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## 1 The type of carbohydrates specifically selects microbial community 2 structures and fermentation patterns

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## 5 Abstract

6 The impact on dark fermentation of seven carbohydrates as model substrates of 7 lignocellulosic fractions (glucose, cellobiose, microcrystalline cellulose, arabinose, 8 xylose, xylan and wheat straw) was investigated. Metabolic patterns and bacterial 9 communities were characterized at the end of batch tests inoculated with manure 10 digestate. It was found that hydrogen production was linked to the sugar type (pentose 11 or hexose) and the degree of polymerisation. Hexoses produced less hydrogen, with a 12 specific selection of lactate-producing bacterial community structures. Maximal 13 hydrogen production was five times higher on pentose-based substrates, with specific 14 bacterial community structures producing acetate and butyrate as main metabolites. 15 Low hydrogen amounts accumulated from complex sugars (cellulose, xylan and wheat 16 straw). A relatively high proportion of the reads was affiliated to *Ruminococcaceae* 17 suggesting an efficient hydrolytic activity. Knowing that the bacterial community 18 structure is very specific to a particular substrate offers new possibilities to design more 19 efficient H<sub>2</sub>-producing biological systems.

### 20 Keywords

21 Biohydrogen; Dark fermentation; Lignocellulosic residues; Microbial consortium

22

#### 1. Introduction

Energetic transition and waste management are ones of the main XXI<sup>th</sup> century 23 24 challenges (UNFCCC. Conference of the Parties (COP), 2015). Among all the 25 renewable energies nowadays available, hydrogen has a promising future. Its energy power by mass unit (122 kJ.g<sup>-1</sup>) is higher than most of other energy sources and its 26 27 combustion produces only water and no carbon dioxide (Saratale et al., 2008). 28 Hydrogen gas can be produced along the bioconversion of organic matter by dark fermentation. This natural biological process is carried out by different types of 29 30 microorganisms widespread in Nature and found in most of anaerobic environments, 31 such as in lakes, guts, urban sludge or manure digestate (Wang and Wan, 2009). First 32 hydrolytic bacteria convert the organic matter into simpler molecules and the 33 monomeric forms can further be assimilated by fermentative hydrogen-producing 34 bacteria. Depending on the type of bacteria and the metabolic pathway used to convert 35 these substrates, accumulation of hydrogenated biochemical intermediates (NADH) in 36 the cell can lead to the production of biohydrogen to recycle the reduced elements in the 37 cell (Cai et al., 2011). More particularly, acetic and butyric acid pathways generate four 38 and two moles of hydrogen per mole of glucose consumed, respectively (Kalia and 39 Purohit, 2008).

In mixed cultures, other metabolic pathways that are detrimental to hydrogen production by consuming carbohydrates, such as lactic acid or alcoholic fermentation, are also observed. Furthermore, the reuse of the hydrogen produced is also possible by other groups of H<sub>2</sub>-consuming bacteria to form other volatile fatty acids such as propionate, caproate but also acetate and methane by homoacetogenesis and methanogenesis, respectively (Saady, 2013). Although heat shock treatment has been well described to get rid of the methanogenic activity, other fermentative pathways are
often carried out by thermo-resistant microorganisms (Wong et al., 2014).

48 Nonetheless, using mixed culture is advantageous since complex substrates such 49 as lignocellulose can also be used. The use of complex microbial consortia will increase 50 the chance to find bacteria with efficient enzymatic materials to degrade complex 51 biomass (Kleerebezem and van Loosdrecht, 2007). Lignocellulosic biomass is a 52 substrate of choice for the production of renewable energies. It is the most abundant 53 organic carbon source because it composes the major part of agricultural waste with a 54 low cost (Guo et al., 2010). But the complex structure of lignocellulosic materials can 55 impact bacterial fermentation and mechanisms are not yet well understood.

56 Lignocellulosic compounds are mainly composed of three polymers linked 57 together to form a complex matrix : lignin, cellulose and hemicelluloses (Menon and 58 Rao, 2012). Lignin is a phenolic polymer known to not be biodegradable for most of 59 bacteria. It is linked to hemicelluloses, bond to cellulose. Whereas hemicelluloses are 60 heteropolymer mainly composed of D-xylose, but also L-arabinose and D-glucose, 61 cellulose is a homopolymer of cellobiose, a dimer of glucose. Because of the polymeric 62 structure, deconstructive pre-treatment are usually applied on lignocellulosic biomass to 63 release fermentable sugars (Nissilä et al., 2014).

64 Depending on the proportion of each sugar, the polymerisation degree and other 65 biochemical parameters, fermentative pathways and performances can be both 66 impacted. This work aims to evaluate the potential of main lignocellulosic biomass 67 fractions to produce hydrogen by dark fermentation. Pure model substrates representing 68 the main fractions of lignocellulosic materials were fermented in batch reactors with the 69 same initial mixed inoculum (manure anaerobic digestate). Through the analysis of metabolites co-produced during the process and the final bacterial community
structures, the impact of the type of carbon source on microbial community function
was evaluated.

73

## 2. Materials and methods

74

#### 2.1. Inoculum preparation and experimental setup

75 The outlet of a solid-state anaerobic reactor fed with bull manure (Montrodat, 76 France), so-called digestate, was used as initial microbial inoculum. Because undigested 77 straw was present in the digestate, microorganisms were first extracted according to a 78 protocole adapted from Zhang et al (2007). For that, 40 ml of sterile physiological water 79 were added to 20 g of digestate in a 500 ml centrifuge tube. After dynamic hand 80 shaking, the tube was centrifuged for 5min at 3000 g (temperature 4°C). The 81 supernatant was recovered in a sterile bottle and the pellet was washed with 40 ml of 82 sterile phosphate buffer at 10 mM. After shaking, the tube was again centrifuged at 83 3000 g during 10 min (4°C). The supernatant was recovered and added to the first one. 84 The residual juice of the pellet was sieved at 1 mm. Heat shock pre-treatment (90°C, 30 85 min followed by ice cooling) was performed before inoculation to get rid of hydrogen 86 consumption by methanogenic archaea.

Biohydrogen production potential (BHP) tests were carried out in batch tests in
quadriplicates in 600 ml plasma bottle with 400 ml of working volume. The medium

89 was composed of 12.5 ml of minimal nutrient solution (in mg. $l^{-1}$  - NH4Cl : 32000 ;

90 K2HPO4 : 20000 ; FeCl2,4H2O : 1500 ; H3BO3,H2O : 60 ; MnSO4,H2O : 117;

91 CoCl2,6H2O : 25 ; ZnCl2 : 70 ; NiCl2,6H2O : 25 ; CuCl2,2H2O : 15 ; NaMoO4,2H2O

92 : 25 ; HCl : 1755) and 100mM of MES (2-[N-morpholino] ethane sulfonic acid buffer).

| 93  | Glucose (Sigma), cellobiose (Fluka), arabinose (Sigma), xylose and xylan (Sigma)                              |
|-----|---|
| 94  | concentrations were fixed at 5 $g_{COD}$ .l <sup>-1</sup> whereas micro-crystalline cellulose (MCC,           |
| 95  | Fluka) and wheat straw (Haussmann common wheat straw) were introduced at 10 $v_{\text{olatile}}$              |
| 96  | $_{solid}$ .l <sup>-1</sup> to increase the production of metabolites and remain above the detection limit of |
| 97  | metabolites. Heat treated inoculum was then added to reach a substrate / inoculum (S/X)                       |
| 98  | ratio of 10 $g_{COD}$ substrate $g^{-1}COD$ inoculum.   |
| 99  | Volatile solid analysis of wheat straw and MCC were performed in  |
| 100 | quadriplicates according to the APHA standard methods (APHA, 1999). Chemical                                  |
| 101 | oxygen demand (COD) was analysed using Spectroquant® kit (Merk) according to                                  |
| 102 | manufacturer's indications.   |
| 100 |   |

103 After inoculation, bottles were sealed with a rubber stopper and locked with an 104 aluminium screw. Head space was then purged with 0.2µm-filtered nitrogen gas to 105 remove oxygen traces and keep the conditions anaerobic. The fermentation was stopped 106 when the hydrogen production stabilized to avoid further consumption.

