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## Dry anaerobic digestion of food waste and cardboard at different substrate loads, solid contents and co-digestion proportions

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### Abstract

The increasing food waste production calls for developing efficient technologies for its treatment. Anaerobic processes provide an effective waste valorization. The influence of the initial substrate load on the performance of batch dry anaerobic co-digestion reactors treating food waste and cardboard was investigated. The load was varied by modifying the substrate to inoculum ratio (S/X), the total solids content and the co-digestion proportions. The results showed that the S/X was a crucial parameter. Within the tested values (0.25, 1 and 4 gVS·gVS<sup>-1</sup>), only the reactors working at 0.25 produced methane. *Methanosarcina* was the main archaea, indicating its importance for efficient methanogenesis. Acidogenic fermentation was predominant at higher S/X, producing hydrogen and other metabolites. Higher substrate conversions ( $\leq 48\%$ ) and hydrogen yields ( $\leq 62\text{ mL}\cdot\text{gVS}^{-1}$ ) were achieved at low loads. This study suggests that different value-added compounds can be produced in dry conditions, with the initial substrate load as easy-to-control operational parameter.

### Keywords

Biomethane; Biohydrogen; Dark fermentation; High-solids; Platform molecules

## 1. Introduction

The production of food waste (FW), which can be defined as the mass of food lost or wasted during the part of the food supply chains leading to edible products for human consumption, is a global problem (Gustavsson et al., 2011). Currently, about 1.3 billion tons of food (one third of the production for human consumption) is wasted every year (FAO, 2012). Moreover, this number is expected to increase in the coming years due to economic and population growth, particularly in developing countries. On a global scale, the production of urban FW is expected to increase by 44 % from 2005 to 2025 (Melikoglu et al., 2013). In Europe, this raise is expected to be from 89 million tons in 2006 to 126 million tons in 2020 (Monier et al., 2010).

Nowadays, most of the FW is disposed in landfills or incinerated, practices associated with different issues, such as rising costs of waste disposal, lack of space, leaching, public environmental concern and emission of toxic and greenhouse effect gases (Curry and Pillay, 2012; Uçkun Kiran and Liu, 2015). Therefore, it is necessary to develop and optimize technologies that allow a proper treatment of this biowaste. Anaerobic processes stand as a well-established technology that permits an effective and environmental-friendly treatment of waste and its valorization in the form of several products, such as biomethane, biohydrogen, alcohols or volatile fatty acids (VFAs) (Banks et al., 2012; Kim et al., 2014; Wang et al., 2015). Particularly, anaerobic digestion (AD) in dry conditions (> 20 % total solids; TS) is a promising alternative, due to several advantages when compared to wet digestion, *e.g.* lower water requirement and/or smaller reactor volume (Karthikeyan and Visvanathan, 2013).

However, AD of FW is a complex process, associated in many cases with the accumulation of ammonia and VFAs, leading to inefficient performances and even to process failure

(Agyeman and Tao, 2014; Dai et al., 2013; El-Mashad and Zhang, 2010; Owamah and Izinyon, 2015; Wan et al., 2013; Wang et al., 2014; L. Zhang et al., 2012). Co-digestion, *i.e.* simultaneous treatment of two or more substrates, has been proved to be an economically feasible option to overcome these complications (Dai et al., 2016; Lin et al., 2011). Co-digestion may favor the methanogenic/acidogenic processes by balancing the nutrient and carbon contents, diluting inhibitory compounds, adjusting the moisture content or increasing the buffering capacity of the system (Mata-Alvarez et al., 2011). Particularly for FW dry AD, a suitable co-substrate should have a high C/N ratio, a high TS content and provide enough buffering capacity to avoid sudden pH drops. Paper/cardboard waste (CB) fulfills all these requirements, with negligible N contents, having high buffering capacities and TS contents and being slowly biodegradable. In addition, CB is a particularly convenient co-substrate for centralized co-digestion with commercial FW in urban areas, where FW and CB are usually the main organic solid waste streams (Kim and Oh, 2011; Y. Zhang et al., 2012). To give an idea of the importance of CB waste streams, in a study dealing with the composition of municipal solid waste in different countries in the 90s, paper and cardboard waste represented up to 36.8 % of the total municipal waste (Hogg et al., 2002).

Besides the great potential of this option, few studies have been carried out to assess the feasibility of FW and CB dry co-digestion. Zhang et al. (2012) co-digested FW and CB in wet AD at a ratio 53:47 g VS·g VS<sup>-1</sup>, achieving effective methane production at a load of 3 g VS·L<sup>-1</sup>·d<sup>-1</sup> and proving that CB addition led to less accumulation of ammonia and VFAs. In a recent study, Asato et al. (2016) co-digested FW and CB (wet AD) at different COD loads and co-digestion proportions. They concluded that concentrations of FW  $\geq 18.75$  g COD·L<sup>-1</sup> caused inhibition, while mixtures with  $\geq 75$  % of CB avoided failure of methanogenesis. In dry conditions, Kim and Oh (2011) achieved a stable methane production (up to 260 ml CH<sub>4</sub>·g COD<sup>-1</sup>·d<sup>-1</sup>) without significant VFA accumulation at OLRs up to 10 g TS·L<sup>-1</sup>·d<sup>-1</sup> and

with a co-digestion ratio FW:paper of 7:1 g TS·g TS<sup>-1</sup>. To our knowledge, no other study has been performed dealing with FW and CB co-digestion at high TS contents. In addition, no study has been performed to optimize critical variables for dry co-digestion of FW and CB, such as the substrate load, the co-digestion ratio or the TS contents. Moreover, taking into account the huge variability of the FW characteristics worldwide, producing comparable experiments (always supplying extensive characterizations of the substrates and the inoculum) is much more important than when using more simple/homogeneous substrates.

Accordingly, the aim of this study was to evaluate the feasibility of FW valorization by dry anaerobic co-digestion with CB using batch systems, which allowed testing different conditions simultaneously. More precisely, the influence of the initial FW load (varied by modifying the substrate to inoculum ratio (S/X), the TS content and the FW:CB proportions) on the performance of a dry batch anaerobic co-digestion system using CB as sole stabilization agent, was investigated for the first time. In addition, the physicochemical characteristics of the substrates and the microbial communities in the reactors were studied extensively.

## 2. Materials and methods

### 2.1. Substrate and microbial inoculum

A model FW was prepared according to the VALORGAS report (VALORGAS, 2010) and used as substrate (Table 1). The FW mixture was finely milled and blended to ensure its homogeneity. Compact cardboard (branded “Cartonnages Michel”) with a density of 1.42 kg·m<sup>-3</sup> was shredded to less than 1 mm and used as co-substrate.

The microbial inoculum consisted on a mixture of (i) a centrifuged granular sludge issued from a mesophilic industrial UASB reactor treating sugar factory effluents and (ii) a dried digestate originated from a thermophilic industrial plant treating the organic fraction of

municipal solid waste used only to increase the TS content. This latter digestate was dried at 105 °C for at least 24 h and the resulting material was finely milled and sieved at 1 cm. Both fractions were mixed in a proportion 1:2 (wet weight basis), to obtain a final TS content of 74.19 % (59.06 % VS/TS; VS standing for volatile solids). This high TS proportion of the inoculum allowed starting the reactors with TS contents up to 35 %. Although this process resulted in a very particular inoculum, this was the only possible way to achieve the desired TS contents in the reactors. This allowed elucidating clearly the influence of this parameter on the AD process. In addition, the dried digestate added was the source of solids closest to those that can be found in a regular high-solid digestate.

