Role of indigenous bacteria in dark fermentation of organic substrates

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Abstract

Hydrogen production by dark fermentation of complex organic substrates, such as biowaste, can naturally take place with indigenous bacteria or by adding an external microbial inoculum issued from various natural environments. This study aims to determine whether indigenous bacteria associated with thermal pretreatment could impact dark fermentation performances. Biochemical hydrogen potential tests were carried out on seven organic substrates. Results showed a strong influence of the indigenous bacteria which are as effective as thermally pretreated exogenous bacteria to produce H_2 and metabolites. High abundance in *Clostridiales* and/or *Enterobacteriales* was associated with high H_2 yield. This study shows that no inoculum nor pretreatment are required to achieve satisfactory dark fermentation performances from organic waste.

Keywords

Biohydrogen, Dark fermentation, Thermal pretreatment, Inoculum, Indigenous bacteria

1. Introduction

Within the coming decades, fossil fuels will be completely depleted. Since their massive use is responsible for greenhouse gases emissions and climate change, renewable energies emerged as real sustainable solutions and a large number of new technologies are currently being developed to limit global warming (Hosseini and Wahid, 2016). Moreover, transportation is an energy-consuming sector and dihydrogen appears as a very promising candidate to meet all these needs. H_2 is an excellent energy carrier and a competitive biofuel since it can be considered as a clean gas with no carbon dioxide released during its combustion (Hosseini and Wahid, 2016). H_2 cannot only be produced from fossil fuels but also from biomass by thermo-chemical and biological technologies. Among them, dark fermentation (DF) appears as one of the most promising routes to produce hydrogen by a biological process. DF of complex organic matter, such as biowaste and effluents, can naturally take place with many sources of micro-organisms from various natural environments (Cabrol et al., 2017). The biowaste

are also rich in fermentative bacteria which are sufficient to perform dark fermentation (Parthiba Karthikeyan et al., 2018).

Although DF process operates with a wide array of organic matter, DF performances may significantly vary according to the substrate structure and its composition. Among the possible substrates, lignocellulosic biomass such as crop residues represents a huge potential to produce hydrogen, as recently reviewed (Bundhoo, 2018). Nonetheless, due to its complex structure and in particular to the lignin content, pretreatments have been extensively investigated to fully exploit the potential of these resources (Kumar et al., 2015). Other biowaste can be used such as food waste (FW) (Parthiba Karthikeyan et al., 2018) and organic fraction of municipal solid waste (OFMSW) (Bru et al., 2012). DF performances are nevertheless limited by several factors such as process stability, a low overall understanding of the process and the low substrate conversion rates into hydrogen (Bundhoo and Mohee, 2016). In order to improve the H_2 yield, substrate pretreatment may be required to release more soluble sugars. Monlau et al. (2012) showed a positive and unique correlation between the soluble sugar content of a complex substrate and the H_2 potential in dark fermentation. Thermal pretreatments were found to be effective for increasing the soluble sugar content and subsequently the H_2 yield, and more especially with food waste (Parthiba Karthikeyan et al., 2018). To optimize the process, an external microbial inoculum is frequently added. It can be pretreated to specifically select spore-forming hydrogen producing bacteria (HPB) and limit hydrogen consuming bacteria (HCB) (Parthiba Karthikeyan et al., 2018). As a result, the impact of substrate or inoculum pretreatment on H_2 yield has been widely studied (Rafieenia et al., 2018), but has more rarely been linked to the microbial community changes occurring in the DF process.

Indigenous bacteria, which are bacteria existing naturally on the substrate can be used to perform DF. Pretreatments have been widely investigated as they impact the substrate structure and the microbial composition of the inoculum. However, pretreatments may also cause changes in the initial indigenous bacteria composition, which could suppress some positive or negative interactions with exogenous bacteria, particularly by suppressing some non-spore-forming bacteria (HPB or HCB). Therefore, the hydrogen yield could be negatively or positively impacted by a thermal pretreatment of the indigenous bacteria. The impact of indigenous bacteria on DF performances and how they are influenced by the substrate pretreatment are not yet clearly understood. Only few studies have tackled this issue. Favaro et al. (2013) reported a higher hydrogen yield for unsterile OFMSW after inoculum addition compared to sterile OFMSW and suggested that a positive interaction between indigenous and exogenous bacteria was suppressed, which probably led to a less efficient hydrolysis. In contrast, Quéméneur et al. (2012) reported a lower H_2 yield for unsterile wheat

straw compared to sterile wheat straw in a two-stage system (hydrolysis of 24 h prior to DF). The authors attributed the difference to the production of other metabolites (acetate and lactate) than hydrogen. Andreani et al. (2019) mentioned a higher yield for a non-inoculated cassava wastewater in an AnSBBR reactor. As a consequence, indigenous bacteria could positively or negatively impact DF by interacting with exogenous bacteria. They could also be further selected by a pretreatment to enrich microbial communities in HPB and to avoid non-hydrogen producing pathways. Other authors reported studies with no strict requirement of external inoculation (for a pretreated substrate) such as Kim et al. (2009) with FW. These authors observed a low hydrogen yield for unpretreated FW (4 mL/gVS_{added}) compared to FW pretreated at 90 °C for 20 min which had a higher hydrogen yield (97 mL/gVS_{added}). Other authors also performed DF experiments with no external inoculation. Nevertheless, they did not investigate the impact of the substrate pretreatment on the indigenous bacteria and the changes in microbial communities. The main objective of this work is therefore to determine whether indigenous bacteria associated with a thermal pretreatment (90 °C - 15 min) could impact DF performances and microbial community composition, from a wide range of organic substrates.

2. Materials and methods

2.1. Inoculum preparation

For all substrates and the inoculum, total solid (TS) and volatile solid (VS) were performed in triplicates according to the APHA standard method (APHA, 1999). For inoculated BHP, the inoculum corresponded to an activated sludge originated from a wastewater treatment plant in Narbonne (France). After sampling, the inoculum was freeze-dried and stored in a closed box at room temperature. Before use, the inoculum was possibly pretreated by thermal pretreatment at 90 °C for 15 min to select spore-forming hydrogen producing bacteria (HPB) (Parthiba Karthikeyan et al., 2018) and to increase H_2 yield.

2.2. Substrate preparation

Biochemical Hydrogen Potential (BHP) tests were carried out on seven different organic substrates: Tunisian dates (pitted), corn silage, sorghum, microalgae (*Scenedesmus quadricauda* and *Pediastrum)*, sewage sludge (same origin as the inoculum but not freeze-dried and sampled at a different time), organic fraction of municipal solid waste (OFMSW) and food waste (FW). The last two substrates were freshly prepared according to the average waste composition found in France (Paillet, 2017). Both compositions are summed up in Table 1. Substrate characteristics and storage conditions are detailed in Table 2. All substrates (excepted microalgae and sewage sludge) were shredded with a Moulinex FP5131.10 Masterchef 5000 shredder in order to obtain a homogenous

mixture. For FW, the food was frozen prior to shredding. For OFMSW, the paper and cardboard fractions were only shredded with a BLIK BB230 shredder (pieces about 1 cm long). The microalgae were cultivated continuously, outside the laboratory, in a raceway outdoor pond of 16 m^3 (hydraulic retention time of 5 days) with synthetic wastewater prepared according to Nopens et al. (2001). The depth and the total surface area of the raceway were 0.3 m and 56 m^2, respectively. The raceway was equipped with a paddle wheel with a rotation speed (linear velocity of 0.2 m^2/s) and a recycling pump (7.6 m^3/h). Incident light at the raceway surface was measured with a PAR probe (PAR 2625 SKYE). The average of the max PAR values measured was about 1700 $\mu mol.m^{-2}.s^{-1}$. Due to their structure, three groups of substrates were distinguished: lignocellulosic substrates (corn silage, sorghum, OFMSW), sugar-rich substrates (FW and dates) and protein-rich substrates (microalgae and sludge).