#### 107 **2.2. Metabolites and gas composition analysis**

108Gas production was monitored every 8 hours with an automatic micro-gas109chromatograph (SRA μ-GC R3000) equipped with two columns: a Molsieve 10m/PPU110at 80°C with Argon as vector gas and a VAR 8m/PPU at 70°C with Helium, for O2-111CH4-H2-N2 and CO2 analysis, respectively. The TCD temperature was set at 90°C.112Gas production was estimated by pressure measurement. From the gas113composition and volume analysis, a modified Gompertz model was used to assess114hydrogen production kinetic parameters (Eq. 1):

115 
$$H(t) = Pexp\left\{-exp\left[\frac{Rm.e}{P}(\lambda - t) + 1\right]\right\}(1)$$

| 116 | Where H is the cumulative volume of hydrogen production (ml) along the   |
|-----|--|
| 117 | incubation time (t), P is the maximum cumulative hydrogen production $(ml_{H2}.g^{-1}_{eq initial})$   |
| 118 | COD), Rm is the maximum hydrogen production rate (ml <sub>H2</sub> .g <sup>-1</sup> <sub>eq initial COD</sub> .d <sup>-1</sup> ), $\lambda$ is the lag |
| 119 | phase (days) and e is exp(1). The values of P, Rm and $\lambda$ were estimated using grofit R  |
| 120 | package (v 1.1.1-1) with nonlinear least square fitting (Kahm et al., 2010).   |
| 121 | Soluble metabolites, i.e., volatile fatty acids (VFAs) and organic acids, solvents   |
| 122 | and residual sugars, were quantified before and after fermentation by Gas  |
| 123 | Chromatography (GC) and High Performance Liquid Chromatography (HPLC),   |
| 124 | respectively. Samples were filtrated at $0.2\mu m$ prior to analysis. The HPLC was coupled   |
| 125 | to refractometric detection (Waters R410). Chemicals were separated by an Aminex   |
| 126 | HPX-87H column (300 x 7.8mm, Biorad) equipped with a protective precolumn  |
| 127 | (Microguard cation H refill catbridges, Biorad). The eluting solution corresponded to 2  |
| 128 | mM H <sub>2</sub> SO <sub>4</sub> under a flow rate of 0.4 ml.min <sup>-1</sup> . The column temperature was set at $35^{\circ}C$                      |
| 129 | and the refractive index detector (Waters 2414) worked at 45°C. The gas chromatograph  |
| 130 | (GC 3900 Varian) was coupled to a flame ionization detector. Conditions were the   |
| 131 | same as previously described by Rafrafi et al. (2013). Measurements errors associated  |
| 132 | to µ-CG, GC and HPLC analysis were 1%, 5% and 3% respectively.   |

133

# 134 amplification and sequencing

Ten mL of liquid sample were collected before and after the fermentation test.
Samples were first centrifuged 10 min at 5,752g. After removing 8ml of the
supernatant, the pellet was resuspended and aliquots of 500 µl were introduced in 4
sterile eppendorf tubes of 2 ml. The tubes were then centrifuged 5min at 16,662g. The
pellets were finally stored at -20°C prior to DNA extraction. Cell lysis and DNA

2.3. Characterisation of microbial community - DNA extraction, PCR

140 extraction was performed according to manual instructions as previously described by 141 Saur et al (2016). DNA purification was performed using the QIAamp DNA minikit 142 (Qiagen, Hilden, Germany) according to manufacturer's instructions. Purified DNA was 143 conserved at -20°C in 50 µl of molecular grade purity water until further use. 144 The V3 region of the 16S rRNA gene were amplified as presented before by 145 Carmona-Martinez et al (2015). The PCR products were purified and loaded onto the 146 Illumina MiSeq cartridge according to the manufacturer's instructions for sequencing of 147 paired 300 bp reads (v3 chemistry). Library preparation and sequencing was done at the 148 GeT PlaGe Sequencing Center of the Genotoul Lifescience Network in Toulouse, 149 France. 150 A modified version of the Standard Operation Procedure for MiSeq data (Kozich 151 et al., 2013) in Mothur version 1.35.0 (Schloss et al., 2009) was used to assemble 152 forward and reverse sequences. Sequences were pre-clustered at 4 differences in 153 nucleotides over the length of the amplicon and chimeras were checked using uchime 154 (Edgar et al., 2011). Scarce sequences that appeared less than three times in the entire 155 data-set were removed. Alignment and taxonomic affiliation from the 16S rRNA 156 sequences was performed with SILVA SSU Ref NR99, release 119, as provided by 157 Mothur (Schloss et al., 2009). Maximum of 44,503 and minimum of 14,941 sequences 158 were obtained from the samples. 14,000 sequences with a mean length of 428 bp were 159 considered per samples for data analysis. As shown in rarefaction curves (see 160 supplementary materials) the number of selected sequences was enough to significantly 161 represent the final communities. Rarefaction curves were drawn with the "rarecurve()" 162 function of the *vegan* R package (Oksanen et al., 2016). Sequences have been submitted 163 to GenBank with accession No. XXXXX.

164

#### 2.4. Statistical data analysis

Principal component analysis (PCA) was performed on reduced and scaled data
using the "*prcomp()*" function from the built-in R stats package. Graphic representation
was performed using "*ggbiplot*" (Vu, 2011).

Bacterial community analysis was performed on unique Operational Taxonomic Units (OTUs). Principal Coordinate Analysis (PCoA) was performed on unique OTU after data transformation by the Hellinger method to avoid noise generated by highly minor OTUs. Hellinger transformation was performed with the "*decostand()*" function from the *vegan* R package (Oksanen et al., 2016) and PCoA with the "*pcoa()*" function in the ape package (Paradis et al., 2004).