## 2.2. Dry batch anaerobic co-digestion

The batch assays were carried out in flasks with a total volume of 600 mL. The initial FW concentrations in the reactors were adjusted by modifying the initial TS content (20, 27.5 and 35 %), the S/X (0.25, 1 and 4 g VS·g VS<sup>-1</sup>) and the FW:CB co-digestion ratio (80:20, 65:35 and 50:50 g TS·g TS<sup>-1</sup>). Thirteen different combinations of these independent variables were defined following an optimal statistical design and varying the initial FW load from 26.4 to 252 g VS·L<sup>-1</sup> (Table 2). After addition of all the components needed (*i.e.*, FW, CB, inoculum and tap water), the flasks were sealed and the volume of the headspace was accurately determined by measuring the pressure in the vessel before and after adding a known volume of gas. The reactors were then flushed with nitrogen to ensure anaerobic conditions and incubated at 35 °C for a maximum of 98 days. Such a long incubation period was necessary to account for the long lag phases in the methane production observed in some reactors. To correct the endogenous contribution to the biogas from the inoculum, three blanks (one per TS content) were carried out. Each condition was performed in triplicate.

## 2.3. Analytical methods

### 2.3.1. Physicochemical characterization of the substrates

Both substrates were characterized before starting the experiments. The TS and VS contents were measured according to the standard methods of the American Public Health Association (APHA, 2005). After acid hydrolysis of the substrate with sulfuric acid (solution 10 % v/v H<sub>2</sub>SO<sub>4</sub> 98 % with 1 g TS·L<sup>-1</sup> of substrate; agitation for 24 h), the protein concentration was determined by the modified Lowry method (Frølund et al., 1996) and the carbohydrate concentration by the Dubois method (Dubois et al., 1956). The lipid content was measured using a gravimetric method (APHA, 2005) based on accelerated solvent extraction with heptane as solvent using an ASE<sup>®</sup>200, DIONEX (100 bar, 105 °C, 5 cycles of 10 min static and 100s purge) coupled to an evaporator MULTIVAPOR P-12, BUCHI. The proportions of cellulose, hemicellulose and lignin-like compounds in the substrates were determined according to the Van Soest procedure (Van Soest, 1963). Total Kjeldahl nitrogen (TKN) and ammonia nitrogen contents were measured with an AutoKjeldahl Unit K-370, BUCHI. Total Organic Carbon (TOC) and Inorganic Carbon (IC) were determined using a Shimadzu TOC-V<sub>CSN</sub> Total Organic Carbon Analyzer coupled to a Shimadzu ASI-V tube rack. The Total Carbon (TC) content corresponded to the sum of TOC and IC. The pH was measured using a WTW pHmeter series inoLab pH720. The chemical oxygen demand (COD) was analyzed using an Aqualytic 420721 COD Vario Tube Test MR (0-1500 mg·L<sup>-1</sup>). Two mL of sample were pipetted into each tube and then they were placed inside a HACH COD reactor at 150 °C for 2 h. COD concentrations were determined using an Aqualytic MultiDirect spectrophotometer. The biochemical methane potentials (BMPs) of the substrates were determined according to Motte et al., (2014).

The concentrations of micro/macro-elements were measured by SAS Laboratoire<sup>®</sup> (Ardon, France) as follows: metallic trace elements were analyzed by water extraction, according to the norm NF EN 13346. The determination of the Cd, Cr, Cu, Ni, Pb, Al, Mo, Co, Zn, Se and As concentrations was performed by plasma emission spectrometry, in accordance with the



NF EN ISO 11885. Hg was measured by elementary analysis (internal method), according to the norm NF EN ISO 12338. The concentrations of total P, K, Mg, Ca, S and Na were measured according to NF EN ISO 11885.

### 2.3.2. Gas quantification and analysis

The amount and composition of the biogas produced were determined as previously described by Cazier et al. (2015). The volumes were normalized (at 0 °C and 1013 hPa) and the endogenous respiration was considered by subtracting the gas generated in the blanks.

### 2.3.3. Analysis of metabolites and final products of the digestion

The concentrations of VFAs, ionic species and other metabolic products (*i.e.* lactic acid or ethanol) were measured according to Cazier et al. (2015) and Motte et al. (2013).

### 2.4. Microbial community analysis

Samples of the initial inoculum and from the batch reactors at the end of the experiments were analyzed to determine the structure of the microbial communities. Polymerase Chain Reaction (PCR), quantitative PCR (qPCR) and DNA sequencing techniques were used. A precise description of the methodology employed can be found elsewhere (Moscoviz et al., 2016).

### 2.5. Data analysis

To evaluate the existence of significant statistical differences between comparable experiments, one-way ANOVA tests were computed. When the differences were found to be significant ( $\alpha = 0.05$ ), Tukey's Post Hoc tests were performed to compare means.

Non-linear regression analyses were performed to adjust some of the obtained data to theoretical models and linear correlations between variables were investigated. The least squares method was used in both cases.

The experimental data corresponding to the methane production were fit to the Gompertz equation (Zwietering et al., 1990) to estimate the kinetic parameters of the process.

Equation 1, derived from Chen et al. (2014), was used to calculate the concentration of free ammonia nitrogen (FAN) as a function of the pH, the temperature (T) and the total ammonia concentration (TAN) in the media.

$$\text{NH}_3 = \text{NH}_4^+ \cdot \frac{K_a}{(10^{-\text{pH}} \cdot [\frac{K_a}{10^{-\text{pH}}} + 1] - K_a)} \quad \text{Eq. 1}$$

where  $K_a$  has a value of  $1.097 \cdot 10^{-9}$  (35 °C) and the concentrations are expressed in  $\text{mg} \cdot \text{L}^{-1}$ .

In order to investigate the relationships between the initial working parameters (TS content, S/X and FW:CB ratio) and the fermentation products, a Principal Component Analysis (PCA) was carried out. The mixOmics R software package was used to perform the PCA.

All the analyses were computed using the statistical software R 3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria). A significance level value of 5 % ( $\alpha = 0.05$ ) and  $N = 3$  were used when needed.

### 3. Results and discussion

#### 3.1. Characterization of substrates

Table 3 shows the characteristics and the composition of both substrates. The synthetic FW was mainly composed of carbohydrates ( $697 \text{ g} \cdot \text{kg TS}^{-1}$ ), with a TS content of 21.6 % (96.2 % VS) and a relatively high BMP ( $498 \text{ mL CH}_4 \cdot \text{g VS}^{-1}$ ; obtained after 35 d). These values are within the range of BMPs ( $210\text{-}648 \text{ mL CH}_4 \cdot \text{g VS}^{-1}$ ) reported in the literature (Capson-Tojo et al., 2016). The results also suggested that CB is a suitable co-substrate to stabilize FW dry digestion, *i.e.* by increasing the C:N ratio and the TS content, supplying trace elements such as Cu, Fe, Mn or Ni or diluting potentially toxic compounds such as  $\text{Na}^+$  or  $\text{K}^+$ , as well as providing alkalinity to the medium.

The results of the Van Soest fractionation (Table 3) pointed out another beneficial characteristic of the CB as co-substrate. The soluble fractions were indeed much lower for CB

(8.53 %) than for FW (66.6 %) and its hydrolysis kinetics should therefore be slower. This may lead to a moderation of the issue of initial VFA accumulation, common in batch AD of FW (Capson-Tojo et al., 2016).