2.3. BHP tests and gas composition analysis

Four different conditions were evaluated in batch tests. (1) No inoculum and no pretreatment of the substrate (NI-NP). (2) No inoculum and a substrate pretreated at 90 °C for 15 min (NI-P). Temperature and time corresponded to the main conditions used for inoculum pretreatment in the literature (Parthiba Karthikeyan et al., 2018; Rafieenia et al., 2018). (3) Inoculum addition and no pretreatment (I-NP), and eventually (4) the inoculum was added and pretreated simultaneously with the substrate (I-P).

BHP tests were carried out in a 550 mL glass bottle with a 350 mL headspace. These tests were conducted in quadruplicates, in batch tests, under mesophilic conditions (37 °C) and with no stirring. The substrate was added into a medium composed of 1 mL of trace element solution whose composition is given by Chatellard et al. (2016), a buffer (100 mM of 2-(N-morpholino)ethanesulfonic acid [MES]) and macronutrients (NH_4Cl at 0.8 g/L and K_2HPO_4 at 0.5 g/L in the BHP flask). For the experiments with inoculum addition, the substrate to inoculum ratio (S/X) was set to 22 ± 1. Then, the pH was adjusted to 6 with HCl or NaOH (if needed). Thereafter, glass bottles were sealed with a rubber stopper and locked with an aluminum screw. In order to reach anaerobic conditions, the headspace was purged with nitrogen gas. BHP experiments were stopped when H_2 production remained constant (constant pressure) to avoid its further consumption by homoacetogenesis reaction.

Gas production was monitored every 2 hours with an automatic micro-gas chromatograph (SRA l-GC R3000) equipped with two columns: a Molesieve 5A 10m column running at 80 °C, 30 PSI with argon as carrier gas (channel A) and a PoraPlot U (PPU) 8m column running at 70 °C, 20 PSI with helium as carrier gas (channel B), for H_2, O_2, N_2, CH_4 and CO_2 analyses, respectively. Both channels

were equipped with a micro-thermal conductivity detector (TCD) set at 90 °C. Gas production was estimated by pressure measurement.

2.4. Metabolite analysis

Volatile fatty acids [VFA], i.e. acetate (C2), propionate (C3), butyrate (C4), iso-butyrate (iC4), valerate (C5), iso-valerate (iC5), caproate (C6) and iso-caproate (iC6) were measured by Gas Chromatography (GC). The Perkin Elmer Clarus 580 GC was equipped with an Alltech-FFAP EC^{TM} 1000 column coupled to a flame ionization detector (FID) set at 280 °C. N_2 was used as carrier gas with a flow of 6 mL/min. Other metabolites (ethanol, succinate, lactate, formate) and residual sugars (glucose, xylose, fructose, arabinose) were analyzed after fermentation by a High Performance Liquid Chromatography (HPLC). Prior to use, samples were centrifuged at 13,000 g for 15 min and filtrated at 0.2 µm. The HPLC was equipped with a protective precolumn (Microguard cation H refill catbridges, Biorad), an HPX-87H column (300 × 7.8 mm, Biorad) running at 35 °C, with a 0.004 M H_2SO_4 solution as eluent (0.3 mL.min^{-1}), and a refractive index detector (Waters R410) running at 45 °C.

2.5. Microbiological analysis

For each quadruplicate, microbial communities of only one BHP bottle (the closest value to the average hydrogen production) were analyzed before (T0) and after fermentation (Tf). Only one replicate among the quadruplicate was measured in order to limit the number of analyses. Firstly, the fermentation medium was incorporated into a 2 mL Eppendorf tube and then centrifuged at 13,000 g for 15 min (in duplicate). The pellets were stored at -20 °C until further use. DNA extraction was performed with a PowerSoil™ DNA isolation sample kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA amount and purity were then measured by spectrophotometry (Infinite NanoQuant M200, Tecan). Extracted DNA was afterward stored at -20 °C before use. The V3 region of the 16S rRNA genes was amplified using universal primers for bacteria as mentioned by Carmona-Martínez et al. (2015). The PCR products were purified and sequenced by Illumina MiSeq at the Genotoul life science network in Toulouse, France (get.genotoul.fr). All sequences were obtained after applying a bioinformatics procedure as described by Carmona-Martínez et al. (2015). Sequences were grouped into operational taxonomic units (OTUs) with 97% similarity. Sequences were submitted to GenBank, with accession No. KX874727 to No. KX878885.

2.6. Statistical analysis

All statistical tests were carried out using R software (version 3.5.1). In order to determine whether there was a statistical difference in DF performances between several BHP for a same substrate. An ANOVA was first carried out using

the Anova function of the car R package. If a significant p-value (<0.05) was found, a Tukey test was then carried out using the glht function from multcomp package. Matrix of Pearson's correlations were obtained with the corrplot package by taking into account the results for all substrates excepted microalgae and sludge due to their different structure and DF performances. For the matrix of Pearson's correlations, only one replicate among each quadruplicate was considered as for microbiological analysis. Moreover, only correlations with a significant p-value ($p<0.05$) were displayed.

3. Results and discussion

3.1. General results

Average DF performances (H_2 yields and total metabolites) are summed up in Table 3 for all substrates and experiments (with or without inoculum / with or without thermal pretreatment) i.e. "I-NP"," I-P","NI-P" and "NI-NP" BHP tests. Hydrogen yield (in mmol/gVS_{ini}) is also presented and compared to a calculated yield. This yield was calculated as the double of the sum of acetate and butyrate concentrations according to acetate and butyrate pathways (for one mole of hexose fermented) (Guo et al., 2010). The substrate performances were classified into 3 groups: (1) the lignocellulosic substrates with corn silage, sorghum and OFMSW (intermediate DF performances), (2) the sugar-rich group with dates and FW (high DF performances), (3) the "protein-rich substrates with microalgae and sludge (low DF performances). For the lignocellulosic group, H_2 yields and the total amount of metabolites (for a same substrate) were similar between all conditions ("I-NP"," I-P","NI-P" and "NI-NP") as indicated by the Tukey tests. For the sugar-rich group, the hydrogen productions of "I-NP" experiments were significantly lower compared to the 3 other conditions. However, the total metabolite amounts were not significantly different. Moreover, the calculated H_2 yield was lower than the experimental yield (as for corn silage). For the protein-rich group, DF performances were very low.

3.2. Influence of substrates on dark fermentation performances

The average H_2 yields for commonly used operating conditions, i.e. inoculated and pretreated experiments ("I-P"), are summed up in Table 3. Almost no hydrogen was produced from the protein-rich substrates. This production is not consistent with Carrillo-Reyes and Buitrón (2016) who mentioned a higher hydrogen yield of 15 mL H_2/gVS_{ini} for untreated microalgae, *Scenedesmus* being also the main species. This difference might be explained by the inoculum impact and by the different culture conditions of the microalgae, which may have significantly impacted their sugar content. The lignocellulosic substrates showed intermediate performances, excepted for corn silage which is a more

fermentable substrate, with H_2 yields of 129 ± 9 mLH_2/gVS_{ini} for corn silage, 47 ± 10 mLH_2/gVS_{ini} for sorghum and 45 ± 12 mLH_2/gVS_{ini} for OFMSW. Consistently, Bru et al. (2012) reported a similar H_2 yield of 57 mLH_2/gVS_{ini} for raw OFMSW. In contrast, Nkemka et al. (2015) observed a significantly lower H_2 yield for corn silage with a production of 56 mLH_2/gVS_{ini}. This difference could be attributed to the inoculum used (granular sludge), the corn variety, the silage method and the process (leach bed reactor connected to a USAB reactor). The sugar-rich substrates showed the highest DF performances due to their high soluble sugar content with hydrogen yields of 306 ± 14 mLH_2/gVS_{ini} and 169 ± 22 mLH_2/gVS_{ini} for dates and FW, respectively. Parthiba Karthikeyan et al. (2018) reported a significantly lower H_2 yield for FW compared to the present study with a hydrogen production of 76 mLH_2/gVS_{ini} and 97 mLH_2/gVS_{ini} for FW pretreated at 90 °C for 30 min and 20 min, respectively. Such difference can be explained by the FW origin and composition. For the dates, Abd-Alla et al. (2011) reported a hydrogen production up to 399 mLH_2/gVS_{ini} with different fermenting conditions (30 °C - stirring – *E. coli* as inoculum).