174The OTUs with relative abundance higher than 5% in at least one sample were175selected to perform further analysis. The average of their relative abundance by176replicate was presented as heatmap using the *"heatmap.2()"* function of the gplots R177package (Warnes et al., 2016). Influence of major OTU identified on metabolites178produced was analysed by the *"envfit()"* function(permutations 9999) which fit179environmental vectors onto ordination, PCA done on major OTUs in this case (Oksanen180et al., 2016)

181

# 3. Results and discussion

### 182 **3.1. Hydrogen production performances**

183 Gas production was periodically analysed until a hydrogen production plateau

184 was observed. It was reached after 4.5, 6.4, 21.5, 6.5, 7.5, 8 and 14.6 days of

185 fermentation for glucose, cellobiose, MCC, arabinose, xylose, xylan and wheat straw,

186 respectively. At this time, experiments were stopped to avoid changes in metabolism 187 due to hydrogen consumption. No methane production was observed during the 188 experiment showing that the heat shock pre-treatment totally supressed the 189 methanogenic activity for the time of experiment. 190 Dynamics of hydrogen production along the experiment are shown in Figure 1. 191 Hydrogen production data were fitted to a modified Gompertz model with a 192 determination of coefficient (R<sup>2</sup>) up to 0.99 and the model parameters are presented in 193 Table 1. Maximum of cumulated hydrogen production (P) on glucose was 31±12 194 ml<sub>H2</sub>.g<sup>-1</sup><sub>eq.initial COD</sub> which was almost a third higher than cellobiose and MCC, two 195 polymers of glucose. The average maximum hydrogen production yield was also different between cellobiose and MCC with 23 and 12 ml<sub>H2</sub>.g<sup>-1</sup><sub>eq.initial COD</sub> obtained 196 197 respectively. In accordance with the degree of polymerisation, hydrogen production rate 198 (Rm) was also different between hexose-based substrates since  $29\pm12 \text{ ml}_{\text{H2}.\text{g}^{-1}eq,initial}$  $COD.d^{-1}$  were obtained on glucose and  $12\pm1$  and  $1.5\pm0.6 \text{ ml}_{H2.g^{-1}}\text{eq.initial COD.}d^{-1}$  were 199 200 calculated for cellobiose and cellulose, respectively. Lag phase ( $\lambda$ ) was similarly 201 impacted since it was 12 days for MCC but only 2 days for glucose and cellobiose. 202 Consistently, several authors showed that the production of hydrogen depends 203 on the degree of polymerisation of the carbohydrates (Danilenko et al., 1993; Monlau et 204 al., 2012a; Quéméneur et al., 2012; Sambusiti et al., 2013). Indeed, a hydrolysis step of 205 carbohydrate polymers is required to produce simpler molecules that can further enter 206 into microbial cells prior to be metabolized. This step impacts directly the bioprocess 207 kinetics (Kumar et al., 2009). However hydrolysis requires also energy in the forms of 208 ATP and NADH equivalents that reduces final hydrogen production. Therefore, high

hydrogen yields are usually observed in presence of high soluble sugars-content
substrates as previously reported (Guo et al., 2014; Monlau et al., 2012b).

The modified Gompertz model parameters were also estimated for pentosebased substrates. The C5 substrates showed higher hydrogen yields than C6 substrates, when comparing substrates with equivalent polymerisation degree. As an illustration, the fermentation of arabinose and xylose led to an increase of the  $P_{max}$  value of 2 and 3 times when compared to glucose, respectively. The maximum hydrogen production rate Rm was also higher with 32 and 40 ml<sub>H2</sub>.g<sup>-1</sup><sub>eq.initial COD</sub>.d<sup>-1</sup> for arabinose and xylose, respectively.

218 These results suggest that pentoses are preferred carbon sources for the 219 production of hydrogen using this inoculum and under these experimental conditions. 220 Such effect was already reported by Prakasham *et al.* (2009) who used a mixed 221 inoculum developed in buffalo dung compost, although the reported differences 222 between hexose and pentose substrates were lower than in the present study. Mäkinen et 223 al.(2012) also obtained the same trend with hot spring culture. All these observations 224 suggest that the metabolic pathway is strongly dependent on the origin of the inoculum 225 especially when considering monomeric carbohydrates. Indeed, the fermentative 226 community developed here is more favourable to produce hydrogen from C5 than from 227 C6.

With C5 substrates, the polymerisation degree had also a negative effect on the overall hydrogen production performances, since lower P and Rm and higher  $\lambda$  were observed for xylan than with xylose.

In the case of wheat straw as model material of complex lignocellulosicbiomass, the complex structure led to very poor hydrogen value which is coherent to

literature (Agbor et al., 2011). Similarly to micro-crystalline cellulose, only 8% of theinitial equivalent COD was converted with a very low hydrogen value.

However, when comparing the quantity of hydrogen produced per unit of COD 235 236 converted during the process, i.e. hydrogen production yield (Table 1), it could be 237 observed that more consumed COD was transformed into hydrogen for MCC and 238 cellobiose than observed for glucose, with 8, 4.2 and 1.7 mmol<sub>H2</sub>.g<sup>-1</sup>ea.COD consumed 239 measured respectively. Hence, even if the degradation was weaker on cellobiose and 240 MCC than on glucose, the metabolisms of the flora converting the polymer seems to be 241 oriented to the production of hydrogen contrary to what observed on glucose. 242 In summary, the hydrogen production correlated well with both the degree of 243 polymerisation and the type of residual sugars (C5 or C6). When considering the 244 relative amount of COD converted to H<sub>2</sub>, cumulated hydrogen ranged from 11 to 2% 245 only. This result should be compared to a theoretical value of 21% for mixed cultures,

as suggested by Hawkes *et al.* (2007). Therefore, the other metabolites co-producedduring fermentation were also considered.

248

#### **3.2. Other fermentation metabolites**

Soluble fermentation products were analysed at the end of batch tests. Amounts of metabolites were expressed in mg  $_{product eq. COD.g^{-1} initial substrate eq.COD}$  and are presented in Table 2. A principal component analysis (PCA) was carried out to illustrate the variability in metabolic pathways between the different substrates (Figure 2).

The COD conversion was evaluated by COD balance according to the metabolites measured. Because batch tests were stopped at the maximum of hydrogen production, the initial substrate COD was not necessarily fully converted at the end of the experiment. In particular, the percentage of COD converted at the final time decreased from 79±3 to 4.68±0.03 according to the degree of polymerisation for C6
substrates. Indeed, the compact structure of micro-crystalline cellulose is an obstacle for
hydrolytic enzymes to access substrate linkage. Complex substrates may not be fully
converted at the end of the process as it could be observed in this study (Kumar et al.,
2009).

Like for hexose substrates, the COD converted at the end of the pentose fermentation was higher for the monomer than for the polymer which supports that the degree of polymerisation also has an impact regardless the sugar residue.

265 The use of xylose and arabinose as different C5 isomer (on carbon 2) showed 266 different conversion yields since only 44% of the initial COD was converted under the 267 form of metabolites for arabinose against 64.4% for xylose. Conversion of arabinose 268 and xylose implies the use of different enzymes. L-arabinose is first converted by via 269 L-arabinose isomerase, L-ribulokinase and L-ribulose-5-P 4- epimerase to L-ribulose, 270 L-ribulose-5- phosphate and D-xylulose-5-phosphate respectively, whereas D-xylose is 271 directly transformed into D-xylulose by D-xylose isomerase (Bettiga et al., 2009). 272 Hence depending on their enzymatic potential, bacteria will have the ability to 273 converted one pentose, the other or both.

Metabolic patterns appear to be strongly linked to the type of carbohydrates (C6 or C5). Hexoses (glucose and cellobiose) were mainly converted into lactate (>70%). The low hydrogen yield was likely linked to the low production of butyrate as second main metabolite, as previously suggested by Guo *et al.* (2014). Acetate and ethanol represent less than 10% of all the metabolites produced. However, the proportion of acetate and butyrate produced was higher for the polymers of glucose than observed for the monomer. This metabolic pattern favourable to the production of hydrogen might explain the high value of hydrogen production yield observed in Table 1 for cellobioseand MCC.