### 3.2. Performance of the dry anaerobic digestion reactors

The performances of the reactors varied widely according to their initial conditions (*i.e.* S/X, TS content and FW:CB ratio). Figure 1 presents a summary of the main results obtained, including the final distribution of metabolic end-products, the final pH values and the final substrate conversion according to the initial load of FW. In this graphic, the vertical axis on the left represents the distribution of products (*i.e.*, gas and soluble metabolites) at the end of the experiments (in COD %) and the vertical axis on the right stands for the substrate conversion. This conversion variable is the result of the sum of the metabolites obtained at the end of the experiment (in COD units) divided by the biodegradable COD of the added substrates ( $\text{COD}_{\text{bio}}$ ), estimated from the BMP tests of both substrates. In Figure 1, these two variables are plotted together with the initial food waste concentration in the reactors ( $\text{g VS}\cdot\text{L}^{-1}$ ). In addition, to underline the importance of the S/X, the reactors were grouped according to this operating parameter (grey rectangular lines).

As it can be appreciated, the substrate conversion was greatly affected by the initial load of FW, observing generally a decreasing trend in the conversion when increasing the FW concentration. These lower conversions at higher FW concentrations occurred likely due to acidification of the reactors, which decreased the final pH of the system (top of Figure 1) and led to modifications in the metabolic pathways. These differences are evident when paying attention to the composition of the final products (Figure 1). In the reactors with an S/X of  $0.25 \text{ g VS}\cdot\text{g VS}^{-1}$ , the final pH was always above 8.0 and methane and carbon dioxide were the only products obtained. These conditions showed the highest substrate degradations,

always over 60 % and in three cases close to 100 % (reactors 2, 3 and 4). At higher values of the S/X (*i.e.* 1 and 4 g VS·g VS<sup>-1</sup>), the final pH was always below 5.5 and no methane was produced. In those conditions, dark fermentation (AD stopped after generation of acids; DF) took place, accumulating hydrogen and different metabolites instead of methane. Basically, at S/X higher than 0.25 g VS·g VS<sup>-1</sup> the alkalinity of the medium was not sufficient to avoid a pH drop when VFAs started to accumulate at the beginning of the AD process, which led to inhibition of the archaea. Thus, the main operating parameter affecting the pH in the reactors, and therefore the metabolic pathways, was found to be the S/X.

### 3.2.1. Methane production at low S/X (0.25 g VS·g VS<sup>-1</sup>)

The obtained results showed that in the reactors with S/X of 0.25 g VS·g VS<sup>-1</sup>, efficient methanogenesis occurred. In those systems, the methane yields were always over 67 % of the BMP (estimated by addition of the BMPs for FW and CB). For reactors 2, 3 and 4, these values were around 100 %, indicating a maximal conversion of the substrate. However, as shown in Figure 1, the substrate conversions and the methane yields were not equal for all the methanogenic reactors. The evolution of the methane yields during the experiments, shown in Figure 2, may help to understand this behavior. The reactors with loads of 26 g VS·L<sup>-1</sup> (26A) and 47 g VS·L<sup>-1</sup> (reactors 1 and 5) showed significantly lower final yields after 98 days of operation (varying from 409.3 mL CH<sub>4</sub>·g VS<sup>-1</sup> in reactor 3 to 306.9 mL CH<sub>4</sub>·g VS<sup>-1</sup> in reactor 1) and much larger lag phases (almost 50 d by adjustment to the Gompertz equation) than the other conditions. These long lag periods might be consequence of a more intense accumulation of VFAs at the beginning of the digestion (Kawai et al., 2014; Liu et al., 2009), causing the need of a stronger adaptation of the microbial consortia.

A change in the structure of the microbial community could also explain the lower methane yields, as a greater amount of substrate would be used for microbial growth and adaptation.

Figure 3 presents the results of the microbial analysis for the reactors producing methane (with an S/X of 0.25 g VS·g VS<sup>-1</sup>). As shown in Figure 3A, representing the relative abundance of archaeal Operational Taxonomic Units (OTUs), the archaeal population clearly varied when compared with the initial inoculum (Inoculum; NA). Starting with the initial inoculum, *Methanosarcina* represented around 5 % of the OTUs and *Methanosaeta* around 20 %. Amongst the reactors producing methane, the only ones in which the archaeal growth led to significant concentrations of archaeal OTUs at the end of the experiments, *Methanosarcina* accounted for more than 50 % of the archaeal OTUs and the proportions of *Methanosaeta* were negligible after the digestion. This suggests that a microbial selection occurred towards *Methanosarcina*. This could be explained by the greater resilience of *Methanosarcina* to high FAN and VFA concentrations when compared to *Methanosaeta* (Batstone et al., 2002). In fact, the concentration of FAN (only significant in the systems producing methane) increased linearly (R<sup>2</sup> of 0.987) with the initial TS content in the reactors, ranging from 360 to 795 mg FAN·L<sup>-1</sup> (2.61-3.70 g TAN·L<sup>-1</sup>). This elevated concentration of FAN/TAN may be responsible for the microbial selection towards *Methanosarcina* mentioned above. As explained by De Vrieze et al. (2012), *Methanosarcina* sp. are more tolerant to ammonia stress than other methanogens, particularly *Methanosaeta* sp., which cannot thrive at TAN concentrations greater than 3 g·L<sup>-1</sup>. Therefore, a population selection according to their resistance to TAN/FAN would favor the digestion. Moreover, while *Methanosaeta* are strict acetotrophs, *Methanosarcina* are able to perform both hydrogenotrophic and acetotrophic methanogenesis. Thus, it can be stated that, due to the high concentrations of FAN observed, the main metabolic pathway for methane production that occurred in the reactors was hydrogen production by acetogenesis and syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis (Qu et al., 2009). The microbial selection observed suggests a great importance of the initial archaeal structure of the inoculum when batch-

digesting FW. Besides non observing inhibition, it is clear that FAN accumulation can become an issue when digesting FW.

However, a quantitative analysis of the microbial population was required to explain the different performances and the lower methane yields obtained in reactors 1 and 5. Figures 3B and 3C show the concentrations of the most significant OTUs in the reactors at the end of the experiments for archaea and bacteria, respectively. The reactor with the lowest FW load (reactor 1) had a significantly higher concentration of *Methanosarcina* than the others and, in both reactors showing significant lag phases and lower methane yields (reactors 1 and 5), the concentration of *clostridiales* OTUs (main responsible for hydrogen and VFA production during fermentation) was significantly higher than in the rest. This can be explained by a more extended initial acidification period, in which acidogenesis took place, leading to a greater growth of *clostridiales* and decreasing the pH, inhibiting methane production until a proper archaeal population (mainly composed of *Methanosarcina*) was developed. This extended fermentation stage and the concomitant bacterial/biofilm growth and COD consumption may aid to explain the lower methane yields observed in reactors 1 and 5. However, although they may contribute, the obtained differences in the microbial growths only cannot explain the significantly lower methane yields obtained (considering the COD consumed). In addition, also the synthesis of other compounds such as soluble microbial products or extrapolymeric substances may have reduced the methane yields. Interestingly, other authors have also reported lower methane yields after initial acidification (with the consequent lag phases) using FW as substrate for wet AD. Liu et al. (2009) obtained lower biogas yields (from 716 to 358 mL CH<sub>4</sub>·g VS<sup>-1</sup>) at higher S/X (from 1.6 to 3.1 g VS·g VSS<sup>-1</sup>) for co-digestion of FW and green waste due to more pronounced acidifications at high loads. As they did not find signs of residual VFAs present at the end of the digestion, they hypothesized that either the acidogenesis or the hydrolysis steps were jeopardized at high S/X.