Fig 1 shows the distribution of metabolites after fermentation for inoculated and pretreated experiments “I-P”, as usual conditions used in the literature. Standard deviations are also given for the main metabolites. Overall, H_2, butyrate, acetate, and ethanol represented the main metabolites for lignocellulosic and sugar-rich substrates with proportions (% of the total COD at Tf) between 13% to 25% for H_2, 41% to 54% for butyrate, 11% to 24% for acetate and 3% to 27% for ethanol (“I-P” experiments). Metabolite concentrations may vary significantly from one substrate to another as shown in Fig 1. For the protein-rich group, acetate, butyrate and propionate were the main metabolites with proportions (% of the total COD at Tf) ranging between 65% to 69% for acetate, 7% to 12% for propionate and of 23% for butyrate (“I-P” experiments). These differences in the protein-rich group might be explained by the very low content in readily accessible carbohydrates, which are highly correlated with H_2 production as reported by Guo et al. (2014). Moreover, the high concentration in acetate might be explained by homoacetogenesis. The low amount of hydrogen produced was very probably consumed to produce acetate by homoacetogenic bacteria as *Clostridium sp.*, which are also HPB (Saady, 2013). For the lignocellulosic and sugar-rich groups, high amounts of ethanol were detected (excepted for OFMSW). Ethanol might be produced through an ethanol-acetate pathway but also by a zero hydrogen producing pathway (Li and Fang, 2007). Other metabolites as propionate or succinate from non-H_2 producing pathways were also detected but in very low amounts (less than 3% of the total COD at Tf). This is consistent with Guo et al. (2014). Indeed, the authors reported low propionate amounts for various solid organic waste.

Fig 2 presents the Pearson correlations of the main metabolites and the main microbial communities at the order level (*Clostridiale*s, *Enterobacteriales*, *Lactobacillales* and *Bacillales*). According to Fig 2, hydrogen is strongly and positively correlated with butyrate and metabolites, slightly and positively correlated with *Clostridiales*, slightly and negatively correlated with *Enterobacteriales*. *Lactobacillale*s is strongly and positively correlated with lactate. However, no correlation was found between H_2 and ethanol, which confirms that ethanol was partially produced by the ethanol pathway. The negative correlation between acetate and ethanol can be explained by the various pathways that produced or consumed acetate. Therefore, this correlation between ethanol and acetate may have been biased by the multiple pathways. Moreover, no correlation between acetate and H_2 was found as acetate can be produced through the acetate or acetate-ethanol pathways, produced by homoacetogenesis (hydrogen consumption) or consumed with lactate to produce H_2 and butyrate (Hashsham et al., 2000). The correlation between hydrogen and butyrate confirms that hydrogen was mainly produced through the butyrate pathway or by lactate consumption. The correlation between hydrogen and butyrate was previously reported by Guo et al. (2014) for solid organic waste. The non-correlation between acetate and hydrogen is also consistent with Guo et al. (2014) who reported that acetate is a wrong indicator for H_2 production.

3.3. Dark fermentation performances and microbial communities

Fig 3 indicates the initial and final microbial community compositions according to the type of substrate (for "I-P" experiments). For all substrates excepted sorghum and OFMSW, *Clostridiales* was the most abundant order. Surprisingly, *Clostridiales* abundance was high in microalgae and sludge-fed reactors, but the H_2 yield was close to zero. As almost no hydrogen was detected and a significant amount of acetate was produced, this result suggests a strong homoacetogenic activity of *Clostridium sp*., as consistently reported by Saady (2013). The relative abundance of *Clostridiales* alone did not explain the differences in H_2 yields between the substrates, as this abundance did not correlate with the most fermentable substrate (dates). It is noteworthy to mention that *Enterobacteriales* was the most abundant order for sorghum and OFMSW (for "I-P" experiments). According to Palomo-Briones et al. (2017), *Enterobacteriales* are HPB but expressing a lower H_2 yield (2 mol H_2 /mol hexose) than *Clostridiales.* The authors reported a positive correlation between *Enterobacteriales* abundance, ethanol and formate production, suggesting that the ethanol-acetate or formate pathways were preferentially used by the *Enterobacteriales* in dark fermentation. In their exhaustive review, Cabrol et al. (2017) suggested that formate could also be further consumed to produce H_2 and CO_2 thanks to a formate hydrogen lyase. This result suggests that formate was firstly produced

and then consumed to produce hydrogen as no formate accumulation was observed in Fig 1.

Maximum calculated H_2 yields (from acetate and butyrate productions) are presented in Table 3. Surprisingly, for some experiments as "Corn silage I-P" and "Date-I-P", the maximum H_2 production was higher than the maximum calculated H_2 yield. This result can be explained by *Lactobacillales* proportions as presented in Fig 4. This figure indicates the cumulative percentage of operational taxonomic units (OTUs) affiliated with *Lactobacillales* order, at T0 and at Tf, for the lignocellulosic group and the sugar-rich group. The OTUs affiliated with *Lactobacillales* order at T0 were different at Tf. As a consequence, bacteria from *Lactobacillales* order have grown and lactate was produced during the fermentation process. Indeed, this order is associated with the lactic acid fermentation as suggested by Hashsham et al. (2000) and lactate can be further consumed to produce H_2 by other species. However, according to Fig 1, no lactate was detected at Tf excepted for "Date-I-NP" experiments. As a consequence, lactate was consumed by lactate consumer species to produce hydrogen and butyrate according to Eq. (1) and Eq. (2) as reported by Hashsham et al. (2000). In a same manner, García-Depraect and León-Becerril (2018) reported that with tequila vinasse as substrate, lactic acid bacteria (LAB) first consumed the substrate to produce lactate, which was further consumed by species as *Clostridium beijerinckii* to produce hydrogen and butyrate.

$$2\ \text{Lactate} + H^+ \rightarrow \text{Butyrate} + 2\ H_2 + 2\ CO_2 \quad \text{Eq. (1)}$$

$$\text{Lactate} + \text{Acetate} + H^+ \rightarrow \text{Butyrate} + 0.8\ H_2 + 1.4\ CO_2 + 0.6\ H_20 \quad \text{Eq. (2)}$$

If reaction of Eq. (1) occurs, the number of moles of hydrogen will be twice higher than the number of moles of butyrate, as for the butyrate pathway. As a consequence, H_2 calculated yield will not change. However, if reaction of Eq. (2) occurs, hydrogen will be produced but the total amount of acetate and butyrate will remain constant. Therefore, Eq. (2) can explain an experimental hydrogen yield greater than the calculated yield (from acetate and butyrate productions). In addition, hydrogen production from the formate pathway can also explain this result as no butyrate and acetate are produced.

3.4. Impact of thermal pretreatments on hydrogen-producing bacteria selection

3.4.1 Thermal pretreatments increase hydrogen production but not the overall conversion of the substrate

In Table 3 are reported DF performances, i.e. hydrogen and total metabolite productions for pretreated and unpretreated substrates, after inoculum addition but also maximum calculated hydrogen yield discussed in the previous section.

For the protein-rich group, no significant difference in maximum H_2 yield was observed between pretreated and unpretreated BHP. This result can be explained by the low soluble sugar content as mentioned in the previous section. Moreover, thermal pretreatment of the inoculum and substrate impacted significantly the overall metabolite production (Table 3). This difference can be attributed to a reduction of the microbial diversity and a *Clostridiales* enrichment at Tf due to the initial inoculum pretreatment (Fig 3). Due to their rich composition in proteins: propionate, acetate and butyrate were the main metabolites (Fig 1).