283 For C5 fermentation (xylose and arabinose), metabolisms were different since 284 very low amount of lactate accumulated for arabinose, and butyrate was the main 285 metabolic pathway (30%). Consistently, hydrogen yield was higher than with hexose. 286 Similar results have been observed in a previous study. Using hot spring culture as 287 fermentative microorganism source, Mäkinen et al.(2012) showed that lactate was 288 preferentially produced during glucose conversion as observed in this study. 289 Interestingly, pentose was converted into butyrate but also formate, metabolite unfound 290 there. 291 Ethanol and acetate were produced in higher quantity from C5 than from C6 292 (x10) and a new metabolite, isobutyrate, was identified. To the authors' knowledge, no 293 information about isobutyrate production from pentose-based sugars is available in the 294 literature. However, the conversion into ethanol has been largely studied in the field of 295 biofuel production. Even if yeast is the main microorganism studied, bacteria are also 296 able to produce bio-ethanol (Bettiga et al., 2009).

Moreover, identification of different by-products depending on the pentose
sugar is observed. While lactate was more produced during arabinose conversion,
propionate was specific of xylose and xylose-based polymers. Indeed, bacteria did not
have the same ability to convert the two pentoses as explained before.

301

#### **3.3. Strong selection in microbial communities**

The differences in metabolic patterns observed for each substrate might be the consequence of different metabolic pathways within the same microbial community or to the selection of specific bacterial communities. Therefore, bacterial DNA was amplified and sequenced by high-throughput amplicon sequencing. Principal coordinate
analysis (PCoA) was used to represent the euclidean distance separating each final
community (Figure 3).

First, it was observed that samples can be distinguished on the first axis according to the polymerisation degree. Communities growing on monomers and dimers are located on the left side whereas polymers-based communities, including wheat straw, are on the right side. The second axis was more related to the carbon content of the substrate. Similarly to the metabolic patterns, it can be concluded that the final bacterial structure was dependent on both the polymerisation degree and the type of substrate.

316 The relative proportion of the major OTUs (relative abundance > 5%) found for 317 each substrate are presented in Figure 4. Main OTUs were affiliated to the 318 Clostridiaceae, Bacillaceae and Peanibacillaceae families when both pentose and 319 hexose simple sugars were used as substrate (xylose, arabinose, glucose and cellobiose). 320 These bacteria have been well described in mixed cultures fermentation. More 321 particularly, members of the *Clostridiaceae* family have generally been associated to 322 hydrogen production, while Bacillaceae and Peanibacillaceae are involved in lactic 323 acid fermentation (Ghimire et al., 2015). 324 Interestingly, these major OTUs were not found in the reactors containing 325 complex substrates such as xylan, cellulose and wheat straw. Here, identified bacteria 326 were affiliated to the *Ruminoccocaceae* family only. Bacteria belonging to this family

327 can be related to *Clostridium* bacteria and are mainly microorganisms found in rumen,

328 reported as efficiently degrading complex biomass (Hung et al., 2011). Because

*Ruminococcaceae* bacteria were not found in reactors fed with simple substrates, this
suggests that *Clostridiaceae*, *Bacillaceae* and *Paenibacillaceae* are more competitive
with regards to readily biodegradable sugars. In counterpart, the hydrolytic activity of
the *Ruminococcaceae* confers to them an ecological advantage to grow on more
complex organic materials.

Three proteobacteria were also identified in reactors fed with complex substrates. The OTU56 affiliated to the *Burkholderia* genus was present in MCC-based reactors. Similar bacteria were already described for their hydrolytic activity during complex organic biomass degradation (Mohana et al., 2008). Their presence was often associated to the production of hydrogen (Porwal et al., 2008).

The OTU7 and OTU199 found in xylan-based reactors were both affiliated to the *Klebsiella* genus (Wong et al., 2014). These facultative anaerobes were previously reported as efficient hydrogen producers. However, they exhibit a concomitant ethanol pathway which may explain the high proportion of ethanol (25±6%) found in these reactors (De Amorim et al., 2012).

344 Metabolites measured at the end of fermentation were fitted onto ordination 345 done with main OTUs to estimate the correlation strength existing between microbial 346 communities and metabolisms. Results show that acetate and lactate correlated better 347 with bacterial community structures than other metabolites (<0.1% of significance, 348 Figure 5). Hence, the production of acetate seems to be explained by the presence of 349 OTU9 and OTU26 affiliated to *Clostridium sp.* and OTU12 and OTU29 affiliated to 350 Paenibacillus sp. developed in C5-based sugar reactors. With lesser significance (<1% 351 and 5%), the emergence of these bacteria also correlated with hydrogen, butyrate and 352 ethanol production.

353 Lactate production is represented by the presence of OTU18 (Paenibacillus sp.), 354 OTU47 (Clostridium sp.) which were mainly identified in hexose-based reactors. 355 Interestingly, OTU11 (Clostridium sp.), OTU9 and OTU20 affiliated to Bacillus sp. are 356 located at in the hexose substrate zone although they are present in both C6 and C5 357 based reactors. Bacillus sp. are facultative anaerobes specially essential at the begging 358 of the fermentation since they allow anaerobic condition by consuming residual oxygen 359 (Huang et al., 2010). Their ability to grow on many carbohydrates may explain their 360 presence in both hexose and pentose-based reactors (Stülke and Hillen, 2000). 361 Few studies also showed that the type of substrate could impact the bacterial 362 community structure (Akutsu et al., 2009; Cheng et al., 2011; Li and Chen, 2007; Qiu et 363 al., 2016; Quéméneur et al., 2011). Table 3 reports hydrogen production yields observed 364 in literature using different bacterial cultures and various substrates. In all cases, acetate 365 and butyrate were the main metabolites observed, which was accompanied with higher 366 hydrogen production yields than measured in the present work. Bacteria affiliated to 367 *Clostridium* sp. were identified as main microorganisms composing the mixed culture 368 whereas in the present study, Bacillus sp. and Paenibacillus sp. represented a large part 369 of the communities. These differences in structures of the bacterial communities led in 370 this case to the production of lactate as main metabolite from hexose-based substrates. 371 Hence, depending on the residue used, bacterial community developed different 372 structures at the end of the fermentation that drove the metabolism to lactate or 373 acetate/butyrate production. Selection of bacteria represents an ecological advantage 374 which favours the development of microorganisms on specific substrate. However, this

into consideration when using continuous systems (Bakonyi et al., 2014). By controlling

sensitivity of bacterial communities structure to substrate composition should be taken

375

377 the substrate input, it could be easier to maintain the community structure in the reactor 378 and, thus, the performance stability for hydrogen production. This could also lead to 379 improve the biomass conversion yield by selecting specific bacteria able to degrade a 380 complex substrate. The use of biological strategies to increase organic matter 381 conversion into biohydrogen has already been performed in dark fermentation (Kotay 382 and Das, 2009; Kuo et al., 2012). Illustratively, the addition of keystone species into the 383 medium as those identified in this study could be a solution to improve the production 384 of hydrogen from lignocellulosic residues (Rafrafi et al. 2013).

385

4. Conclusion

386 This study shows that fermentative hydrogen yields from model carbohydrates 387 was linked to the type of sugars (pentose or hexose) and the degree of polymerisation. 388 Hydrogen production was strongly linked to a specific selection of bacterial community 389 structures with different metabolic patterns. Species affiliated to *Paenibacillus sp.*, 390 Bacillus sp. were associated to lactate production from hexoses, whereas Clostridium 391 sp. were dominant in hydrogen-producing reactors fed with pentoses. The presence of 392 *Ruminococcaceae sp.* on polymeric sugars (cellulose, xylan and wheat straw) suggests 393 that these bacteria play an important role in the hydrolytic activity.