In addition, Kawai et al. (2014) studied the mono-digestion of FW at different loads, concluding also that the S/X was inversely proportional to the methane yield due to reversible initial VFA accumulation. Furthermore, they achieved yields over 400 mL CH<sub>4</sub>·g VS<sup>-1</sup> only at S/X lower than 1.0 g VS·g VS<sup>-1</sup>. They attributed the lower yields to the initial pH drop caused by the initial accumulation of VFAs. In this study, no residual VFAs were detected after AD. The fate of the COD not becoming VFAs nor methane in FW AD after long lag phases (and initial VFA accumulations) must be elucidated and further research should be performed on this topic. Hypotheses that can be drawn to explain the lower methane yields after VFA accumulation are lower degrees of hydrolysis or more intense synthesis of soluble microbial under stressful AD conditions, such as high VFA concentrations or relatively low pH values. The long lag phases in the methane production occurring in reactors 1 and 5 might have been caused by a more intense initial acid accumulation, due to: (i) the high proportion of FW in the substrate in reactor 1 (only reactor producing methane with 80 % of FW in the experimental design) and (ii) the high FW load and TS content in reactor 5 (47 g VS FW·L<sup>-1</sup> and 35 % TS). These characteristics may have caused a rapid increase in the VFAs concentrations during the first days in both reactors. In reactor 1, the FW concentration was low due to dilution with water, which did not supply any extra alkalinity to the medium. In addition, this reactor had the lowest proportion of CB, which might have also led to a lower alkalinity when compared to the other reactors and might have also favored a faster VFA accumulation at the beginning of the AD process. In addition, as demonstrated by Abbassi-Guendouz et al. (2012), increasing the TS contents may cause the first-order hydrolysis rates to decrease in batch experiments. Thus, in the case of reactor 1, its low TS content (20 %) may also have prolonged the acidification effect due to faster hydrolysis kinetics. However, as the high TS content (and associated FW concentration) in reactor 5 might have been responsible for the lag phase, it can be hypothesized that both the TS content and the substrate



composition might have led to excessive VFAs concentrations, decreasing the methane yields and slowing down the kinetics of the methanogenesis. An optimum combination of the TS content of the system and the FW:CB proportions of the substrate remains to be found. At this point, it must be mentioned that the physicochemical characteristics of the inoculum are also of critical importance. The results presented in this study were obtained using an inoculum with relatively high TS and VS contents, meaning that the initial substrate concentrations for a defined S/X were also relatively high. This led to a system easier to acidify when compared to other processes using an inoculum with lower contents of solids and higher alkalinities. A previously adapted inoculum with a higher proportion of *Methanosarcina* and lower initial TS content should be tested at different S/X. This would allow the optimization of the AD process as well as the comparison of experiments using different inoculums. In addition, optimal combinations of the FW:CB ratio and the TS content at different S/X should be found to assess the impact of these operating parameters at different loads.

### 3.2.2. Production of hydrogen and metabolites at high S/X (1 and 4 g VS·g VS<sup>-1</sup>)

In the reactors with an S/X higher than 0.25 g VS·g VS<sup>-1</sup> (i.e. 1 and 4 g VS·g VS<sup>-1</sup>), no methane was produced and a much lower substrate conversion was achieved (Figure 1). Moreover, the substrate conversion was found to be negatively correlated to the initial FW concentration. As shown in Figure 4A, the final substrate conversion showed a decreasing trend when increasing the FW charge.

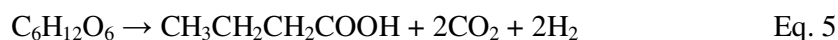
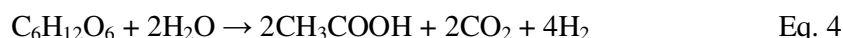
In these reactors, the hydrogen yields ranged from 22.1 to 61.2 mL·g VS<sup>-1</sup>. The highest values obtained were in agreement with results presented in the literature (Ghimire et al., 2015), confirming an efficient FW conversion by DF. As for the substrate conversion, a generally decreasing trend was found when plotting the obtained hydrogen yields against the FW



concentration. This occurred due to a greater accumulation of organic acids and alcohols in the reactors at higher charges of substrate, which led to changes in the pH of the system, modifying the microbial pathways followed. At low pH values (~5.0), non-hydrogen producing pathways, such as those related to ethanol and lactate production (Eqs. 2 and 3; Batstone et al., 2002) were favored.



In fact, lactate accumulation was found to be the reason for the decreasing hydrogen yields in the reactors with an S/X of 4 g VS·g VS<sup>-1</sup> (Motte et al., 2013). A directly proportional correlation was found between the lactate concentrations and the initial FW concentration (R<sup>2</sup> of 0.944). This means that less substrate was available for hydrogen production and, moreover, as lactic acid has a relatively low pKa (3.08), the accumulation of this compound affected greatly the pH, favoring the acidification of the medium. In the reactors with an S/X of 1 g VS·g VS<sup>-1</sup>, the production of lactic acid was observed only in the 2 most heavily charged reactors (number 10 and 11), showing also the lowest hydrogen yields. Moreover, it was also observed that the ratio butyric/acetic acid increased linearly with the FW charge in those conditions (R<sup>2</sup> of 0.914). As butyric acid production yields less hydrogen than that of acetic acid (Eqs. 4 and 5) (Ghimire et al., 2015), an increase of this ratio represents a detriment on the hydrogen yields when homoacetogenesis (Eq. 6; Batstone et al., 2002) does not take place. Thus, the metabolic shift towards butyric acid production instead of acetic acid was responsible for the decreasing hydrogen yields in the reactors with an S/X of 1 g VS·g VS<sup>-1</sup>.





However, special attention must be paid when considering the butyric/acetic acid ratio. Although this ratio has been reported to be a good indicator of the biohydrogen yields associated with the metabolic pathways followed (Khanal et al., 2004), controversy exists when applying this approach (Ghimire et al., 2015). The reason for that is that, if homoacetogenesis takes place, hydrogen is also consumed for production of acetic acid. Therefore, some authors have found direct correlations between the hydrogen yields and the yields of butyric acid, and not those of acetic (Guo et al., 2014). Thus, our results suggest that, in these experiments, homoacetogenesis did not occur in a great extent.

Finally, another possible explanation for the lower substrate conversion at higher FW loads is inhibition of hydrolysis due to local accumulation of hydrogen (Abbassi-Guendouz et al., 2012; Cazier et al., 2015), an issue that has been previously reported at high TS contents. To evaluate the general influence of each initial working parameter (TS content, S/X and FW:CB ratio) on the synthesis of the different products, a PCA analysis was carried out: the products were expressed as  $\text{g COD} \cdot \text{g COD}_{\text{bio}}^{-1}$ , the TS content in %, the S/X in  $\text{g VS} \cdot \text{g VS}^{-1}$ , the FW:CB ratio in  $\text{g TS} \cdot \text{g TS}^{-1}$  and the initial FW concentration in  $\text{g VS} \cdot \text{L}^{-1}$ . Figure 5 shows the corresponding correlation circles. As it can be observed, both the TS content and the FW:CB ratio of the substrate (as well as the initial FW concentration) were negatively correlated to the hydrogen yield and positively correlated to the yields of lactate and non-degraded sugars (glucose, fructose, xylose and lactose) left in the media. In agreement with equations 4 and 5, the hydrogen yield was correlated to the production of acetic acid.

Surprisingly, it was also positively related to the ethanol and the caproic acid yields, meaning that probably equation 7 (Ghimire et al., 2015) was a mayor pathway for the production of ethanol.



