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1 **Co-ensiling as a new technique for long-term storage of agro-industrial waste prior**
2 **to anaerobic digestion**

3 Marie-Lou Hillion^{a,b}, Roman Moscoviz^a, Eric Trably^a, Yoann Leblanc^b, Nicolas
4 Bernet^a, Michel Torrijos^a, Renaud Escudie^{a,*}

5 ^aLBE, INRA, 102 avenue des Etangs, 11100 Narbonne, France

6 ^bVol-V Biomasse, 45 impasse du Petit Pont, 76230 Isneauville, France

7 * Corresponding author: tel. +33 468 425 173, e-mail: renaud.escudie@inra.fr

8 **Abstract**

9 Biodegradable wastes produced seasonally need an upstream storage, because of
10 the requirement for a constant feeding of anaerobic digesters. In the present article, the
11 potential of co-ensiling biodegradable agro-industrial waste (sugar beet waste) and
12 lignocellulosic agricultural residue (wheat straw) was evaluated for long-term storage
13 prior to anaerobic digestion. Co-ensiling was tested in bags at lab-scale during 180 days.
14 Characterization of the reaction by-products and microbial communities showed a
15 succession of metabolic pathways. Even though the low initial sugars content was not
16 sufficient to lower the pH under 4.5 and avoid undesirable fermentations, the methane
17 potential was not substantially impacted all along the experiment. No lignocellulosic
18 damages were observed during the silage process. Overall, it was shown that co-ensiling
19 was effective to store highly fermentable fresh waste and offers new promising
20 possibilities for constant long-term supply of industrial anaerobic digesters.

21
22 **Keywords**

23 Storage; Silage; Wheat straw; Lactic acid fermentation; Mixed culture; BMP

24 **1. Introduction**

25 Over the past decades, human population growth has led to increasing quantities
26 of waste and a stronger demand in energy and more especially in fossil fuels. The total
27 energy consumption is expected to increase by 48% from 2012 to 2040 (U.S. Energy
28 Information Administration, 2016). As a consequence, the production of renewable
29 energy from waste represents a sustainable alternative to fossil fuel consumption with
30 concomitant waste treatment. Among the other renewable technologies, anaerobic
31 digestion (AD) has recently received an increasing amount of attention worldwide. AD
32 consists in the biological conversion of organic matter into a methane-rich biogas that
33 can further be used for energetic purposes (i.e., heat, steam, electricity, biofuel). A
34 digestate that can be further used as organic fertilizer in agriculture is also generated.
35 Anaerobic digestion presents the advantage to treat various kinds of organic wastes,
36 such as co-products generated from industrial or agricultural productions. Among them,
37 waste issued from the agri-food industry presents a high potential through high
38 availability, large quantities and high performances in anaerobic digestion processes,
39 with an average biochemical methane potential (BMP) of about $400 \text{ NL}_{\text{CH}_4} \cdot \text{kg}_{\text{VS}}^{-1}$
40 (Fisgativa et al., 2017). However, agro-industrial waste is most often produced
41 seasonally. Considering that an anaerobic digester must be continuously fed throughout
42 the year, such seasonal waste has to be stored prior to use. This type of feedstock is
43 characterized by a high moisture content ($77.2 \pm 10.0 \%$) and a high amount of
44 carbohydrates and proteins ($36.4 \pm 20.8\%_{\text{VS}}$ and $21.0 \pm 13.0\%_{\text{VS}}$, respectively) (Fisgativa
45 et al., 2016), resulting in a high degradability and low stability at ambient temperature.
46 As a consequence, controlling long-term storage is crucial to prevent biological
47 degradation and avoid losses in methane potential.