394

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- 401 **References**
- Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment:
   Fundamentals toward application. Biotechnol. Adv. 29, 675–685.
- Akutsu, Y., Lee, D.-Y., Li, Y.-Y., Noike, T., 2009. Hydrogen production potentials and
   fermentative characteristics of various substrates with different heat-pretreated
   natural microflora. Int. J. Hydrogen Energy 34, 5365–5372.
- 407 3. APHA, 1999. Standard Methods for the Examination of Water and Wastewater, 20th
  408 ed.
- Bakonyi, P., Nemestóthy, N., Simon, V., Bélafi-Bakó, K., 2014. Review on the start-up
   experiences of continuous fermentative hydrogen producing bioreactors. Renew.
   Sustain. Energy Rev. 40, 806–813.
- 412 5. Bettiga, M., Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2009.
- 413 Arabinose and xylose fermentation by recombinant Saccharomyces cerevisiae
- 414 expressing a fungal pentose utilization pathway. Microb. Cell Fact. 8, 40.
- 415
  6. Cai, G., Jin, B., Monis, P., Saint, C., 2011. Metabolic flux network and analysis of
  416
  fermentative hydrogen production. Biotechnol. Adv. 29, 375–387.
- 417 7. Carmona-Martínez, A.A., Trably, E., Milferstedt, K., Lacroix, R., Etcheverry, L., Bernet,
- 418 N., 2015. Long-term continuous production of H2 in a microbial electrolysis cell (MEC)
- 419 treating saline wastewater. Water Res. 81, 149–156.
- 420 8. Cheng, J., Su, H., Zhou, J., Song, W., Cen, K., 2011. Microwave-assisted alkali
- 421 pretreatment of rice straw to promote enzymatic hydrolysis and hydrogen production
- 422 in dark- and photo-fermentation. Int. J. Hydrogen Energy 36, 2093–2101.

| 423 | 9.  | Danilenko, A.N., Bogomolov, A.A., Yuryev, V.P., Dianova, V.T., Bogatyrev, A.N., 1993.      |
|-----|-----|--|
| 424 |     | Effect of the Polymerization Degree, Moisture Content, and Temperature on Kinetics         |
| 425 |     | of Hydrolysis of Corn Starch by Alpha-Amylase. Starch - Stärke 45, 63–65.                  |
| 426 | 10. | De Amorim, E.L.C., Sader, L.T., Silva, E.L., 2012. Effect of substrate concentration on    |
| 427 |     | dark fermentation hydrogen production using an anaerobic fluidized bed reactor. Appl.      |
| 428 |     | Biochem. Biotechnol. 166, 1248–1263.   |
| 429 | 11. | Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves     |
| 430 |     | sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.                  |
| 431 | 12. | Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., |
| 432 |     | 2015. A review on dark fermentative biohydrogen production from organic biomass:           |
| 433 |     | Process parameters and use of by-products. Appl. Energy 144, 73–95.                        |
| 434 | 13. | Guo, X.M., Trably, E., Latrille, E., Carrere, H., Steyer, JP., 2014. Predictive and        |
| 435 |     | explicative models of fermentative hydrogen production from solid organic waste:           |
| 436 |     | Role of butyrate and lactate pathways. Int. J. Hydrogen Energy 39, 7476–7485.              |
| 437 | 14. | Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, JP., 2010. Hydrogen production   |
| 438 |     | from agricultural waste by dark fermentation: A review. Int. J. Hydrogen Energy 35,        |
| 439 |     | 10660–10673.   |
| 440 | 15. | Hawkes, F., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D., 2007. Continuous dark         |
| 441 |     | fermentative hydrogen production by mesophilic microflora: Principles and progress.        |
| 442 |     | Int. J. Hydrogen Energy 32, 172–184.   |
| 443 | 16. | Huang, Y., Zong, W., Yan, X., Wang, R., Hemme, C.L., Zhou, J., Zhou, Z., 2010.             |
| 444 |     | Succession of the bacterial community and dynamics of hydrogen producers in a              |
| 445 |     | hydrogen-producing bioreactor. Appl. Environ. Microbiol. 76, 3387–3390.                    |
| 446 | 17. | Hung, CH., Chang, YT., Chang, YJ., 2011. Roles of microorganisms other than                |
| 447 |     | Clostridium and Enterobacter in anaerobic fermentative biohydrogen production              |

448 systems – A review. Bioresour. Technol. 102, 8437–8444.

- 449 18. Kahm, M., Kahm, M., Hasenbrink, G., Lichtenberg-Frate, H., Ludwig, J., Kschischo, M.,
  450 2010. Grofit: Fitting biological growth curves. Nat. Preced. 33, 1–21.
- 451 19. Kalia, V.C., Purohit, H.J., 2008. Microbial diversity and genomics in aid of bioenergy. J.
  452 Ind. Microbiol. Biotechnol. 35, 403–419.
- 453 20. Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for
  454 bioenergy production. Curr. Opin. Biotechnol. 18, 207–212.
- 455 21. Kotay, S.M., Das, D., 2009. Novel dark fermentation involving bioaugmentation with

456 constructed bacterial consortium for enhanced biohydrogen production from

457 pretreated sewage sludge. Int. J. Hydrogen Energy 34, 7489–7496.

458 22. Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013.

- 459 Development of a dual-index sequencing strategy and curation pipeline for analyzing
- 460 amplicon sequence data on the miseq illumina sequencing platform. Appl. Environ.
- 461 Microbiol. 79, 5112–5120.

462 23. Kumar, P., Kumar, P., Barrett, D.M., Barrett, D.M., Delwiche, M.J., Delwiche, M.J.,

- 463 Stroeve, P., Stroeve, P., 2009. Methods for Pretreatment of Lignocellulosic Biomass for
- 464 Ef cient Hydrolysis and Biofuel Production. Ind. Eng. Chem. Analytical Ed. 3713–3729.
- 465 24. Kuo, W.-C., Chao, Y.-C., Wang, Y.-C., Cheng, S.-S., 2012. Bioaugmentation Strategies to
  466 Improve Cellulolytic and Hydrogen Producing Characteristics in CSTR Intermittent Fed
  467 with Vegetable Kitchen Waste and Napiergrass. Energy Procedia 29, 82–91.
- 468 25. Li, D., Chen, H., 2007. Biological hydrogen production from steam-exploded straw by
  469 simultaneous saccharification and fermentation. Int. J. Hydrogen Energy 32, 1742–
- 470 1748.
- 471 26. Mäkinen, A.E., Nissilä, M.E., Puhakka, J.A., 2012. Dark fermentative hydrogen
- 472 production from xylose by a hot spring enrichment culture. Int. J. Hydrogen Energy 37,

473 12234–12240.

- 474 27. Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuels, platform
  475 chemicals & biorefinery concept. Prog. Energy Combust. Sci. 38, 522–550.
- 476 28. Mohana, S., Shah, A., Divecha, J., Madamwar, D., 2008. Xylanase production by
- 477 *Burkholderia* sp. DMAX strain under solid state fermentation using distillery spent
  478 wash. Bioresour. Technol. 99, 7553–7564.
- 479 29. Monlau, F., Barakat, A., Steyer, J.P., Carrere, H., 2012a. Comparison of seven types of
  480 thermo-chemical pretreatments on the structural features and anaerobic digestion of
  481 sunflower stalks. Bioresour. Technol. 120, 241–7.
- 482 30. Monlau, F., Sambusiti, C., Barakat, A., Guo, X.M., Latrille, E., Trably, E., Steyer, J.-P.,
- 483 Carrere, H., 2012b. Predictive Models of Biohydrogen and Biomethane Production
- 484 Based on the Compositional and Structural Features of Lignocellulosic Materials.