However, the yields of these two products in the reactors (and therefore their final concentrations) were of minor importance when compared to others, such as acetic or butyric acids.

In accordance to equations 8 and 9 (Motte et al., 2013), the yields of valeric and caproic acids were strongly correlated to the yields of ethanol (for both) and those of propionic and butyric acids, respectively.



These results were in agreement with the previous statements, showing that the increasing FW concentrations led to greater degrees of acidification, decreasing the pH, raising the lactate yields and jeopardizing the hydrogen production. The yields for the different metabolites were also in accordance with the literature (Wang et al., 2015). Maximum yields of 0.127, 0.231, 0.121, 0.0612 and 0.0295 g COD·g COD<sub>bio</sub><sup>-1</sup> for acetic acid, butyric acid, lactic acid, caproic acid and ethanol respectively, suggest that FW could be used for production of these value-added products at a large scale. Due to the aforementioned acidification effect, greater yields of all the organic acids other than lactate were observed at S/X of 1 g VS·g VS<sup>-1</sup> when compared to the S/X of 4 g VS·g VS<sup>-1</sup>. In the reactors with an S/X of 1 g VS·g VS<sup>-1</sup>, increasing the FW load decreased the acetic acid yields, increasing at the same time those of butyric, valeric and caproic acids and ethanol.

The results shown above suggest that a two-stage system coupling DF and AD, with an intermediate extraction process for recovery of value-added metabolites (therefore not entering the AD system) is an interesting option that should be tested using FW and CB as substrates. In the first stage, as hydrogen and organic acids are to be produced, high S/X should be applied, aiming at the optimal conditions (*i.e.* FW:CB ratios and TS %) to obtain

the highest yields of the desired products. After recovery of the useful products, the remaining COD leaving the DF process (between 50 to 85 % of the input COD<sub>bio</sub>) could enter the AD stage (at much lower loads) for final waste treatment and stabilization, producing methane at the same time. This is a very interesting option in urban areas, where FW and CB are the main components of solid waste. In addition, these wastes are generally taken to the same treatment facilities, which facilitates their centralized co-digestion.

Finally, the microbial communities in the reactors where DF took place (S/X of 1 and 4 g VS·g VS<sup>-1</sup>) were also analyzed. The concentrations of microbial OTUs at the end of the experiments were lower than those found in the reactors where AD occurred. While in the methanogenic reactors the average concentration of bacterial 16S OTUs was  $4.25 \cdot 10^{10}$  OTUs·gr<sup>-1</sup>, this value was  $5.80 \cdot 10^9$  OTUs·gr<sup>-1</sup> for the DF reactors. This might be explained by the much lower substrate conversion obtained by DF when compared to AD, which was translated into a lower microbial growth. Moreover, as methanogenesis did not occur in those systems, negligible amounts of archaeal OTUs were detected. Regarding the composition of the bacterial communities in these reactors, the relative abundance of OTUs is shown in Figure 6. The predominant order in all the reactors at the end of the DF was *Clostridiales*, with proportions ranging from 61 % to 93 %. The abundance of *Clostridiales* was much lower in the inoculum (13 %), indicating a clear microbial selection towards the growth of these microorganisms. Moreover, the microbial diversity was also much lower after the fermentation (see “Others” in Figure 6) and barely no *Anaerolineales* (important in the inoculum and after AD, see Figure 3) were detected. The relevance of *Clostridiales* for DF has been previously presented in the literature (Ghimire et al., 2015), which confirms that these bacteria were the main responsible for the production of hydrogen and other metabolites.

#### 4. Conclusions

By varying the selected operational parameters, different metabolic pathways occurred. The S/X was a critical parameter, affecting the pH due to lack of alkalinity and thus affecting the final products. Efficient methane production was achieved at low S/X ( $0.25 \text{ gVS} \cdot \text{gVS}^{-1}$ ), with *Methanosarcina* as essential archaea for AD. At higher S/X, hydrogen and metabolites were produced, obtaining lower substrate degradations. For DF, higher TS contents, FW:CB or S/X resulted into lower degradations and greater lactate proportions, decreasing the hydrogen yields. This study shows that different value-added compounds can be produced in dry conditions by anaerobic co-digestion of FW and CB.

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### Figure and table captions

**Figure 1.** Distribution of metabolic end-products and substrate conversion according to the initial concentration of FW. The initial S/X and the final pH values are also presented

**Figure 2.** Methane yields in the reactors with an S/X ratio of 0.25 g VS·g VS<sup>-1</sup> (1 to 5). The legend represents the initial food waste concentrations (g VS·L<sup>-1</sup>) applied

**Figure 3.** Relative abundance of Archaeal OTUs (A), concentrations of archaeal 16S OTUs (B) and concentrations of bacterial 16S OTUs (C) in the inoculum and in reactors 1 to 5 (S/X of 0.25 g VS·g VS<sup>-1</sup>) at the end of the batch experiments. NA stands for “not applicable”

**Figure 4.** Influence of the initial FW concentration on the substrate conversion (A) and the hydrogen yields (B) in the reactors 6 to 13 (S/X of 1 and 4 g VS·g VS<sup>-1</sup>)

**Figure 5.** Correlation circle of the initial working parameters and the final yields of metabolites. It is based on the projection in plans formed by the two first principal components, accounting for 72.5 % of the variance

**Figure 6.** Relative abundance of Bacterial OTUs in the inoculum and in reactors with S/X of 1 and 4 g VS·g VS<sup>-1</sup> at the end of the batch experiments

**Table 1.** Components of the model food waste



**Table 2.** Experimental design used

**Table 3.** Characteristics and composition of the substrates

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**Table 1.** Components of the model food waste

Component	Ingredient	Proportion (% in wet basis)
Fruits and vegetables	Apples	25.9
	Lettuce	25.9
	Potato	25.9
Pasta/rice/flour/cereals	Couscous	4.80
Bread and bakery	Bread	6.20
Meat and fish	Chicken	4.10
	Beef	4.10
Dairy products	Cheese	1.90
Confectionery/snacks	Biscuits	1.50