For the lignocellulosic group (excepted OFMSW), the non-significant difference in maximum H_2 yield is probably due to the lignocellulosic structure of the substrates which might have selected the same microbial communities for both inoculated experiments ("I-P" and "I-NP") as reported by Chatellard et al. (2016) for carbohydrates. This hypothesis is supported by the *Clostridiales* proportions, which were quite similar for both corn silage experiments and both sorghum experiments (see Fig.3). *Clostridiales* are efficient H_2 producers that could explain the non-significant difference in maximum H_2 yield for the lignocellulosic group (Palomo-Briones et al., 2017). Moreover, for OFMSW, H_2 yields were significantly different between both inoculated experiments contrary to other substrates of the lignocellulosic group (Table 3). This difference can be justified by Fig 3 with a high percentage of *Clostridiales (44%)* and *Enterobacteriales (54%)* for "I-P" (no data for "I-NP" experiment). Moreover, excepted for corn silage, the non-significant difference in maximum total metabolites was found between both inoculated experiments of the lignocellulosic group. This result might be explained by a similar overall biodegradability of the substrates in both cases even if different microbial communities were selected. Indeed, *Enterobacteriales* percentage is much higher in pretreated experiments for lignocellulosic substrates (excepted for corn silage) (Fig 3). However, the difference in metabolite production between corn silage inoculated experiments was attributed to a decrease in microbial diversity after a thermal pretreatment, and more particularly, in the initial (T0) *Clostridiales* diversity as consistently reported elsewhere (Chatellard, 2016). Initial and final Simpson indexes decreased respectively from 0.96 to 0.82 and from 0.58 to 0.14 for "I-P" experiments compared to "I-NP" experiments.

For the sugar-rich group, inoculation without pretreatment was very detrimental to hydrogen production as reported in Table 3. Indeed, those substrates are rich in sugars and without pretreatment, the initial microbial diversity was highly diverse. As a consequence, some non-hydrogen-producing bacteria as *Lactobacillales* were able to growth due to a high availability in sugars. According to Fig 3, *Lactobacillales* proportion was very high for "FW-I-NP" and "Dates-I-NP" experiments (compared to pretreated experiments) with percentages of 40% and

93%, respectively. This lower H_2 yield might also be due to lactate and formate pathways which are less efficient in hydrogen production than the acetate pathway as explained in the previous section. Fig 1 confirms that some bacteria used metabolic pathways that do not produce hydrogen. Indeed, for FW, metabolites from non-hydrogen producing pathways as butanol and caproate were only measured for "FW-I-NP" experiments. For "Dates-I-NP" experiments, a very high concentration in lactate (5.57 ± 0.29 mmol/gVS_{ini}) was measured, which is consistent with a high *Lactobacillales* relative abundance and a low hydrogen production. Indeed, when lactate is produced, the substrate availability to produce hydrogen decreases as reported by Dessì et al. (2018). Moreover, there was only a significant statistical difference in total metabolites for dates. For FW, thermal pretreatment did not increase overall biodegradability of the substrate. For "Dates-I-NP" experiments, the lower total metabolite amount (compared to "Dates-I-P") is due to the high lactate concentration and the highly final acidic pH (<4). As a consequence, residual fructose was detected but not taken into account in the total metabolites. In general, the impact of thermal pretreatment on total metabolite production for inoculated experiments was very limited due to a similar overall conversion of the substrates in both experiments (excepted for protein-rich substrates). Nevertheless, hydrogen production was mainly impacted by the thermal pretreatment for sugar-rich substrates due to a high microbial diversity and the presence of *Lactobacillales* in significant proportion for inoculated experiments.

3.4.2 Thermal pretreatments impact microbial community selection but are inefficient to increase H_2 yield

In addition, other experiments were carried out on uninoculated substrates ("NI-NP" and "NI-P") to evaluate the impact of pretreatment on indigenous bacteria (Table 3 and Fig 3). For lignocellulosic and sugar-rich substrates, H_2 production did not statistically differ. Such similar H_2 productions for both uninoculated experiments show that the thermal pretreatment performed at 90 °C for 15 min was ineffective to increase the H_2 yield. This ineffectiveness could be attributed to the complex structure of most of the substrates. Indeed, 90 °C is probably a too low temperature and 15 min a too short duration to break down lignocellulosic material of organic substrates in a significant way in order to release more soluble sugars. Monlau et al. (2012) showed that hydrogen production was strongly and uniquely correlated with the content in soluble sugars. Nonetheless, Elbeshbishy et al. (2011) showed an increase in H_2 production of uninoculated FW after a thermal pretreatment carried out at 70 °C for 30 min (70 mL/gVS_{ini} compared to 40 mL/gVS_{ini} for untreated FW). Such difference can probably result from the FW origin, composition and nature as suggested by Parthiba Karthikeyan et al. (2018). Wei et al. (2014) also observed

an increase in H_2 yield for kitchen waste pretreated at 90 °C for 30 min (76 mL/gVS_{ini} compared to 35 mL/gVS_{ini} for untreated kitchen waste). The inefficiency of thermal pretreatments for uninoculated experiments was also attributed to a decrease in non-spore-forming HPB or hydrolytic bacteria, as suggested elsewhere (Kraemer and Bagley, 2007), but also to a decrease in diversity which could suppress some positive interactions between microbial communities that promote hydrogen production (Chatellard, 2016).

Nevertheless, and according to Fig 3, thermal pretreatments favored at T0 the emergence of HPB in the uninoculated experiments. For the experiments "Corn silage-NI-NP" and "Corn silage-NI-P", the *Clostridiales* proportion increased from 26% to 58% and *Lactobacillales* percentage decreased from 35% to 0%. Similar results were observed for other substrates among the lignocellulosic and sugar-rich groups. Despite different initial microbial compositions for uninoculated experiments, similar bacterial proportions were observed at Tf as indicated in Fig 3 for all substrates of the lignocellulosic and sugar-rich groups (excepted for dates). Indeed, for "OFMSW NI-NP" and "OFMSW NI-P", *Enterobacteriales* and *Clostridiales* relative abundances were 43% - 55% and 39% - 56% respectively. This could be explained by the selection of bacteria through the operating conditions and also resulted from the substrate structure (Chatellard et al., 2016). The storage method may also be responsible for a strong bacterial selection (silage for corn and freezing for sorghum). Interestingly, for uninoculated experiments at Tf, *Enterobacteriales* were almost exclusively composed of bacteria affiliated to *Escherichia-Shigella* at the genus level (data not shown), excepted for sorghum and "Dates NI-P" experiments. Paillet et al. (2019) associated the presence of *Escherichia-Shigella* to H_2 production through the formate and ethanol pathways. As no formate was detected at the end of the experiments, this supports the assumption of formate production followed by its degradation to produce hydrogen.

Moreover, as shown in Fig 1, ethanol production can partially be explained by *Enterobacteriales* as for uninoculated FW experiments. Indeed, a substantial amount of ethanol was detected compared to "FW-I-P" BHP tests, where only *Clostridiales* were detected at Tf. For uninoculated experiments with dates, Fig 3 shows a real impact of thermal pretreatment on final microbial composition with a significant decrease in *Enterobacteriales*, *Lactobacillales* percentages and a huge increase in *Clostridiales* proportion with a shift from 29% to 82% for pretreated dates. However, H_2 yields were not statistically different for those experiments. This indicates that *Enterobacteriales* play a key role in hydrogen production of complex substrates. This result also implies that an enrichment in *Clostridiales* percentage is not always correlated to an increase in H_2 yield. This result is not consistent with Kim et al. (2014) who reported an increase in hydrogen yield with an increase in *Clostridiales* percentage. This result might also

be explained by a decrease in OTUs diversity and in synergetic effects between bacteria after the thermal pretreatment (Chatellard, 2016). To conclude, thermal pretreatments were efficient to increase H_2 yields when an inoculum was added for sugar-rich substrates due to a bacterial selection of HPB of the inoculum. However, thermal pretreatments were inefficient on the lignocellulosic group probably due to a stronger bacterial selection by the lignocellulosic structure. When no inoculum was added, the thermal pretreatment (90 °C - 15 min) was inefficient to break down the substrate, to increase the H_2 yield and the overall amount of metabolites, but it had only an effect on microbial communities. In uninoculated experiments, thermal pretreatment was indeed efficient to enrich in HPB at T0 with similar microbial communities at Tf, probably due to a bacterial selection by operating conditions or substrate structure.