485 Environ. Sci. Technol. 46, 12217–12225.

- 486 31. Nissilä, M.E., Lay, C.-H., Puhakka, J. a., 2014. Dark fermentative hydrogen production
- 487 from lignocellulosic hydrolyzates A review. Biomass and Bioenergy 67, 145–159.
- 488 32. Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B.,
- 489 Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2016. vegan: Community
- 490 Ecology Package. R package version 2.3-3. http://CRAN.R-project.org/package=vegan.
- 491 33. Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of Phylogenetics and Evolution
  492 in R language. Bioinformatics 20, 289–290.
- 493 34. Porwal, S., Kumar, T., Lal, S., Rani, A., Kumar, S., Cheema, S., Purohit, H.J., Sharma, R.,
- 494 Singh Patel, S.K., Kalia, V.C., 2008. Hydrogen and polyhydroxybutyrate producing
- 495 abilities of microbes from diverse habitats by dark fermentative process. Bioresour.
  496 Technol. 99, 5444–5451.
- 497 35. Qiu, C., Zheng, Y., Zheng, J., Liu, Y., Xie, C., Sun, L., 2016. Mesophilic and Thermophilic

| 498 |     | Biohydrogen Production from Xylose at Various Initial pH and Substrate                        |
|-----|-----|---|
| 499 |     | Concentrations with Microflora Community Analysis. Energy & Fuels 30, 1013–1019.              |
| 500 | 36. | Quéméneur, M., Hamelin, J., Barakat, A., Steyer, JP., Carrère, H., Trably, E., 2012.          |
| 501 |     | Inhibition of fermentative hydrogen production by lignocellulose-derived compounds            |
| 502 |     | in mixed cultures. Int. J. Hydrogen Energy 37, 3150–3159.                                     |
| 503 | 37. | Quéméneur, M., Hamelin, J., Benomar, S., Guidici-Orticoni, MT., Latrille, E., Steyer, J       |
| 504 |     | P., Trably, E., 2011. Changes in hydrogenase genetic diversity and proteomic patterns         |
| 505 |     | in mixed-culture dark fermentation of mono-, di- and tri-saccharides. Int. J. Hydrogen        |
| 506 |     | Energy 36, 11654–11665.   |
| 507 | 38. | Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., Giudici- |
| 508 |     | Orticoni, MT., Steyer, JP., 2013. Sub-dominant bacteria as keystone species in                |
| 509 |     | microbial communities producing bio-hydrogen. Int. J. Hydrogen Energy 38, 4975–               |
| 510 |     | 4985.   |
| 511 | 39. | Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed                     |
| 512 |     | cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38, 13172–          |
| 513 |     | 13191.  |
| 514 | 40. | Sambusiti, C., Monlau, F., Ficara, E., Carrère, H., Malpei, F., 2013. A comparison of         |
| 515 |     | different pre-treatments to increase methane production from two agricultural                 |
| 516 |     | substrates. Appl. Energy 104, 62–70.  |
| 517 | 41. | Saratale, G.D., Chen, S., Lo, Y., Saratale, R.G., Chang, J., 2008. Outlook of biohydrogen     |
| 518 |     | production from lignocellulosic feedstock using dark fermentation – a review. J. Sci.         |
| 519 |     | Ind. Res. 67, 962–979.  |
| 520 | 42. | Saur, T., Escudié, R., Santa-Catalina, G., Bernet, N., Milferstedt, K., 2016. Conservation    |
| 521 |     | of acquired morphology and community structure in aged biofilms after facing                  |
| 522 |     | environmental stress. Water Res. 88, 164–172.   |

| 523 | 43. | Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B.,    |
|-----|-----|--|
| 524 |     | Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B.,      |
| 525 |     | Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: Open-source,    |
| 526 |     | platform-independent, community-supported software for describing and comparing          |
| 527 |     | microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.                          |
| 528 | 44. | Stülke, J., Hillen, W., 2000. Regulation of Carbon Catabolism in Bacillus Species. Annu. |
| 529 |     | Rev. Microbiol. 54, 849–880.   |
| 530 | 45. | UNFCCC. Conference of the Parties (COP), 2015. Adoption of the Paris Agreement.          |
| 531 |     | Proposal by the President., in: Paris Climate Change Conference - November 2015, COP     |
| 532 |     | 21. Paris, p. 32.  |
| 533 | 46. | Vu, V.Q., 2011. ggbiplot: A ggplot2 based biplot. R package version 0.55.                |
| 534 |     | http://github.com/vqv/ggbiplot.  |
| 535 | 47. | Wang, J., Wan, W., 2009. Factors influencing fermentative hydrogen production: A         |
| 536 |     | review. Int. J. Hydrogen Energy 34, 799–811.   |
| 537 | 48. | Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A., Lumley,    |
| 538 |     | T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., Venables, B., 2016.          |
| 539 |     | Package "gplots", Various R Programming Tools for Plotting Data.                         |
| 540 | 49. | Wong, Y.M., Wu, T.Y., Juan, J.C., 2014. A review of sustainable hydrogen production      |
| 541 |     | using seed sludge via dark fermentation. Renew. Sustain. Energy Rev. 34, 471–482.        |
| 542 | 50. | Zhang, B., He, PJ., Lü, F., Shao, LM., Wang, P., 2007. Extracellular enzyme activities   |
| 543 |     | during regulated hydrolysis of high-solid organic wastes. Water Res. 41, 4468–78.        |
| 544 |     |  |

### 545 **Figure captions**

546 Figure 1: Production of hydrogen analysed for each substrate during the 547 experiment  $ml_{H2}$ .g-1 of initial substrate eq.COD for a) glucose ( $\bigcirc$ ), cellobiose ( $\bigcirc$ ), 548 arabinose (•), xylose (•), xylan (•), and wheat straw (•), and b) for MCC (•).Error 549 bars correspond to standard deviation observed between each replicate. 550 Figure 2: Principal component analysis done on metabolite production. Symbols 551 correspond to each substrate, Glucose ( $\bigcirc$ ), Cellobiose ( $\square$ ), Microcrystalline cellulose ( •), Arabinose ( $\Delta$ ), Xvlose ( $\nabla$ ), Xvlan ( $\diamond$ ) and Wheat Straw ( $\overset{\frown}{\prec}$ ). 552 553 Figure 3: Principal coordinate analysis (PCoA) done on OTUs after Hellinger 554 transformation. Symbols correspond to each substrate, Glucose ( $\bigcirc$ ), Cellobiose ( $\square$ ), Microcrystalline cellulose ( $\bigcirc$ ), Arabinose ( $\triangle$ ), Xylose ( $\bigtriangledown$ ), Xylan ( $\diamondsuit$ ) and Wheat 555 Straw ( 🛣 ). 556 557 Figure 4: Major OTUs identified in reactors for each substrate (relative 558 abundance >5%). White colour corresponds to absence of identified OTU, light blue a 559 low relative abundance, and black relative abundance close to the maximum measured 560 (38%). 561 Figure 5: Principal component analysis ordination plot of major bacteria 562 composing the sample bacterial community (a) with metabolite variables as predictors 563 onto the ordination (b) analysed by the *envfit* function (permutations 9999, R package 564 *vegan*). Symbols correspond to substrates: Glucose ( $\bigcirc$ ), Cellobiose ( $\square$ ), Microcrystalline cellulose ( $\bigcirc$ ), Arabinose ( $\triangle$ ), Xylose ( $\bigtriangledown$ ), Xylan ( $\diamondsuit$ ) and Wheat 565 Straw ( $\bigstar$ ); and to number of Operation Taxonomic Unit (OTU, +). Stars following 566 metabolite name represent the significance of the fit (\*\*\*<0.001; \*\*<0.01, \*<0.05). 567

| 568 | <b><u>Table 1</u></b> : Kinetic parameters of hydrogen production in batch tests according to |
|-----|---|
| 569 | the initial substrate concentration determined from modified Gompertz equation with F         |
| 570 | the maximum cumulative hydrogen production (mlH2.g-1eq initial COD), Rm the                   |
| 571 | maximum hydrogen production rate (mlH2.g-1eq initial COD.d-1), $\lambda$ the lag phase        |
| 572 | (days) and $R^2$ the coefficient of determination of the model. Values correspond to the      |
| 573 | average of four replicates $\pm$ standard deviation observed between these replicates.        |
| 574 | Values in % in the H2 yield column correspond to the proportion of the maximum                |
| 575 | theoretical value suggested by Hawkes et al. (2007), i.e 2.5 molH2.mol-1 degraded             |
| 576 | substrate in glucose equivalent.  |