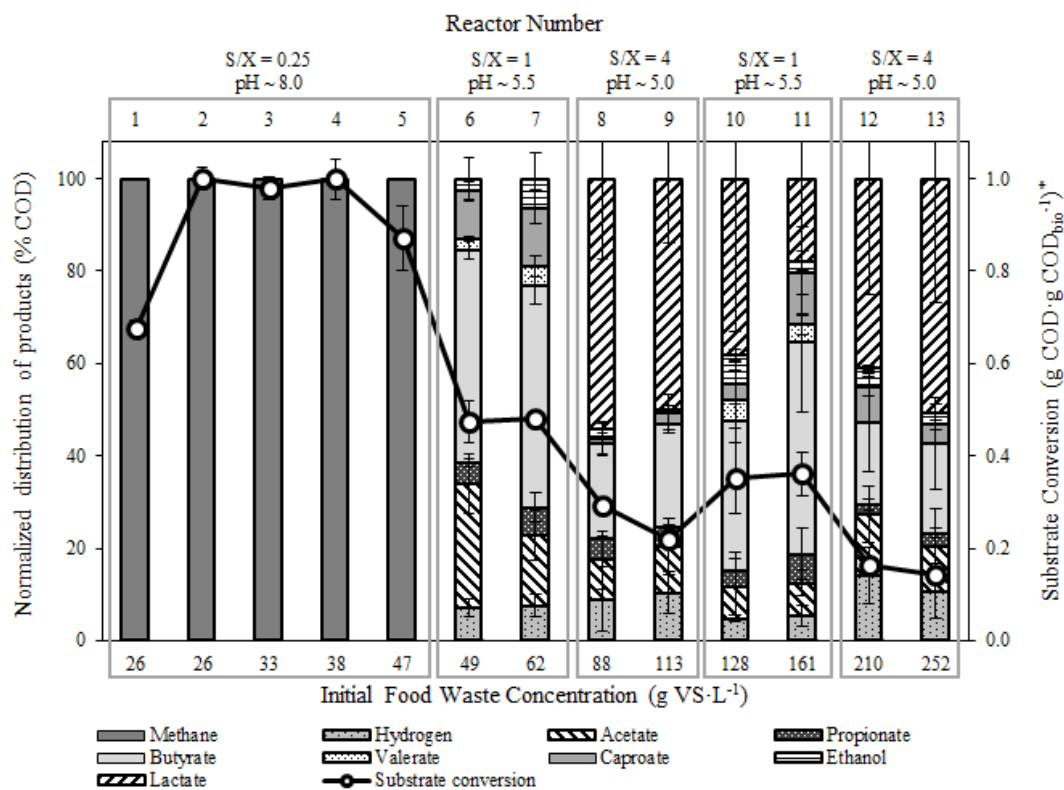
**Table 2.** Experimental design used

Reactor number	TS content (%)	Co-digestion ratio (g TS:g TS <sup>-1</sup> )	S/X (g VS:g VS <sup>-1</sup> )	Initial FW concentration (g VS·L <sup>-1</sup> )
1	20	80:20	0.25	26.4
2	27.5	50:50	0.25	26.5
3	27.5	65:35	0.25	33.4
4	35	50:50	0.25	37.7
5	35	65:35	0.25	47.0
6	20	50:50	1	48.6
7	20	65:35	1	62.0
8	20	50:50	4	87.6
9	20	65:35	4	113
10	27.5	80:20	1	128
11	35	80:20	1	161
12	27.5	80:20	4	210
13	35	80:20	4	252

**Table 3.** Characteristics and composition of the substrates

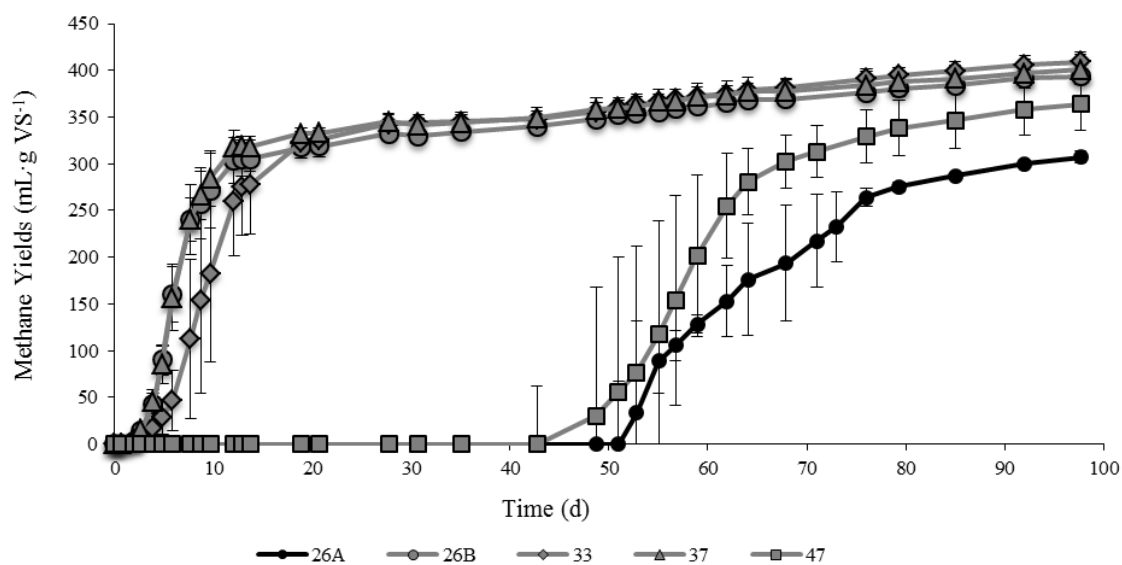
Parameter/Element	Unit	Model Food Waste	Cardboard
TS	% (w. b.)	21.6±0.7	92.7±3.7
VS	% TS	96.2±0.1	77.5±0.2
pH	Unit pH	5.60	7.10
COD	g COD·g TS <sup>-1</sup>	1.37±0.05	1.19±0.05
BMP	mL CH <sub>4</sub> ·g VS <sup>-1</sup>	498±42	250±3
NH <sub>4</sub>	g·kg TS <sup>-1</sup>	0.051	0.002
TKN	g·kg TS <sup>-1</sup>	27.08±1.64	2.00±0.02
TC	g·kg TS <sup>-1</sup>	442±7	366±6
C:N	g·g <sup>-1</sup>	16.3	183
Carbohydrates	g·kg TS <sup>-1</sup>	687±15	958±5
Proteins	g·kg TS <sup>-1</sup>	169±10	0
Lipids	g·kg TS <sup>-1</sup>	72.3±1.5	0
Water soluble fraction*	%	66.6±0.3	8.5±0.9
Soluble fraction*	%	11.5±0.9	11.8±1.3
Hemicellulose fraction*	%	18.2±0.5	9.5±0.6
Cellulose fraction*	%	2.0±0.2	52.1±0.3
Lignin fraction*	%	0.7±0.1	10.7±0.1
Ash fraction*	%	1.0±0.0	7.4±0.3
Total P <sub>2</sub> O <sub>5</sub>	g·kg TS <sup>-1</sup>	7.02	0.45
Total CaO	g·kg TS <sup>-1</sup>	3.80	68.2
Total MgO	g·kg TS <sup>-1</sup>	1.21	1.88
Total K <sub>2</sub> O	g·kg TS <sup>-1</sup>	14.6	< 0.53
Total Na	g·kg TS <sup>-1</sup>	3.27	0.56
B	mg·kg TS <sup>-1</sup>	7.49	14.4
Co	mg·kg TS <sup>-1</sup>	< 9.07	< 8.55
Cu	mg·kg TS <sup>-1</sup>	23.5	43.5
Fe	mg·kg TS <sup>-1</sup>	421	866
Mn	mg·kg TS <sup>-1</sup>	16.4	34.4
Mo	mg·kg TS <sup>-1</sup>	0.408	0.976
Zn	mg·kg TS <sup>-1</sup>	38.7	35.0
Cd	mg·kg TS <sup>-1</sup>	< 0.186	< 0.175
Cr	mg·kg TS <sup>-1</sup>	8.19	7.87
Hg	mg·kg TS <sup>-1</sup>	0.012	0.013
Ni	mg·kg TS <sup>-1</sup>	2.32	4.18
Pb	mg·kg TS <sup>-1</sup>	< 4.59	14.8