48 Up to now, the preservation of biomass nutrient and energy was ensured by three
49 main types of storage technologies: freezing, drying and ensiling (Egg et al., 1993).
50 However, freezing and mechanical drying are rather uneconomic and are not suitable
51 for long-term waste storage (Madhukara et al., 1993). A way to reduce costs is to carry
52 out natural drying (i.e., field wilting). However this process is weather dependent and
53 inefficient to conserve the methane potential, as shown by Teixeira Franco et al. (2017).
54 As last option, ensiling was first developed and applied to preserve the nutritive value of
55 animal feed such as forages in anaerobic conditions, and could represent a feasible and
56 cost-effective technology for conservation and storage of similar feedstock prior to AD
57 (Teixeira Franco et al., 2016). The ensiling process is divided in four main biochemical
58 and microbiological conversion steps (Rooke and Hatfield, 2003). First, the aerobic
59 phase consists in fast consumption of residual oxygen. Second, when all oxygen is
60 removed, an anaerobic phase occurs where lactic acid fermentation takes place. This
61 lactic acid fermentation converts soluble carbohydrates into lactic acid, with a
62 concomitant drop of pH below 4.5. Such acidification of the medium inhibits the
63 microbial activity (Ambye-Jensen et al., 2013b) favoring thus long-term preservation
64 and stabilization of the organic matter, all along this third step (i.e., storage period).
65 Finally, during the “feed-out step”, silos are opened to use silage and partial aerobic
66 degradation can occur (Herrmann et al., 2011). The objective of ensiling before
67 anaerobic digestion is to rapidly reach the stabilization phase to minimize energy losses
68 and conserve the methane potential. According to Kafle and Kim (2013), ensiling
69 agricultural or food processing co-products represents an adequate solution to store
70 seasonal and perishable feedstocks. Success of the silage process is impacted by the
71 intrinsic biochemical characteristics of the feedstock. For this reason, substrates with

72 high water-soluble carbohydrates and low buffering capacities are preferred (Teixeira
73 Franco et al., 2016). In addition, ensiling is commonly operated at TS contents ranging
74 between 25% and 35% (Liu et al., 2016) to limit the release of leachate and avoid
75 undesirable microbial activities (Teixeira Franco et al., 2016).

76 Because of their high water content and high fermentability, agro-industrial
77 organic waste alone are rather not suitable for ensiling (Piltz and Kaiser, 2004).
78 Incorporating a second substrate characterized by a high TS content could represent a
79 solution to absorb the excess of water and provide a physical structure to the silage
80 mixture. As an illustration, tomato pomace was well preserved when co-ensiled with
81 wheat grain or straw as bio-sorbent (Denek and Can, 2006). In this context, a bio-
82 sorbent (i.e., co-substrate) has not only to be available at a low cost, but also provide a
83 positive (i.e., stabilizing) effect on the silage mixture. According to Haigh and Farmers
84 (1998), straw can be one of the best absorbent. Indeed, with a worldwide production of
85 760.1Mt in 2016 (Trade and Market Division, 2017), wheat is one of the most widely
86 cultivated crop and the net worldwide production of wheat straw reached about 988Mt
87 in 2016 (Zahoor and Tu, 2014). Moreover wheat straw is known as a recalcitrant
88 biomass for anaerobic digestion due to its lignocellulosic structure (Zhao et al., 2012).
89 The silage process could therefore improve its bioconversion into gas through partial
90 acid hydrolysis known to partly damage the lignocellulosic matrix (Ambye-Jensen et
91 al., 2013a).

92 This work aims to assess the feasibility of co-ensiling a mixture of fresh seasonal
93 agro-industrial waste (i.e., sugar beet waste) together with agricultural waste (i.e., wheat
94 straw) for long-term storage prior to anaerobic digestion. The specific objective of
95 ensiling these co-substrates were (1) to preserve the methane potential of the agro-

96 industrial organic waste throughout the storage period and (2) to partially destructure
97 the lignocellulosic materials through the acidification of the medium. This experimental
98 work was performed at laboratory-scale with plastic vacuum-packed bags as silos.
99 Dynamical changes of the fermentative pathways as well as the impact on the methane
100 potential all along the ensiling process were more particularly investigated.

101 **2. Materials and Methods**

102 *2.1. Substrate origin*

103 The agro-industrial waste was collected from a Sugar Company in Calvados,
104 France. This waste consisted of leaves, weeds and small pieces of roots resulting from
105 sugar beet processing. The sugar beet residue was cut by hand before being mixed with
106 wheat straw.

107 The agricultural waste consisted of wheat straw (*Triticum aestivum*) harvested
108 with a rotary combine in an agricultural field at La Marne, France. Wheat straw was
109 then grinded by a hammer-cylinder mill to ensure a final particle size ranging from 0.1
110 mm to 10 mm (Lessines industries). Such grinding is recommended to reduce the
111 particle size and enhance lactic acid fermentation during ensiling (Herrmann et al.,
112 2012).