577 <u>**Table 2**</u>: Metabolites accumulated at the end of the fermentation tests (in mg 578  $_{product \ eq. \ COD.\ g_{initial \ substrate \ eq. \ COD}^{-1}.)$  Values correspond to the average of four replicates ± 579 standard deviation. Values in italic and in parenthesis correspond to the proportion (in 580 %) of each product among all the metabolites produced in a same batch test ± standard 581 deviation.

582**Table 3**: Hydrogen yields, metabolic patterns and main identified bacteria583obtained from dark fermentation performed on different inocula and substrates in the584literature. Units of hydrogen yield, H2 column, correspond to a)  $mol_{H2}.mol^{-1}substrate$ , b)585 $mmol_{H2}.g^{-1}vs$ , c)  $mmol_{H2}.g^{-1}substrate$ , d)  $mmol_{H2}.g^{-1}Tvs$  and e)  $mmol_{H2}./g^{-1}DCO$ .586Metabolites are expressed in % of COD converted and abbreviations correspond to587Acetate (HAc), Butyrate (HBut); Caproate (HCap), Ethanol (EtOH), Lactate (HLac),588Propionate (Hpr), Formate (Hfor), Valerate (HVal) and Succinate (HSuc).

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Figure Click here to download Figure: FIG3.pptx







**Table 1**: Kinetic parameters of hydrogen production in batch tests according to the initial substrate concentration determined from modified Gompertz equation with P the maximum cumulative hydrogen production (mlH2.g-1eq initial COD), Rm the maximum hydrogen production rate (mlH2.g-1eq initial COD.d-1),  $\lambda$  the lag phase (days) and R<sup>2</sup> the coefficient of determination of the model. Values correspond to the average of four replicates ± standard deviation observed between these replicates. Values in % in the H2 yield column correspond to the proportion of the maximum theoretical value suggested by Hawkes et al. (2007), i.e 2.5 molH2.mol-1 degraded substrate in glucose equivalent.

| Cubatrata  | Go  | H <sub>2</sub> yield  |           |             |                  |  |
|------------|---|---|-----------|-------------|------------------|--|
| Substrate  | P<br>(ml <sub>H2</sub> .g <sup>-1</sup> <sub>eq.COD initial</sub> ) | $\begin{array}{c} Rm \\ (ml_{H2}.g^{-1}_{eq.COD initial}.d^{-1}) \end{array}$ | λ (days)  | R²          | COD consumed)    |  |
| Glucose    | 31±12   | 29±12   | 1.9±0.2   | 0.999±0.001 | 1.7±0.6 (13±5 %) |  |
| Cellobiose | 23±2  | 12±1  | 2.1±0.2   | 0.999±0.001 | 4.2±0.6 (32±4 %) |  |
| Cellulose  | 12±5  | 1.5±0.6   | 12.1±0.5  | 0.997±0.004 | 8±4 (62±31 %)    |  |
| Arabinose  | 67±6  | 32±4  | 2.04±0.06 | 0.999±0.001 | 6.4±0.4 (49±3 %) |  |
| Xylose     | 94±17   | 40±11   | 2.4±0.3   | 0.999±0.001 | 6±1 (44±8 %)     |  |
| Xylan      | 32±5 1±4  |   | 3.2±0.2   | 0.999±0.001 | 4.1±0.6 (31±5 %) |  |
| Straw      | 10±9  | 13±13   | 3±1       | 0.998±0.002 | 1.3±0.6 (10±4 %) |  |

<u>**Table 2**</u>: Metabolites accumulated at the end of the fermentation tests (in mg product eq. COD.ginitial substrate eq. COD-1.) Values correspond to the average of four replicates  $\pm$  standard deviation. Values in italic and in parenthesis correspond to the proportion (in %) of each product among all the metabolites produced in a same batch test  $\pm$  standard deviation.

| Sample       | Acetate           | Butyrate          | Isobutyrate        | Propionate           | Lactate          | Ethanol                        | Hydrogen           | COD converted (%) |
|--------------|-------------------|-------------------|--------------------|----------------------|------------------|--------------------------------|--------------------|-------------------|
| Glucose      | 20±8<br>(2.4±0.9) | 116±42<br>(14±5)  | /                  | /                    | 592±11<br>(76±6) | 44±4<br>(5.5±0.4)              | 21±8<br>(2.5±0.9)  | 79±3              |
| Cellobiose   | 11±4<br>(5±2)     | 11±11<br>(5±5)    | /                  | /                    | 165±12<br>(73±2) | 23±3<br>(11±1)                 | 15±2<br>(6.8±0.7)  | 23±1              |
| MC-Cellulose | 25±5<br>(54±7)    | 7±2<br>(19±9)     | /                  | 8±3<br>(15±5)        | /                | $0.6{\pm}0.6 {(1{\pm}1)}$      | 6±3<br>(11±6)      | 4.68±0.03         |
| Arabinose    | 86±7<br>(20±3)    | 146±46<br>(31±8)  | 54±6<br>(12.5±0.6) | /                    | 48±17<br>(13±5)  | 61±30<br>(13±5)                | 45±3<br>(10.5±0.8) | 44±6              |
| Xylose       | 118±14<br>(19±2)  | 247±82<br>(34±10) | 35±18<br>(5±2)     | 72±35<br>(14±7)      | 6±6<br>(0.9±0.9) | 104±16<br>(18±4)               | 62±11<br>(9.6±0.7) | 64.4±0.9          |
| Xylan        | 111±20<br>(35±7)  | 88±50<br>(26±14)  | 8±2<br>(2.4±0.4)   | 12±7<br>(4±2)        | /                | 83±19<br>(25±6)                | 21±3<br>(7±1)      | 32±2              |
| Wheat Straw  | 38±7<br>(50±5)    | 26±3<br>(35±5)    | 4±2<br>(5±2)       | 4.7±0.6<br>(6.3±0.8) | /                | $0.7{\pm}0.7$<br>(1 ${\pm}1$ ) | 1.6±0.7<br>(2±1)   | 8±1               |

**Table 3**: Hydrogen yields, metabolic patterns and main identified bacteria obtained from dark fermentation performed on different inocula and substrates in the literature. Units of hydrogen yield, H<sub>2</sub> column, correspond to a)  $mol_{H2}.mol_{substrate}^{-1}$ , b)  $mmol_{H2}.g_{VS}^{-1}$ , c)  $mmol_{H2}.g_{substrate}^{-1}$ , d)  $mmol_{H2}.g_{TVS}^{-1}$  and e)  $mmol_{H2}./g_{DCO}^{-1}$ . Metabolites are expressed in % of COD converted and abbreviations correspond to Acetate (HAc), Butyrate (HBut); Caproate (HCap), Ethanol (EtOH), Lactate (HLac), Propionate (Hpr), Formate (Hfor), Valerate (HVal) and Succinate (HSuc).