\* Calculated by Van Soest fractionation

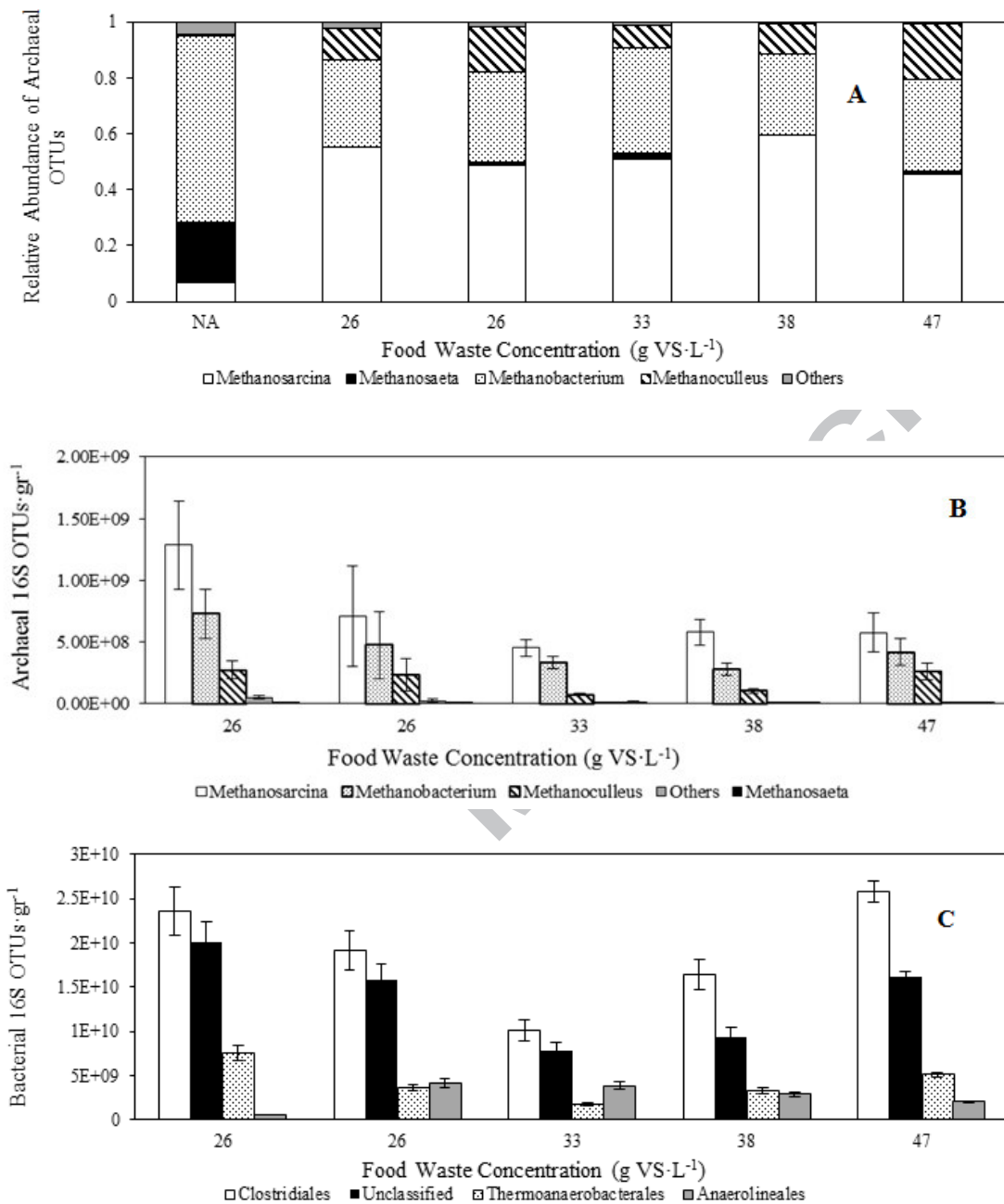


\* The substrate conversion was calculated according to the initial amount of biodegradable COD added as substrate (estimated from the BMPs)

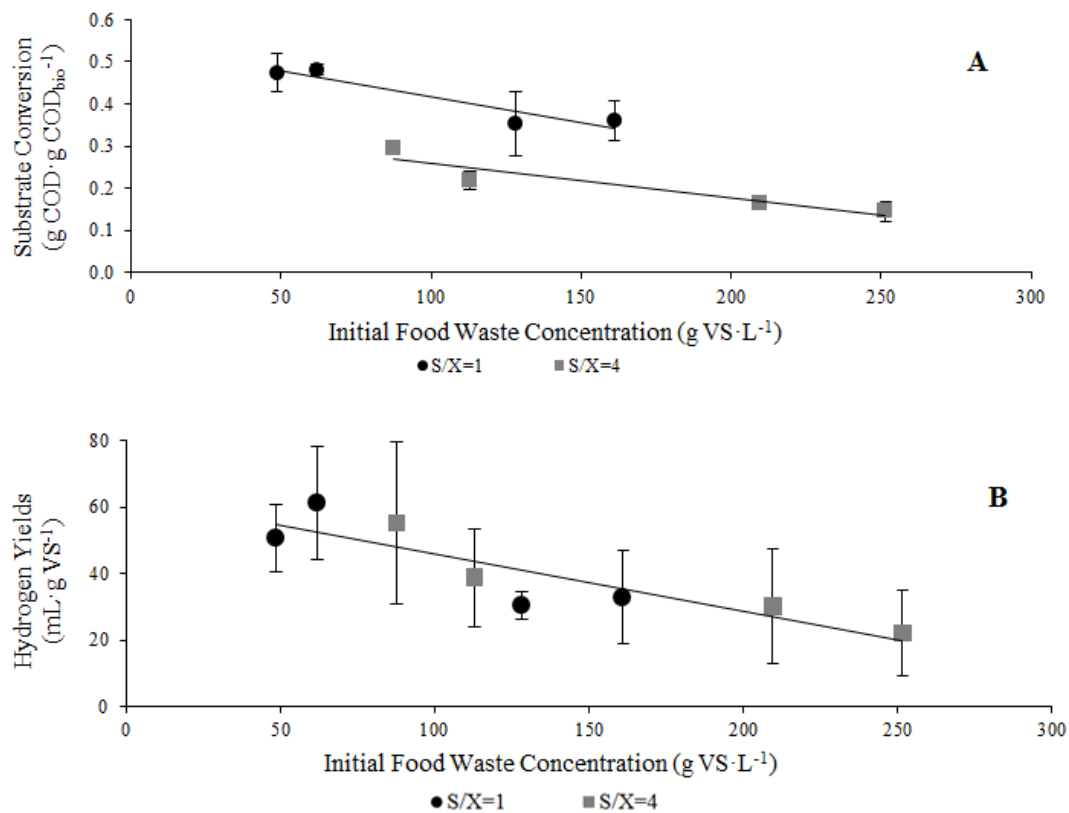
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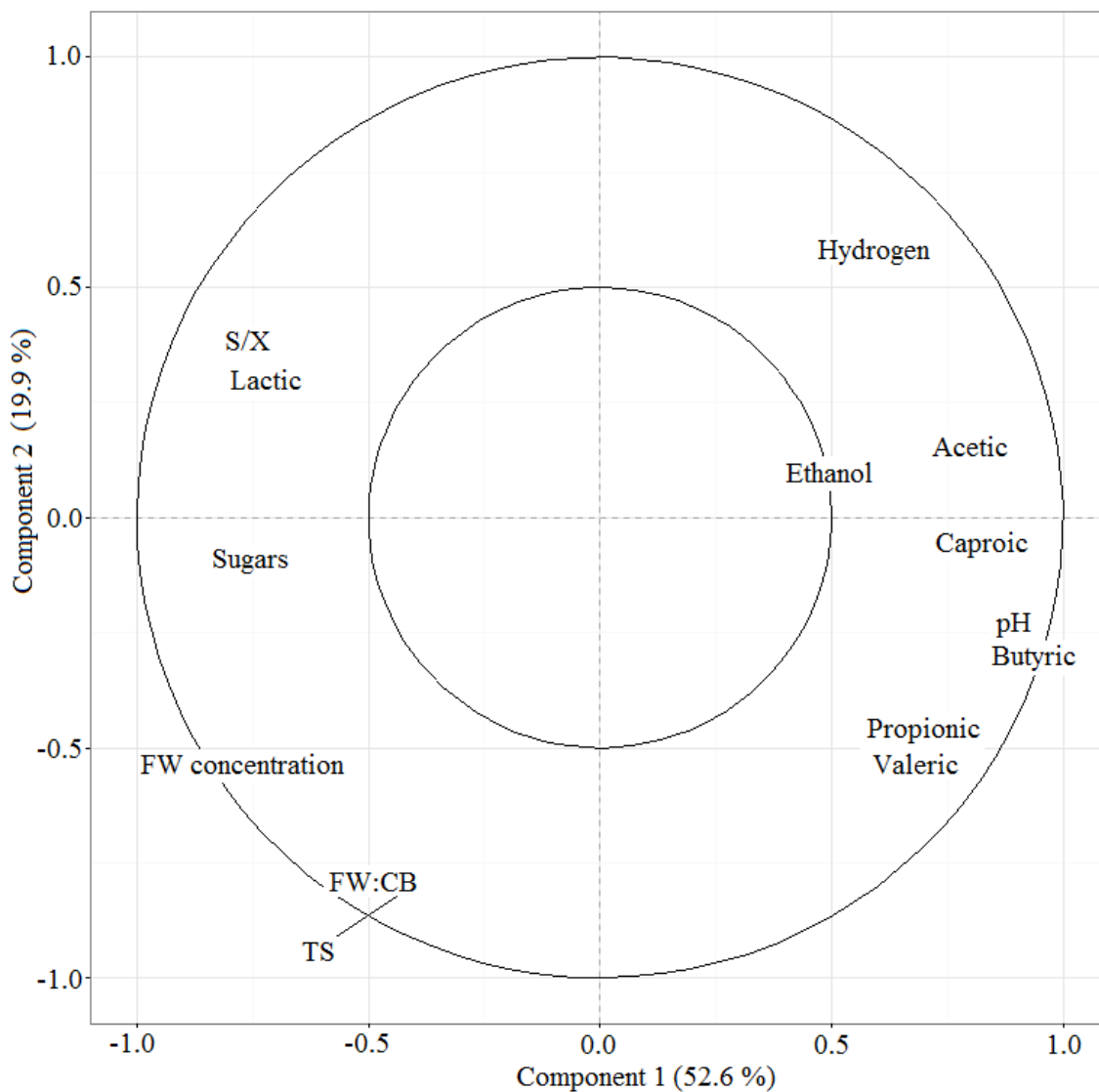