113 *2.2. Experimental set-up*

114 In order to reach a final TS content of 32.7%, 157.7 ± 0.3 g of wheat straw and
115 42.3 ± 0.1 g of sugar beet waste were mixed together. 200g/bag of this mixture was then
116 packed into 50 plastic vacuum-packed bags (18.4cm x 28.9cm) to be representative of a
117 typical ensiling process (Cherney et al., 2004). Johnson et al. (2005) have previously
118 demonstrated that vacuum packing constitutes a reliable and accurate model system for
119 laboratory-scale silage. The vacuum-packed bags were then sealed by a “Golden star”

120 chamber vacuum packaging machine to remove oxygen, and stored in a dark room at
121 15°C.

122 In order to investigate the dynamics of the silage process, three bags were
123 periodically frozen (-20°C) prior to analysis. Nine different storage times were
124 characterized: 0, 2, 7, 14, 21, 30, 60, 120 and 180 days. As ensiling takes less than one
125 week to start-up, frequent analyses were carried out at the beginning of the experiment
126 (Vargas-Ramirez et al., 2016). All analyses were performed at each storage time on
127 three plastics bags considered as replicates.

128 2.3. Silage characterization

129 Before analyses, silage samples were thoroughly mixed in a blender and
130 homogenized.

131 Total Solid (TS) content was measured in triplicate on initial substrates and on
132 each replicated bag, by drying about 5g of fresh matter (FM) during 48 hours at 105°C.
133 Volatile Solid (VS) content was then measured by calcination at 550°C during 3 hours.
134 Because of the loss of volatile compounds during the drying operation, the TS content
135 value was corrected as proposed by Weissbach et al. (2008) with the following equation
136 (Eq. 1):

$$137 \quad TS_{Corrected}(g.kg_{FM}^{-1}) = TS_{measured}(g.kg_{FM}^{-1}) + 0.95 \times$$
$$138 \quad volatile\ fatty\ acids(g.kg_{FM}^{-1}) + 1.00 \times n\ alcohols(g.kg_{FM}^{-1}) + 0.77 \times$$
$$139 \quad 1.3propanediol(g.kg_{FM}^{-1}) + 0.08 \times lactic\ acid(g.kg_{FM}^{-1}) \text{ (Eq. 1)}$$

140 In the same way, VS contents were also corrected. All results were thus expressed
141 according to these corrected values of TS and VS contents.

142 Fibre distributions of soluble, hemicellulose, cellulose and lignin were
143 determined in duplicate on the initial substrates. Substrates were dried and grounded at

144 0.4-1mm, according to the modified Van Soest procedure (Van Soest and Wine, 1967)
145 as described in Motte et al. (2014). The soluble fraction was corrected by taking into
146 account the evaporation of soluble compounds.

147 The pH was measured with a SenTix®41 electrode plugged on a WTW
148 inoLab®pH7110, on the soluble phase obtained on initial substrates and on each
149 replicated bag after diluting the silage with milliQ water at around 10-15% TS during
150 1h. Before pH analysis, leachate was centrifuged at 18 592g for 15 min and filtrated
151 through a 40 µm nylon filter.

152 2.4. *Biological by-products characterization*

153 The biogas production was estimated and collected with a water displacement
154 method, before opening the replicated plastic vacuum-packed bags. The biogas
155 composition was determined by gas chromatography (Perkin Elmer Clarus®580). After
156 injection of 200 µL of gas sample, CO₂ was separated from other gases on a capillary
157 column R-Q-bond (30 m length for 0.32 mm of internal diameter). Meanwhile H₂, O₂,
158 N₂ and CH₄ were separated on a capillary column Rt-Molsieve 5Å (30 m length for 0.32
159 mm of internal diameter). Injector was set at 250°C, the thermal conductivity detector at
160 150°C and the carrier gas corresponded to argon (350 kPa at 34 mL.min⁻¹). Because of
161 the low diffusivity of H₂ and CO₂ through the plastic bags, the biogas production could
162 not have been accurately quantified, and biogas amounts and compositions are thus only
163 indicative.