3.5. Equal DF performances of indigenous bacteria compared to external microbial inoculum

To better understand the roles of the indigenous bacteria and the external microbial inoculum, BHP tests were carried out with pretreatment and with or without inoculum (NI-P and I-P). The average DF performances of these experiments are summed up in Table 3. For all substrates of the lignocellulosic and sugar-rich groups, no significant difference was observed in maximum H_2 yield, excepted for dates with a lower hydrogen yield with no inoculum addition. This observation shows the low influence of external inoculum, suggesting a high adaptation of the indigenous bacteria to the substrate, already reported by Turhal et al. (2019). For dates, the improvement of H_2 production with inoculum addition is consistent with Turhal et al. (2019). The authors also used thermally pretreated sugar-rich substrates such as melon and watermelon waste and reported a better H_2 yield for experiments with a pretreated inoculum (142 mLH_2/gVS_{ini}) compared to uninoculated experiments (114 mLH_2/gVS_{ini}). As shown in Fig 3, the final microbial communities of “Dates NI-P” and “Dates I-P” experiments were very similar with a *Clostridiales* proportion of 82% and 83%, and a *Lactobacillales* abundance of 16% and 14%, respectively. For “Dates NI-P” experiments, *Clostridiales* diversity at Tf was very low with a Simpson index of 0 (only one OTU belonging to *Clostridiales*) compared to “Dates I-P” experiments with a Simpson index of 0.51 for *Clostridiales* (data not shown). As a consequence, the difference in H_2 yield for pretreated dates was not related to the microbial community composition but to the *Clostridiales* diversity.

For the lignocellulosic group, the non-significant difference observed in maximum H_2 yield is not consistent with Favaro et al. (2013) who reported a lower H_2 yield for untreated indigenous bacteria (42 mLH_2/gVS) compared to experiments carried out with a pretreated inoculum (70 mLH_2/gVS) with OFMSW as substrate. The authors also noticed a positive interaction between indigenous

bacteria and exogenous bacteria as the H_2 production for sterilized OFMSW with inoculum addition was lower (57 mLH_2/gVS) compared to unsterilized OFMSW (70 mLH_2/gVS). This underlines the key role of indigenous bacteria in hydrogen production as suggested by the present study regarding the lignocellulosic group. The low impact of inoculation on hydrogen yield for the lignocellulosic and sugar-rich groups was also noticed by comparing "NI-NP" and "I-P" experiments as there was also no significant difference in H_2 production for all substrates. In Table 3, no significant difference in total metabolite concentration was shown between both pretreated experiments (for a same substrate). For the sugar-rich group, this result is not consistent with Turhal et al. (2019) who reported a twice higher concentration in VFA for inoculated experiments (melon and water melon as substrate). Fig 1 confirms this result as concentrations in main metabolites were similar. This also suggests that the same metabolic pathways were used between pretreated experiments for a same substrate.

Microbial composition at T0 and Tf are given in Fig 3 for inoculated and pretreated experiments (I-P) and for uninoculated and pretreated experiments (NI-P). In some cases, similar microbial compositions explain the similar DF performances between both experiments for a same substrate. For others, a decrease in *Enterobacteriales* population associated with an increase in *Clostridiales* proportions was not detrimental to hydrogen production which confirms the key role of *Enterobacteriales*, especially without inoculation. For lignocellulosic substrates (excepted corn silage), final microbial communities were very similar with *Clostridiales* and *Enterobacteriales* proportions as main orders (>95% relative abundance for OFMSW). However, at Tf, *Enterobacteriales* abundance was only 15% higher for "OFMSW I-P" experiments compared to "OFMSW NI-P" experiments, hence the similar H_2 yields for the lignocellulosic group. The equal DF performance of sugar-rich substrates is a surprising result for FW as microbial compositions at Tf differ from both pretreated experiments (with a same substrate). Interestingly, *Clostridiales* abundance was higher for inoculated and pretreated experiments (I-P) for corn silage and FW at T0 and at Tf, with only *Clostridiales* at Tf for "FW I-P" experiments. For FW, indigenous bacteria at T0 were almost only consisted of *Lactobacillales*. Despite a higher proportion of *Clostridiales* for "I-P" experiments, H_2 yields were not statistically different from "NI-P" BHP tests for corn silage and FW. Surprisingly, for corn silage, no *Enterobacteriales* were detected after an inoculum addition. In some case, inoculation can be detrimental to the *Enterobacteriales* population. This result is consistent with Marone et al. (2019) experiments on solid-state fermentation of raw corn stover. Indeed, the authors noticed a significant decrease of *Enterobacteriales* abundance from 35.6% to 10.2% at Tf for inoculated experiments. As a consequence, an enrichment in *Clostridiales* abundance associated with a decrease in *Enterobacteriales* population led to similar DF performances.

According to Fig 2, there is a positive Pearson's correlation between butyrate and H_2 yield, with a significant p-value (below 0.05). Nonetheless, butyrate and hydrogen could have been produced through the butyrate pathway or by lactate consumption as described in a previous section with Eq. 1 and Eq. 2, which have similar yields as the formate or acetate-ethanol pathways (used by *Enterobacteriales*) as described by Cabrol et al. (2017). As a consequence, a shift from *Enterobacteriales* order to *Clostridiale*s order (using mainly butyrate pathway) explains the non-correlation between H_2 yield and *Clostridiales* abundance at Tf for a single substrate. Indeed, the increase in *Clostridiales* percentage is generally associated with a decrease in *Enterobacteriales* percentage and both orders have used pathways with similar hydrogen yield (mainly butyrate pathway). This suggests that *Enterobacteriales* and *Clostridiale*s are playing a key role to obtain a high hydrogen yield with complex organic matter.

Nevertheless, Fig 2 also points out a positive Pearson's correlation between *Clostridiales* relative abundance and H_2 yield with a significant p-value. This result is consistent with Kim et al. (2014) who noticed an increase in *Clostridiales* proportion of the fermentative broth and in H_2 yield (98.1%; 152 mLH_2/gVS_{added}) after an acid pretreatment at pH = 1 of FW compared to untreated FW (9.1%; 54 mLH_2/gVS_{added}). Etchebehere et al. (2016) also reported a direct link between *Clostridium sp*. proportion and a high H_2 yield (soluble sugars and biowaste as substrate). However, this correlation is no longer so clear when an increase in *Clostridiales* percentage is associated with a decrease in *Enterobacteriales* as mentioned in the previous paragraph. For instance, for corn silage, *Clostridiales* proportions at Tf were higher for inoculated experiments but H_2 yield was similar to uninoculated experiments.

Fig 2 shows a negative correlation between *Enterobacteriales* and hydrogen production. This statistical result can be explained by the substrate influence on H_2 yield. Low proportions of *Enterobacteriales* for most fermentable substrates as dates were detected and only one replicate among the quadruplicate was considered. This statistical analysis may have been biased for *Enterobacteriales*. Indeed, this finding is not consistent with Palomo-Briones et al. (2017) who reported *Enterobacteriales* as HPB. Interestingly, Fig 3 shows that for all complex substrates, even after a thermal pretreatment of indigenous bacteria, *Enterobacteriales* were detected in a significant amount at Tf excepted for "Dates NI-P" experiments. This suggests that *Enterobacteriales* were selected among indigenous bacteria during the DF process. This study confirms that *Enterobacteriales* are playing an important role in hydrogen production of complex substrates and especially when no inoculum is added as a decrease in *Enterobacteriales* proportions associated with an increase in *Clostridiales* proportions led to similar H_2 yields. This section also shows that the addition of a

pretreated inoculum has a low impact on the DF performances of complex organic substrates due to the high performances of indigenous bacteria.