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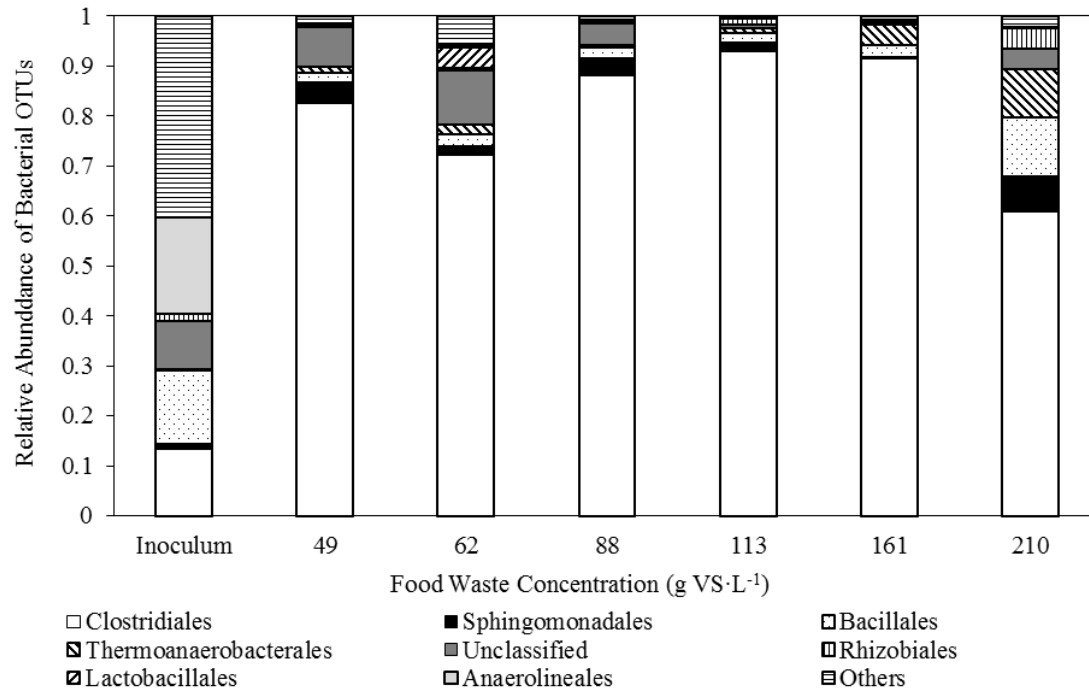


**Figure 4.** Influence of the initial FW concentration on the substrate conversion (A) and the hydrogen yields (B) in the reactors 6 to 13 (S/X of 1 and 4 g VS · g VS<sup>-1</sup>)



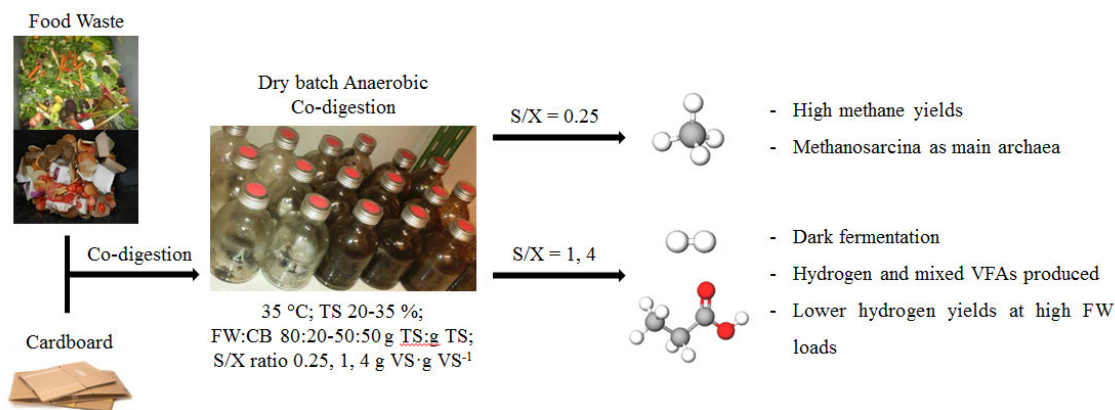


**Figure 5.** Correlation circle of the initial working parameters and the final yields of metabolites. It is based on the projection in plans formed by the two first principal components, accounting for 72.5 % of the variance



**Figure 6.** Relative abundance of Bacterial OTUs in the inoculum and in reactors with S/X of 1 and 4 g VS·g VS<sup>-1</sup> at the end of the batch experiments

## Graphical abstract



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**Highlights**

- The initial substrate to inoculum ratio is a critical parameter in dry anaerobic co-digestion
- Methanogenesis was favored at low substrate to inoculum ratios (0.25 g VS·g VS<sup>-1</sup>)
- *Methanosarcina* as main archaea after digestion
- Efficient acidogenic fermentation occurred at higher substrate to inoculum ratios
- Lower hydrogen yields at higher food waste loads

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