164 NH₄⁺, NO₃⁻ and NO₂⁻ were measured on the soluble phase, after dilution and
165 filtration through 0.2 µm nylon syringe filter, by ion chromatography (ICS3000,
166 Dionex) as presented before by Mendez et al. (2016). Total Kjeldahl nitrogen (TKN)
167 was analyzed with an AutoKjehdahl Unit K-370, BUCHI. The Kjeldahl method

168 consisted in a first mineralization of samples during 3 hours at 420°C with H₂SO₄ 96%
169 and a kjeldahl catalyzer following by a distillation and titration done by hydrochloric
170 acid (0.02 N).

171 Volatile Fatty Acids (VFA), metabolites and sugars were also quantified in the
172 soluble phase. VFA were measured after filtration on a 0.45µm nylon syringe filter with
173 a gas chromatograph Perkin Clarus VARIAN 580 (PerkinElmer[®], Waltham, USA) on
174 an Elite-FFAP crossbond[®] carbowax[®] 15 m column connected to a FID detector at
175 280 °C and nitrogen at 6 mL.min⁻¹ as gas carrier. For analysis of others metabolic by-
176 products (1.3-propanediol, ethanol, lactate and succinate) and monosaccharides
177 (glucose, fructose, arabinose and xylose), the soluble phase was further filtered at 0.2
178 µm (nylon syringe filter). This filtrate was then injected into a High Performance Liquid
179 Chromatograph (HPLC) equipped with an Aminex 4PX-87H column (Biorad) at 45°C,
180 running with 0.005 M H₂SO₄ water solution as eluent (0.3 mL.min⁻¹).

181 2.5. *Biochemical analyses*

182 Biomethane Potentials (BMP) were estimated on silage samples at different
183 storage times by Near Infrared Spectroscopy (NIRS) according to Lesteur et al. (2011),
184 in so-called flash BMP test. Analyses were performed in triplicate on each replicated
185 bag. Samples were previously dried and grounded in order to get a homogeneous
186 sample and remove water before NIRS analysis. Following the same reasoning as for
187 TS and VS correction, BMP values obtained by NIRS were also corrected according to
188 the metabolites that evaporated during the drying step.

189 2.6. *Weight loss estimation*

190 In this study, weight losses were not directly measured by monitoring weight of
191 silage samples in each vacuum-plastic bag. Nonetheless, knowing that weight loss arose

192 from conversion of volatile solid into gases, the maximum gas production (i.e., CO₂ and
 193 H₂) was estimated to determine the maximum of weight loss of each storage time. To
 194 ensure this, maximum gas production was assessed based on associated metabolites
 195 productions as follows (Eq. 2 and 3):

$$\begin{aligned}
 196 \quad CO_2(g_{loss} \cdot g_{TS}^{-1}) &= [Acetic\ acid\ (mol.\ kg_{TS}^{-1}) + Ethanol\ (mol.\ kg_{TS}^{-1}) + \\
 197 \quad Propionic\ acid(mol.\ kg_{TS}^{-1}) &+ 2\ Butyric\ acid(mol.\ kg_{TS}^{-1}) + \\
 198 \quad Isobutyric\ acid(mol.\ kg_{TS}^{-1}) &+ Valeric\ acid(mol.\ kg_{TS}^{-1}) + \\
 199 \quad Isovaleric\ acid(mol.\ kg_{TS}^{-1}) &+ Caproic\ acid(mol.\ kg_{TS}^{-1})] \times CO_2\ Molar\ Mass/1000 \\
 200 \quad (Eq. 2)
 \end{aligned}$$

$$\begin{aligned}
 201 \quad H_2(g_{loss} \cdot g_{TS}^{-1}) &= 2 \times [Acetic\ acid\ (mol.\ kg_{TS}^{-1}) + Butyric\ acid(mol.\ kg_{TS}^{-1}) + \\
 202 \quad Isobutyric\ acid\ (mol.\ kg_{TS}^{-1}) &+ Valeric\ acid\ (mol.\ kg_{TS}^{-1}) + \\
 203 \quad Isovaleric\ acid\ (mol.\ kg_{TS}^{-1}) &] \times H_2\ Molar\ mass/1000\ (Eq. 3)
 \end{aligned}$$

204 Taking into account the maximum dry weight losses during storage, the theoretical
 205 initial TS and VS contents were estimated. The theoretical maximal losses of energetic
 206 methane potential of the silage mixture, corresponding to the maximum hydrogen losses
 207 and therefore methane potential reduction, were also estimated at each storage time.