4 Conclusions

This study showed that H_2 production by dark fermentation with indigenous bacteria from varied organic substrates can be achieved with satisfactory performances. In most cases, hydrogen yields were not significantly different when a pretreated inoculum was added. Thermal pretreatments performed at 90 °C for 15 min were inefficient to increase H_2 yields but efficient to select HPB among indigenous and exogenous bacteria before DF. Nevertheless, further investigations are needed to better understand the impacts of pretreatments on indigenous bacteria selection which could be detrimental to DF performances.

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Figure captions

Fig. 1 Main metabolite concentrations after dark fermentation (Tf) of the seven organic substrates (average of 4 samples for each experiment) with inoculum or not and with a thermal pretreatment (90 °C - 15 min) or not as indicated by the following abbreviation: NI = No Inoculum / I = Inoculum / P = Pretreatment / NP = No Pretreatment. Standard deviations are only given for hydrogen, acetate, butyrate and ethanol.

Fig. 2 Matrix of Pearson's correlations with the main microbial communities for all substrates (excepted microalgae and sludge) and for all experiments (with or without pretreatment and with or without inoculum). One replicate among each quadruplicate was considered. Only correlations with a significant p-value ($p<0.05$) are displayed.

Fig. 3 Microbial communities at the order level for all substrates and for all experiments (with or without pretreatment and with or without inoculum) (relative abundance > 3%) at T0 (a) and after dark fermentation (Tf) (b). Percentages are indicated for Clostridiales, Enterobacteriales, Lactobacillales and Bacillales. Percentages have been rounded off to the nearest whole number. One replicate among the quadruplicate was measured. NI = No Inoculum / I = Inoculum / P = Pretreatment / NP = No Pretreatment.

Fig. 4 Lactobacillales proportion at the genus level (relative abundance > 4%) at T0 and after dark fermentation (Tf). For each experiment, microbial communities of one replicate among the quadruplicate were measured. NI = No Inoculum / I = Inoculum / P = Pretreatment / NP = No Pretreatment.

Tables and Figures

Table 1. Composition of synthetic OFMSW and FW (*cooked).

Synthetic OFMSW	**Percentage (%)**	**Synthetic FW**	**Percentage (%)**
Meat*	6.03	Meat	15.00
Frozen carrots*	4	Yoghurt	10.00
Coffee grounds*	3.88	Red berries	15.00
Rice*	4.31	Breaded fish	10.00
Potatoes*	17.89	French fries	20.00
Rusk	5.12	Vegetables	15.00
Grass	5.10	Bread	15.00
Yoghurt	1.98		
Office paper	31.64		
Cardboard	20.05		

Table 2. Substrate characteristics.

Substrate	**Storage**	**Origin/ Variety**	**TS (g/g)**	**VS (g/g)**
Dates	4 °C	Tunisia / Deglet Nour variety (pitted)	0.767 ± 0.001	0.749 ± 0.001
Corn silage	4 °C in an airtight bag	Early variety - Crops harvested in October 2016 and ensiled for 2 years	0.310 ± 0.001	0.299 ± 0.001
Sorghum	-20 °C	Collected in Saint Thibery in 2011 in the South of France (Sambusiti et al., 2013)	0.379 ± 0.007	0.359 ± 0.016
Microalgae	No storage (continuous production)	Cultivated outside the laboratory in a raceway / *Scenedesmus quadricauda* and *Pediastrum*	0.104 ± 0.002	0.082 ± 0.001
Sewage sludge	No storage	Wastewater treatment plant in Narbonne	0.111 ± 0.005	0.088 ± 0.004
OFMSW	No storage	Prepared in the laboratory	0.655 ± 0.001	0.514 ± 0.009
FW	No storage	Prepared in the laboratory	0.360 ± 0.004	0.348 ± 0.020

Table 3

Maximum cumulative H_2 production for all substrates and BHP conditions (average of a quadruplicate). NI = No Inoculum / I = Inoculum / P = Pretreatment / NP = No Pretreatment. Letters indicate Tukey's test results (for a same substrate), i.e. if two samples share a common letter (a, b or c), there is no significant difference. # Indicates an H_2 yield greater than the maximum calculated H_2 yield.

Experiment	**H_2 (mL/gVS$_{ini}$)**	**H_2 (mmol/gVS$_{ini}$)**	**Calculated H_2 yield (mmol/gVS$_{ini}$)**	**Metabolites (gCOD/gVS$_{ini}$)**
Corn silage				
I-NP	125 ± 3 ^{a}	5.57 ± 0.14	6.56 ± 0.34	0.48 ± 0.01 ^{b}
I-P	129 ± 9 ^{ab}	5.78 ± 0.4 #	4.95 ± 0.51	0.43 ± 0.01 ^{a}
NI-P	131 ± 11 ^{ab}	5.75 ± 0.47 #	4.73 ± 0.18	0.45 ± 0.02 ^{a}
NI-NP	148 ± 11^{b}	6.58 ± 0.49 #	5.36 ± 0.49	0.49 ± 0.01 ^{b}
Sorghum				
I-NP	34 ± 13 ^{a}	1.52 ± 0.58	3.28 ± 0.12	0.23 ± 0.03 ^{a}
I-P	47 ± 10 ^{ab}	2.11 ± 0.44	2.83 ± 0.17	0.23 ± 0.02 ^{a}
NI-P	49 ± 9 ^{ab}	2.20 ± 0.41	2.45 ± 0.44	0.22 ± 0.02 ^{a}
NI-NP	60 ± 3 ^{b}	2.69 ± 0.13	2.78 ± 0.36	0.25 ± 0.02 ^{a}
OFMSW				
I-NP	22 ± 7 ^{a}	0.96 ± 0.30	4.32 ± 0.32	0.24 ± 0.01 ^{a}
I-P	45 ± 12 ^{b}	2.02 ± 0.53	3.96 ± 0.8	0.24 ± 0.02 ^{a}
NI-P	35 ± 5 ^{ab}	1.45 ± 0.24	4.41 ± 0.99	0.26 ± 0.02 ^{a}
NI-NP	33 ± 5 ^{ab}	1.56 ± 0.23	4.29 ± 0.76	0.26 ± 0.02 ^{a}
Dates				
I-NP	55 ± 26 ^{a}	2.45 ± 1.17 #	2.02 ± 0.75	0.74 ± 0.04 ^{a}
I-P	306 ± 14 ^{c}	13.67 ± 0.60 #	8.59 ± 0.61	0.82 ± 0.02 ^{ab}
NI-P	254 ± 23 ^{b}	11.35 ± 1.01 #	5.23 ± 1.22	0.78 ± 0.07 ^{ab}
NI-NP	288 ± 9 ^{bc}	12.83 ± 0.38 #	6.61±0.69	0.86 ± 0.04 ^{b}
FW				
I-NP	79 ± 13 ^{a}	3.51 ± 0.58	9.25 ± 0.82	0.61 ± 0.08 ^{a}
I-P	169 ± 22 ^{b}	7.55 ± 0.98	9.19 ± 1.27	0.64 ± 0.04 ^{a}
NI-P	142 ± 36 ^{b}	6.33 ± 1.58 #	4.52 ± 0.42	0.54 ± 0.11 ^{a}
NI-NP	156 ± 23 ^{b}	6.96 ± 1.01 #	5.96 ± 1.18	0.58 ± 0.07 ^{a}
Microalgae				
I-NP	1 ± 1 ^{a}	0.03 ± 0.06	1.48 ± 0.34	0.1 ± 0.01 ^{a}
I-P	0 ± 1 ^{a}	0.02 ± 0.03	4.74 ± 0.74	0.18 ± 0.02 ^{b}
Sludge				
I-NP	0 ± 0 ^{a}	0 ± 0	0 ± 0	0 ± 0 ^{a}
Sludge I-P	1 ± 0 ^{b}	0.04 ± 0.01	3.12 ± 0.22	0.14 ± 0 ^{b}

