph × TDF
Environment	Methane	g/ animal/ day	I	2.7	2.2	2.7	2.3	2.5^{k}	0.85^{1}	<0.01	0.23	0.14	0.42	0.82
			II	3.5	3.5	4.2	2.8	3.5^{k}	0.59^{2}					
			III	8.5	6.5	8.3	6.8	7.6^{j}	0.48^{3}					
			mean	4.9	4.1	5.0	4.0							
Ammonia			I	2.3	1.2	2.1	1.4	1.8^{l}	0.54	<0.01	<0.01	0.28	0.02	0.91
			II	5.7	4.0	5.0	4.7	4.8^{k}	0.38					
			III	10.8^{a}	6.2^{b}	8.7	8.3	8.5^{j}	0.31					
			mean	6.3^{a}	3.8^{b}	5.3	4.8							

[1,2,3]Standard error of the mean for comparisons between CP or TDF content within phases, between phases and between CP or TDF content, respectively.

Different upper case superscripts (j,[k,l]) within columns denote differences between phases ($P < 0.05$). Different upper case superscripts [(a, b)] within rows denote differences among high/low CP and different upper case superscripts [(e, f)] within rows denote differences among high/low TDF content ($P < 0.05$).

Table 6

Values of methane expressed as g/an/day or g/m^3 of slurry per day as the main greenhouse gases emitted at slurry pits.

Gas	Unit	Phase	CP		TDF		mean	SEM	P values				
			HP	LP	HF	LF			Ph	CP	TDF	Ph × CP	Ph × TDF
Slurry pit Methane	g/ m^3 slurry	I	1.86^{k}	0.33.1^{k}	1.10^{l}	1.09^{l}	1.09^{l}	0.876^{1}	<0.01	0.35	0.2	0.49	0.01
		II	4.03^{k}	3.04^{k}	4.66^{k}	2.42^{l}	3.54^{k}	0.621^{2}					
		III	11.99^{j}	12.51^{j}	9.72^{f,j}	14.78^{e,k}	12.25^{j}	0.507^{3}					
		mean	5.97	5.29	5.16	6.10							
	g/ animal/ day	I	0.14	0.02	0.04 ^{k}	0.13 ^{k}	0.08 ^{l}	0.087	<0.01	0.94	0.02	0.47	0.01
		II	0.28	0.35	0.37 ^{k}	0.26 ^{k}	0.32 ^{k}	0.062					
		III	1.25	1.32	1.03 $^{f, j}$	1.54 $^{e, j}$	1.28^{j}	0.050					
		mean	0.56	0.56	0.48 ^{f}	0.64 ^{e}							

[1,2,3]Standard error of the mean for comparisons between CP or TDF content within phases, between phases and between CP or TDF content, respectively.

Different upper case superscripts (j,$^{k,l)}$ within columns denote differences between phases (P < 0.05). Different upper case superscripts $^{(e, f)}$ within

rows denote differences among high/low TDF content (P < 0.05).

Table 7

Chemical composition of slurry (g/kg DM) stored into the pits at the end of each collection period.

	Phase	CP		TDF		mean	SEM	P values					
		HP	LP	HF	LF			Ph	CP	TDF	CP × TDF	Ph × CP	Ph × TDF
pH	I	7.5	7.2	7.4	7.2	7.3	0.161	0.73	0.04	0.16	0.34	0.53	0.59
	II	7.3	7.2	7.4	7.1	7.2	0.112						
	III	7.5	7.0	7.2	7.2	7.2	0.093						
	mean	7.4a	7.1b	7.4	7.2								
DM, g/ kg FM	I	85.0	62.4	85.3	62.2	73.7	20.3	0.61	0.12	0.13	<0.01	0.91	0.98
	II	79.6	54.5	81.5	52.6	67.0	14.35						
	III	106.0	68.5	102.7	71.8	87.3	11.89						
	mean	90.2	61.8	89.8	62.2								
OM	I	778.3	788.3	793.9	772.7	783.3	21.87	0.81	0.65	0.09	0.32	0.84	0.4
	II	786.8	808.1	803.5	791.3	797.4	15.46						
	III	795.5	789.8	826.3	759.1	792.7	12.81						
	mean	786.9	795.4	807.9	774.4								
Total N	I	88.6	86.3	89.3	85.6	87.5	10.00	0.17	0.66	0.95	0.12	0.49	0.4
	II	73.9	81.9	83.2	72.6	77.9	7.07						
	III	77.9	60.8	61.3	77.4	69.4	5.86						
	mean	80.1	76.4	77.9	78.5								
N-NH3	I	52.4	52.1	52.0	52.5	52.3	11.56	0.56	0.95	0.81	0.08	0.88	0.6
	II	40.6	45.9	47.7	38.9	43.3	8.18						
	III	44.8	38.0	33.7	49.0	41.4	6.77						
	mean	45.9	45.4	44.5	46.8								
Phosphorous	I	41.2	42.0	46.0	37.2	41.6	4.17	0.85	0.62	0.7	0.45	0.58	0.3
	II	39.0	46.0	40.3	44.7	42.5	2.95						
	III	45.0	42.4	43.5	43.9	43.7	2.44						
	mean	41.7	43.5	43.3	41.9								
Potassium	I	73.3	68.0	72.5	68.9	70.7	14.16	0.34	0.92	0.92	0.15	0.75	0.32
	II	50.3	63.0	68.3	45.0	56.7	10.01						
	III	57.3	46.5	40.4	63.4	51.9	8.29						
	mean	60.3	59.2	60.4	59.1								

Different upper case superscripts $^{(a, b)}$ within rows denote differences among high/low CP (P < 0.05).

[1,2,3]Standard error of the mean for comparisons between CP or TDF content within phases, between phases and between CP or TDF content, respectively.

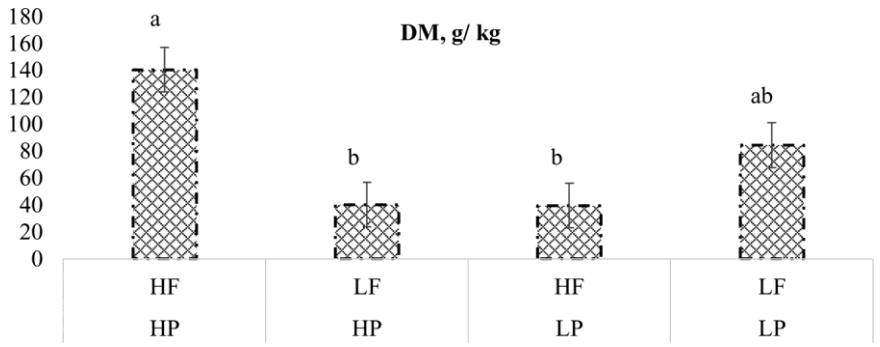

Fig. 3. The interactions between CP level (high or low) and TDF content (high or low) of the experimental diets on DM content of the slurry stored into the pits.