208 2.7. Microbial community characterization

209 At each time of operation, 300 mg of silage sample were introduced into two
 210 sterile Eppendorf tubes of 2 ml and stored at -20°C before DNA extraction. DNA
 211 extraction was carried out on one replicated bag for each storage time as previously
 212 described by Saur et al.(2016), and DNA was then purified using the QIAamp DNA
 213 Mini Kit in accordance with manufacturer's recommendations. Purified DNA was
 214 stored at -20°C before use. Amplification of the V4-V5 region of the archaeal and
 215 bacterial 16S rRNA genes were performed as described in Venkiteshwaran et al. (2016).

216 Sequencing was performed at the technology platform Genome and Transcriptome
217 (GeT) of the Génopole Toulouse, France. Sequences are referenced under the accession
218 numbers n° MF373844 to MF374289 at the NCBI database (GenBank). Finally,
219 Quantitative PCR (qPCR) was performed to estimate the 16S rRNA gene copy number,
220 following the procedure of Braun et al. (2011), i.e., an initial enzyme activation step of
221 2 min at 95°C followed by 40 cycles of denaturation (95°C, 7 s; 60°C, 25 s). Bacterial
222 community analysis was based on unique Operational Taxonomic Units (OTUs). Only
223 OTUs with a relative abundance higher than 1.5% in at least one sample were selected
224 for further data analysis. 16S rRNA gene sequences of the OTUs were compared to
225 sequence database on the National Center for biotechnology Information (NCBI)
226 website via the Basic Local Alignment Search Tool (BLAST).

227 2.8. *Statistical data analysis*

228 Statistical analysis were performed on major Operational Taxonomic Units
229 (OTUs) having a relative abundance higher than 1.5% in at least one condition. Raw
230 abundancies were transformed using Hellinger transformation corresponding to the
231 “decostand ()” function from the vegan R package (Oksanen et al., 2016). Using these
232 transformed data, a principal component analysis (PCA) was carried out using the
233 “prcomp()” function from the built-in R stats package (R Core Team, 2014). The
234 package “ggbiplot” (Vu, 2011) was used for graphical representation. Correlations
235 between principal components and produced metabolites were assessed using the
236 “envfit()” function of the vegan R package. Moreover, correlations between individual
237 OTU abundancies and metabolite concentrations were calculated with the “rcorr”
238 function of the R package Hmisc. For both correlation calculations, significance (p-

239 value) was determined by performing 9999 random permutations. P-values lower than
240 0.05 were considered as statistically significant.

241 **3. Results and Discussion**

242 *3.1. Characteristics of the initial co-substrates*

243 Both agro-industrial and agricultural waste were characterized before ensiling.
244 Table 1 summarizes the main characteristics of these organic materials. As expected,
245 sugar beet waste had a high water content and contained high amount of soluble sugars
246 as required by the fermentation process. In contrast, wheat straw had a poor nutrient
247 content and a high solid content providing a structuring effect to the mixture. These
248 results confirm the interest of co-ensiling these two substrates to achieve high-quality
249 silage.

250 Sugar beet waste was basically composed of soluble elements (46.8% of TS)
251 consisting of pectins, proteins, sugars, lipids and small parts of the cell wall (Motte et
252 al., 2014; Cherney, 2000). It is well known that the soluble phase is more easily
253 degradable, and thus sugar beet waste can be considered as an interesting substrate for
254 anaerobic digestion. In contrast, wheat straw contained only few soluble elements and
255 was mainly composed of cellulose (45.2 % of TS), hemicellulose (29.3% of TS) and
256 lignin (7.98% of TS) linked together. Despite interesting methane potentials of cellulose
257 and hemicellulose (415 and 424 mL_{CH₄}.g VS⁻¹ respectively), chemical and structural
258 properties of this complex network are unfavorable to its bioconversion of
259 lignocellulosic into biogas (Zheng et al., 2014).