| Inoculum                      | Substrate   | H2                | HAc | HBut | EtOH | HPr | HLac | HFor | HVal | НСар | HSuc | HiBut | Major Bacteria   | References                  |
|-------------------------------|-------------|-------------------|-----|------|------|-----|------|------|------|------|------|-------|--|-----------------------------|
|                               | fructose    | 1.84 <sup>a</sup> | 34% | 59%  | 6%   | /   | 1%   | /    | /    | 0%   | /    | /     | C. sporogenes  |                             |
| _                             | glucose     | 1.79 <sup>ª</sup> | 33% | 60%  | 6%   | /   | 1%   | /    | /    | 0%   | /    | /     | C. sporogenes  |                             |
| _                             | sucrose     | 1.67 <sup>ª</sup> | 86% | 13%  | 1%   | /   | 0%   | /    | /    | 0%   | /    | /     | C. sporogenes  |                             |
| anaorohically                 | maltose     | 1.65 <sup>a</sup> | 36% | 58%  | 4%   | /   | 0%   | /    | /    | 1%   | /    | /     | C. acetobutylicum  | (Quámánour et al            |
| digested sludge               | cellobiose  | 1.56°             | 35% | 52%  | 9%   | /   | 2%   | /    | /    | 1%   | /    | /     | C. cellulolyicum; C.<br>sporogenes; C.<br>Saccharobutylicum<br>; C. kluyveri; C.<br>acetobutylicum | (Quemeneur et al.,<br>2011) |
|                               | maltotriose | 1.38 <sup>ª</sup> | 31% | 48%  | 17%  | /   | 3%   | /    | /    | 1%   | /    | /     | C. acetobutylicum  |                             |
| hot spring culture            | xylose      | 0.7 <sup>ª</sup>  | 25% | 34%  | 5%   | 0%  | 1%   | 34%  | /    | /    | /    | /     | C. acetobutylicum;<br>C. tyrobutyricum   | (Mäkinen et al.,            |
|                               | arabinose   | 0.42 <sup>a</sup> | 23% | 31%  | 13%  | 0%  | 8%   | 25%  | /    | /    | /    | /     | /  | 2012)                       |
| seed sludge                   | xylose      | 2.24 <sup>ª</sup> | 17% | 62%  | 13%  | 8%  | /    | /    | /    | /    | /    | /     | C.<br>saccharobutylicum;<br>Clostridiales sp   | (Qiu et al., 2016)          |
| anaerobic<br>activated sludge | rice straw  | 6 <sup>d</sup>    | 67% | 25%  | 7%   | /   | /    | /    | /    | /    | /    | /     | Clostridium sp.  | (Cheng et al.,<br>2011)     |
| C. butyricum<br>AS1.209       | Corn straw  | 2.6 <sup>c</sup>  | 46% | 28%  | 7%   | 19% | /    | /    | /    | /    | /    | /     | C. butyricum<br>AS1.209 (%)  | (Li and Chen, 2007)         |
| Mesophilic<br>digested sludge |             | 6.1 <sup>e</sup>  | 15% | 80%  | 2%   | 0%  | 0%   | 3%   | /    | /    | 0%   | /     | C. acetobutylicum;<br>C. butyricum   |                             |
| Soybean meal                  |             | 5.6 <sup>e</sup>  | 27% | 40%  | 31%  | 0%  | 0%   | 2%   | /    | /    | 0%   | /     | C. acetobutylicum;<br>C. butyricum   |                             |
| Kitchen Waste                 | Starch      | 5.1 <sup>e</sup>  | 20% | 71%  | 2%   | 0%  | 0%   | 6%   | /    | /    | 1%   | /     | C. paraputrificum;<br>Citrobacter freufii  | (Akutsu et al.,<br>2009)    |
| Cattle Manure                 |             | 4.6 <sup>e</sup>  | 23% | 49%  | 22%  | 0%  | 0%   | 7%   | /    | /    | 0%   | /     | C. acetobutylicum;<br>C. butyricum   |                             |
| Activated Sludge              |             | 3.5 <sup>e</sup>  | 36% | 5%   | 54%  | 2%  | 0%   | 3%   | /    | /    | 0%   | /     | Bacteroides<br>eggerthii; C.   |                             |

| Inoculum                          | Substrate   | H2                | HAc  | HBut | EtOH | HPr  | HLac | HFor | HVal | HCap | HSuc | HiBut | Major Bacteria   | References |
|-----------------------------------|-------------|-------------------|------|------|------|------|------|------|------|------|------|-------|--|------------|
|                                   |             |                   |      |      |      |      |      |      |      |      |      |       | pasteurianum   |            |
| Thermophilic<br>Digested Sludge   |             | 2.2 <sup>e</sup>  | 42%  | 7%   | 51%  | 0%   | 0%   | 0%   | /    | /    | 0%   | /     | Clostridium sp.;<br>Bacillus sp.   |            |
| Soil                              |             | 2 <sup>e</sup>    | 31%  | 2%   | 51%  | 10%  | 0%   | 2%   | /    | /    | 4%   | /     | Bacteroides<br>eggerthii   |            |
| Thermophilic<br>Acidogenic Sludge |             | 0.7 <sup>e</sup>  | 22%  | 0%   | 66%  | 0%   | 1%   | 4%   | /    | /    | 7%   | /     | Bacteroides sp.; C.<br>aminovalericum                                      |            |
|                                   | Glucose     | 0.60 <sup>a</sup> | 2.4% | 14%  | 5.5% | /    | 76%  | /    | /    | /    | /    | /     | <i>Clostridium</i> sp.;<br><i>Bacillus</i> sp.<br><i>Paenibacillus</i> sp. |            |
|                                   | Cellobiose  | 0.36 <sup>a</sup> | 5%   | 5%   | 11%  | /    | 73%  | /    | /    | /    | /    | /     | Bacillus sp.<br>Paenibacillus sp.  |            |
|                                   | MCC         | 6 <sup>e</sup>    | 54%  | 19%  | 1%   | 8%   | /    | /    | /    | /    | /    | /     | Ruminococcaceae  |            |
| Manure anaerobic                  | Arabinose   | 0.45 <sup>ª</sup> | 20%  | 31%  | 13%  | /    | 13%  | /    | /    | /    | /    | 12.5% | <i>Clostridium</i> sp.;<br><i>Bacillus</i> sp.<br><i>Paenibacillus</i> sp. | This study |
| ugestate                          | Xylose      | 0.62 <sup>ª</sup> | 19%  | 34%  | 18%  | 14%  | 0.9% | /    | /    | /    | /    | 5%    | <i>Clostridium</i> sp.;<br><i>Bacillus</i> sp.<br><i>Paenibacillus</i> sp. |            |
|                                   | Xylan       | 21 <sup>e</sup>   | 35%  | 26%  | 25%  | 4%   | /    | /    | /    | /    | /    | 2.4%  | Ruminococcaceae;<br>Clostridium sp.;<br>Bacillus sp.<br>Paenibacillus sp.  |            |
|                                   | Wheat straw | 1.6 <sup>e</sup>  | 50%  | 35%  | 1%   | 6.3% | /    | /    | /    | /    | /    | 5%    | Ruminococcaceae  |            |