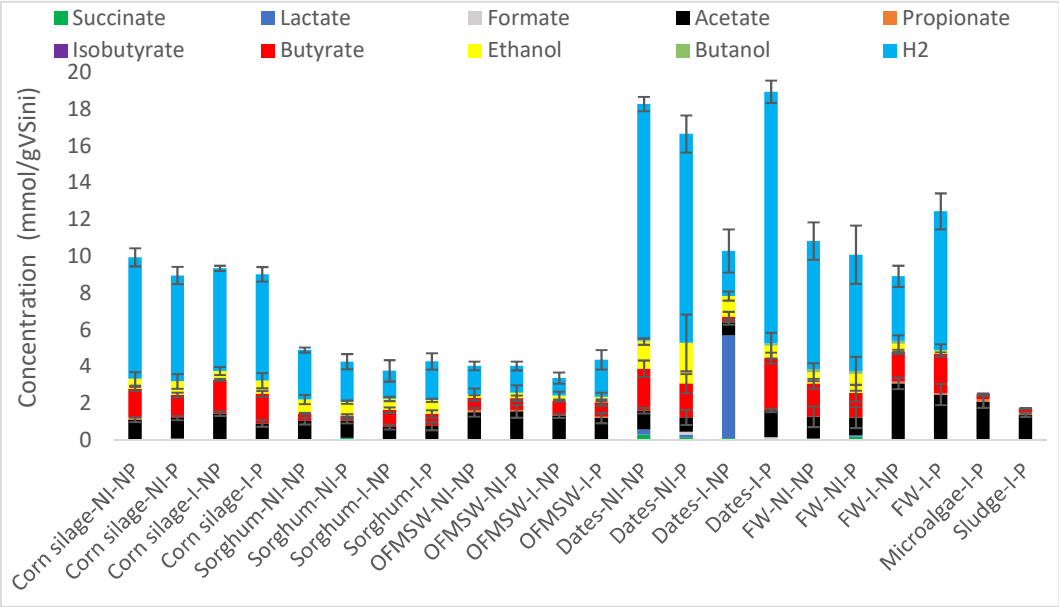

Succinate
Lactate
Formate
Acetate
Propionate
Isobutyrate
Butyrate
Ethanol
Butanol
H2
Concentration (mmol/gVSini)
20
18
16
14
12
10
8
6
4
2
0
Corn silage-NI-NP
Corn silage-NI-P
Corn silage-I-NP
Corn silage-I-P
Sorghum-NI-NP
Sorghum-NI-P
Sorghum-I-NP
Sorghum-I-P
OFMSW-NI-NP
OFMSW-NI-P
OFMSW-I-NP
OFMSW-I-P
Dates-NI-NP
Dates-NI-P
Dates-I-NP
Dates-I-P
FW-NI-NP
FW-NI-P
FW-I-NP
FW-I-P
Microalgae-I-P
Sludge-I-P

Fig. 1

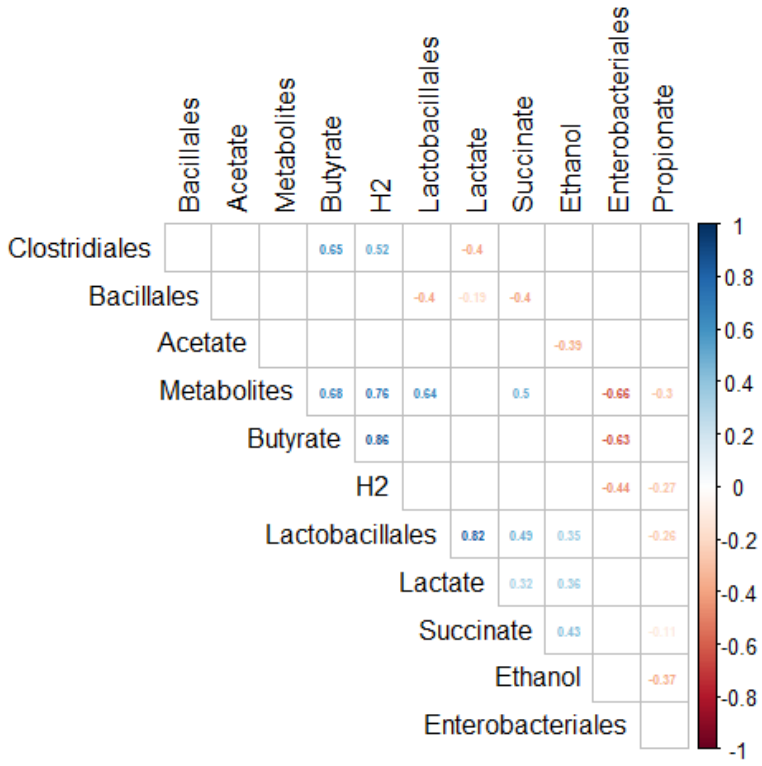
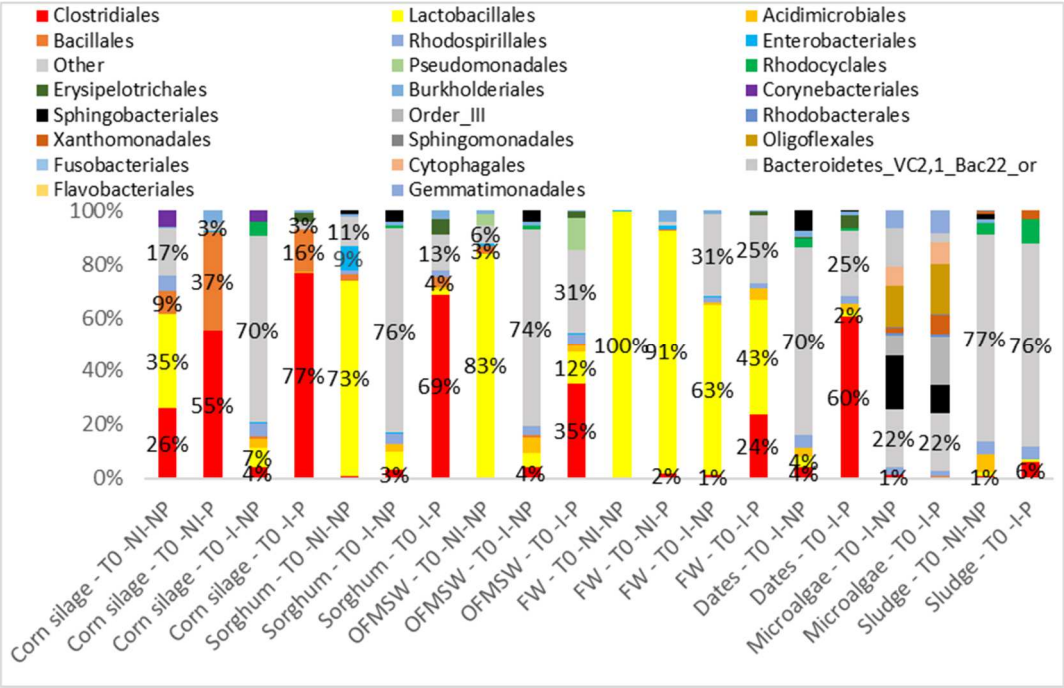

Bacillales
Acetate
Metabolites
Butyrate
H2
Lactobacillales
Lactate
Succinate
Ethanol
Enterobacteriales
Propionate
Clostridiales
Bacillales
Acetate
Metabolites
Butyrate
H2
Lactobacillales
Lactate
Succinate
Ethanol
Enterobacteriales
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Fig. 2

(a)