260 Interestingly, sugar beet waste did not only contain high soluble sugars (58.2g
261 Total soluble sugar.kg TS⁻¹), but also significant amount of microbial metabolites (42.1
262 g_{Lactate}.kg TS⁻¹ ; 35.6 g_{VFA}.kg TS⁻¹ ; 6.4 g_{Ethanol}.kg TS⁻¹). The presence of these compounds

263 together with a relatively low initial pH (5.47) indicate that partial lactic fermentation
264 occurred during industrial storage before substrate collection (Table 2). Abundances of
265 main OTUs (with relative abundance > 1.5%) in the initial silage are presented in Figure
266 1. At the beginning of the experiment, the main orders in the silage mixture were
267 affiliated to *Lactobacillales* with $32.4 \cdot 10^8$ 16S rRNA copy number.g_{FM}⁻¹ (i.e., relative
268 abundance of 29.1%) including 71.8% of OTU3 affiliated to *Leuconostoc*
269 *mesenteroides* and 27.7% of OTU2 affiliated to *Enterococcus sp.*. These results
270 confirmed that preliminary partial lactic fermentation of sugar beet waste occurred
271 before the experiment.

272 3.2. Dynamics of metabolic pathways

273 All along the 180 days of experimentation, metabolites and monosaccharides
274 content were periodically determined (Figure 2). Until day 14, most of the soluble
275 sugars, i.e., glucose, fructose, xylose and arabinose (figure 2a) were degraded, which
276 explains the concomitant production of fermentative metabolites (figure 2b). During the
277 following days, the slight sugars consumption could not explain alone the metabolites
278 accumulation. It is important to stress that only soluble simple sugars were measured in
279 this study. Consequently, metabolites accumulation clearly indicated that complex
280 sugars of the silage mixture were continuously converted.

281 In addition, a succession of different metabolic pathways was observed during
282 the ensiling process. Four main steps can be distinguished based on sugar consumption
283 and metabolites accumulation related to microbial reactions, as shown in Table 2. The
284 first 2 days, an aerobic phase was observed through oxygen consumption (data not
285 shown). Lactic acid fermentation then occurred until day 14. Butyric and acetic acid
286 fermentations prevailed from day 14 to 120. Finally, after day 120 and until the end of

287 the experiment, a low metabolic activity occurred and methanogenesis started, but at
288 trace levels. To better visualize the relationship existing between microbial communities
289 dynamics and metabolite accumulation, a Principal Component Analysis (PCA) is
290 provided in Figure 3. Lactic and succinic acids were anti-correlated with other
291 metabolites, and few OTUs co-correlated with different metabolites accumulation
292 suggesting that several metabolic phases carried by specific microorganisms occurred
293 along the storage time. These phases are described here below.

294 *3.2.1. First step from 0 to 2 days: Aerobic phase*

295 As expected, a first aerobic step took place in the experimental silos during the
296 first two days, by consuming part of the sugars, all of the trapped oxygen and producing
297 carbon dioxide (data not shown). This aerobic respiration ended once all oxygen was
298 consumed. Abundances of main OTUs (with relative abundance > 1.5% at almost one
299 storage time) in the silage mixture are presented in Figure 1. As predicted, total amount
300 of 16S rRNA gene copy related to OTUs affiliated to aerobic bacteria decreased from
301 40.48 to 4.63 10^8 copy number.g_{FM}⁻¹ after the second day up to the end of the
302 experiment.

303 *3.2.2. Second step from 0 to 14 days: Lactic fermentation*

304 When anaerobic conditions were reached, a lactic fermentation pathway was
305 favored as commonly reported during ensiling. Lactic fermentation consists in
306 converting monosaccharides (e.g., glucose, fructose, xylose) and disaccharides (e.g.,
307 sucrose, lactose), into lactic acid as well as ethanol, acetic acid and carbon dioxide
308 depending on the fermentation type (Table 2). During this second step lasting 14 days,
309 most of the soluble sugars were rapidly consumed. This degradation was concomitant
310 with the accumulation of lactic acid (+20.29 g.kg_{TS}⁻¹), acetic acid (+9.26 g.kg_{TS}⁻¹),