Clostridiales
Lactobacillales
Acidimicrobiales
Bacillales
Rhodospirillales
Enterobacteriales
Other
Pseudomonadales
Rhodocyclales
Erysipelotrichales
Burkholderiales
Corynebacteriales
Sphingobacteriales
Order_III
Rhodobacterales
Xanthomonadales
Sphingomonadales
Oligoflexales
Fusobacteriales
Cytophagales
Bacteroidetes_VC2,1_Bac22_or
Flavobacteriales
Gemmatimonadales
100%
80%
60%
40%
20%
0%
Corn silage - T0 -NI-NP
Corn silage - T0 -NI-P
Corn silage - T0 -I-NP
Corn silage - T0 -I-P
Sorghum - T0 -NI-NP
Sorghum - T0 -I-NP
Sorghum - T0 -I-P
OFMSW - T0 -NI-NP
OFMSW - T0 -I-NP
OFMSW - T0 -I-P
FW - T0 -NI-NP
FW - T0 -NI-P
FW - T0 -I-NP
FW - T0 -I-P
Dates - T0 -I-NP
Dates - T0 -I-P
Microalgae - T0 -I-NP
Microalgae - T0 -I-P
Sludge - T0 -NI-NP
Sludge - T0 -I-P

(b)

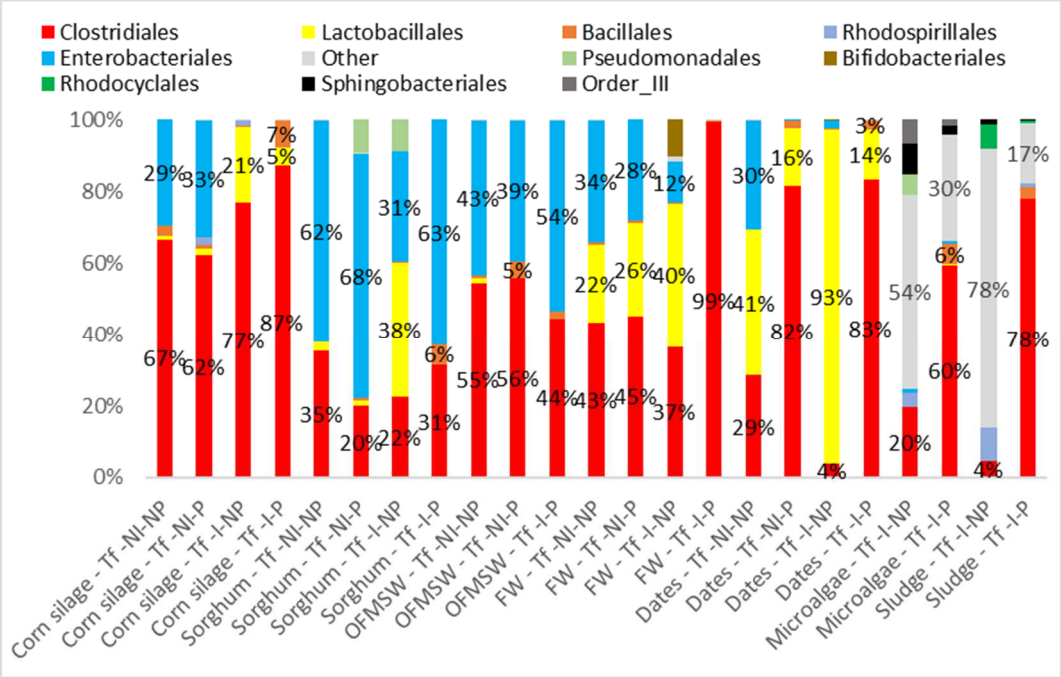

Clostridiales
Lactobacillales
Bacillales
Rhodospirillales
Enterobacteriales
Other
Pseudomonadales
Bifidobacteriales
Rhodocyclales
Sphingobacteriales
Order_III
100%
80%
60%
40%
20%
0%
Corn silage - Tf -NI-NP
Corn silage - Tf -NI-P
Corn silage - Tf -I-NP
Corn silage - Tf -I-P
Sorghum - Tf -NI-NP
Sorghum - Tf -NI-P
Sorghum - Tf -I-NP
Sorghum - Tf -I-P
OFMSW - Tf -NI-NP
OFMSW - Tf -NI-P
OFMSW - Tf -I-P
FW - Tf -NI-NP
FW - Tf -NI-P
FW - Tf -I-NP
FW - Tf -I-P
Dates - Tf -NI-NP
Dates - Tf -NI-P
Dates - Tf -I-NP
Dates - Tf -I-P
Microalgae - Tf -I-NP
Microalgae - Tf -I-P
Sludge - Tf -I-NP
Sludge - Tf -I-P

Fig. 3

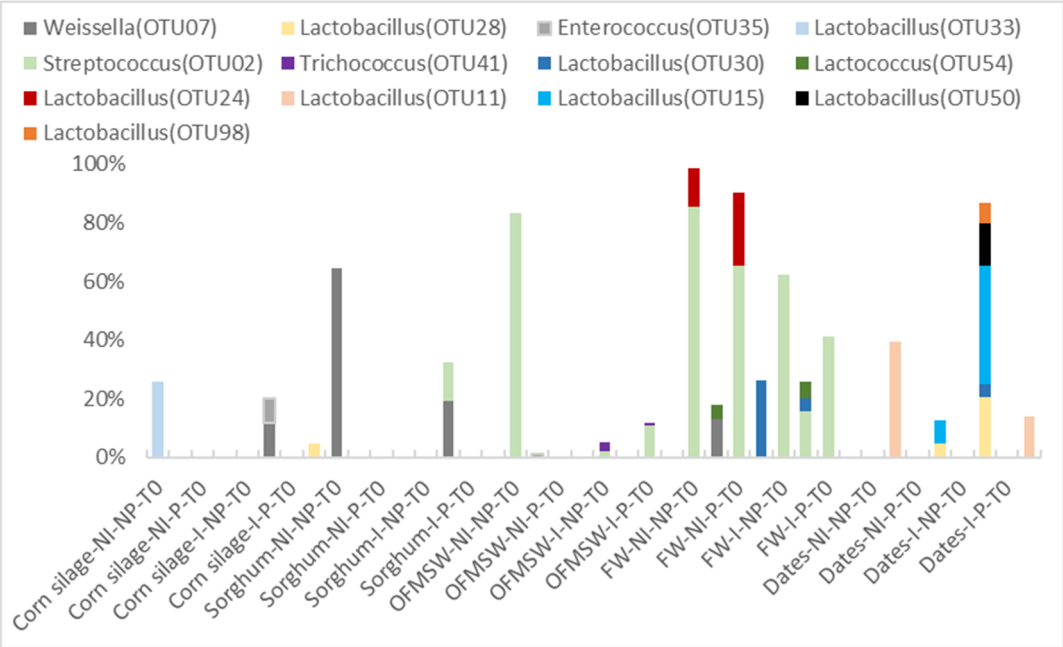

Weissella(OTU07)
Lactobacillus(OTU28)
Enterococcus(OTU35)
Lactobacillus(OTU33)
Streptococcus(OTU02)
Trichococcus(OTU41)
Lactobacillus(OTU30)
Lactococcus(OTU54)
Lactobacillus(OTU24)
Lactobacillus(OTU11)
Lactobacillus(OTU15)
Lactobacillus(OTU50)
Lactobacillus(OTU98)
100%
80%
60%
40%
20%
0%
Corn silage-NI-NP-T0
Corn silage-NI-P-T0
Corn silage-I-NP-T0
Corn silage-I-P-T0
Sorghum-NI-NP-T0
Sorghum-NI-P-T0
Sorghum-I-NP-T0
Sorghum-I-P-T0
OFMSW-NI-NP-T0
OFMSW-NI-P-T0
OFMSW-I-NP-T0
OFMSW-I-P-T0
FW-NI-NP-T0
FW-NI-P-T0
FW-I-NP-T0
FW-I-P-T0
Dates-NI-NP-T0
Dates-NI-P-T0
Dates-I-NP-T0
Dates-I-P-T0

Fig 4