311 ethanol (+2.65 g.kg TS⁻¹), carbon dioxide (data not shown) and the pH dropped from
312 5.38 to 5.04 (Figure 4a) on day 14. Since lactic acid was produced with other
313 metabolites, the type of fermentation was suspected to be principally heterolactic. The
314 metabolic pathways were confirmed by the microbial community composition. Initial
315 presence of OTU3 affiliated to *Leuconostoc mesenteroides*, due to preliminary
316 fermentation before sample collection, which is well known to start ensiling with
317 heterolactic acid fermentation and then being replaced by more acid-tolerant LAB
318 (Daeschel et al., 1987). In this way, from day 0 to 7, the growth of OTU1 affiliated to
319 *Lactobacillus sp.* and OTU4 affiliated to *Lactobacillus brevis*, a heterofermentative
320 bacterium, was observed (+48.1 10⁸ and +31.6 10⁸ 16S rRNA copy number.g FM⁻¹,
321 respectively) (Figure 1). Emergence of OTU1 (r=0.81, P_{value}<0.01) and OTU4 (r=0.80,
322 P_{value}<0.01) were highly correlated to lactic acid production, as shown in PCA analysis
323 (figure 3).

324 Lactic and also succinic acid fermentations could also be explained by others
325 OTUs activities. Indeed, OTU24 affiliated to *Prevotella sp.*, correlated to lactic acid
326 production (r=0.83, P_{value}<0.01). In the same way, the presence of OTU5 affiliated to
327 *Dysgonomonas sp.*, correlated with lactic acid (r=0.78, P_{value}<0.01) and succinic acid
328 (r=0.73, P_{value}<0.05) production. Consistently, fermentation of soluble sugars into
329 short chain acids such as acetic, propionic, lactic and succinic acid, without gas
330 production from *Dysgonomonas sp.*, facultative anaerobic bacteria from *Bacteroidales*
331 order, was already reported by other authors (Chen and Dong, 2005).

332 In order to obtain stable and high-quality silage, it is required to rapidly lower
333 the pH below 4.5 by homolactic fermentation (Ambye-Jensen et al., 2013b). During the
334 lactic acid fermentation step, the pH drop stopped around 5.04. When compared to other

335 silage experiments reported in the literature, this slight drop can be explained by several
336 parameters: First, sugars were already partially consumed by preliminary lactic acid
337 fermentation of sugar beet waste during its industrial storage and by aerobic respiration
338 during transportation. As a result, the remaining sugars content at the beginning of the
339 silage test represented only a low fraction of the initial biomass, with 27.4 g_{Total Soluble}
340 sugar·kg_{TS}⁻¹ (i.e., 2.7% of the TS). According to Yang et al. (2006), an initial water-
341 soluble carbohydrate content higher than 7.0% of TS is required to reduce and stabilize
342 the pH under 4.5. Thus, the initial soluble sugars content in the silage mixture was not
343 sufficient to produced enough acids and lower the pH down to 4.5 (Ambye-Jensen et al.,
344 2013b). Furthermore, competition for sugar consumption could occur through the first
345 aerobic step. Moreover, heterolactic fermentation occurred, yielding acetic acid and
346 ethanol production in addition to lactic acid. Because acetic acid (pKa of 4.76) is a
347 weaker acid than lactic acid (pKa of 3.86), acetic acid accumulation was less efficient
348 and induced a lower pH drop. In addition, as reported on figure 4b, during the first 14
349 days, an increase of Total Ammonia Nitrogen (TAN) from 51.35 to 81.2 g_N·kg_{TotalN}⁻¹
350 was observed suggesting a proteolytic activity. TAN has a buffering capacity and
351 prevent also the lowering of the pH (Piltz and Kaiser, 2004). Hence, in this study, the
352 increasing content of TAN in the silage contributed to prevent the pH drop.

353 *3.2.3. Third step from 14 to 120 days: undesirable fermentations*

354 Since the pH value never dropped below 4.5, microorganisms were not fully
355 inhibited and undesirable fermentations prevailed on lactic acid fermentation from day
356 14 to day 120. During this period, lactic acid was consumed (-24.13 g·kg_{TS}⁻¹) with
357 concomitant production of hydrogen (data not shown), butyric acid (+24.84 g·kg_{TS}⁻¹),
358 acetic acid (+11.13 g·kg_{TS}⁻¹), propionic acid (+4.25 g·kg_{TS}⁻¹) and ethanol (+5.60 g·kg_{TS}⁻¹)

359 ¹). Throughout this storage time, the evolution of metabolic pathways from lactic acid
360 fermentation to others undesirable fermentations by-products, such as butyric,
361 propionic, acetic acids and ethanol is presented in figure 2. Meanwhile, during this third
362 step, results indicated the emergence of the OTU10 (+ 3.8 10⁸ 16S rRNA copy
363 number.g_{FM}⁻¹) and OTU41 (+0.9 10⁸ 16S rRNA copy number.g_{FM}⁻¹) affiliated to
364 *Prevotella sp.*, and to OTU36 (+0.7 10⁸ 16S rRNA copy number.g_{FM}⁻¹) affiliated to
365 *Clostridium sp.* (Figure 1).

366 The emergence of these OTUs correlated with metabolite production as shown
367 in PCA analysis (figure 3). Indeed, OTU10 and OTU41 (*Prevotella sp.*), correlated with
368 ethanol production (r=0.88, Pvalue<0.001 and r=0.77, Pvalue<0.001 respectively),
369 acetic acid production for OTU10 (r=0.66, Pvalue=0.05) and butyric acid production for
370 OTU41 (r=0.71, Pvalue<0.05). Accordingly, Takahashi (2003) reported that some
371 species within the genus *Prevotella* could produce butyric acid from amino acids, in
372 addition to other end-products such as ammonia, acetic and succinic acid. Finally, the
373 OTU36 related to the genus *Clostridium*, correlated with butyric acid (r=0.71,
374 Pvalue=0.03) and ethanol production (r=0.77, Pvalue=0.007). No correlations could be
375 found between other major OTUs such as OTU8 affiliated to *Erwinia sp.* and metabolite
376 production. This could be due to functional redundancy or non-linear phenomenon
377 resulting from both the production and consumption of metabolites.

378 Acetic, propionic and butyric acid production from lactic acid led to an increase
379 of the pH value because they are much weaker acids (pKa of 4.76, 4.87 and 4.82,
380 respectively) than lactic acid (pKa of 3.86), and because butyric acid production
381 required two moles of lactic acid to produce only one mole of butyric acid (Teixeira
382 Franco et al., 2016). As a result, the pH increased to 5.25 at day 21 and then stabilized

383 around this value (5.20) until the end of the experiments. Moreover, the level of total
384 ammonium-N (TAN) increased from 81.25 to 124.7 $\text{g}_\text{N}.\text{kg}_\text{Ntotal}^{-1}$ between days 14 and
385 120, suggesting deamination activities (Figure 4b). Such production of TAN, as an
386 indicator of poor silage quality, likely contributed to the slight increase of the pH.

387 *3.2.4. Final step from 120 to 180 days: Decline of the bacterial activity and* 388 *initiation of methanogenesis*

389 From day 120 until the end of the experiment, a decline of the microbial activity
390 was observed. Indeed, results showed only low butyric (+5.60 $\text{g}.\text{kg}_\text{TS}^{-1}$), propionic
391 (+1.77 $\text{g}.\text{kg}_\text{TS}^{-1}$) and acetic acid accumulation (+1.67 $\text{g}.\text{kg}_\text{TS}^{-1}$). This could be explained
392 by the low sugar and lactic acid contents remaining, and as a consequence less substrate
393 was available. As shown in figure 1, the reduction of OTUs affiliated to *Lactobacillales*
394 order ($-6.82 \cdot 10^8$ 16S rRNA copy number. g_FM^{-1}), to *Prevotella sp.* ($-3.90 \cdot 10^8$ 16S rRNA
395 copy number. g_FM^{-1}) and to *Clostridium sp.* ($-0.42 \cdot 10^8$ 16S rRNA copy number. g_FM^{-1})
396 supported this observation. However, the slight increase of isobutyric acid (+0.49 $\text{g}.\text{kg}_\text{TS}^{-1}$),
397 isovaleric acid (+0.49 $\text{g}.\text{kg}_\text{TS}^{-1}$) and Total Ammonia Nitrogen content (+18.31
398 $\text{g}_\text{N}.\text{kg}_\text{Ntotal}^{-1}$) highlighted a remaining proteolytic activity through amino acids
399 deamination (Table 2). In the same way, valeric acid (+1.97 $\text{g}.\text{kg}_\text{TS}^{-1}$) and caproic acid
400 (+0.44 $\text{g}.\text{kg}_\text{TS}^{-1}$) production suggested also carbon-chain elongation. Finally, traces of
401 methane were found at the end of the ensiling, indicating that methanogenesis also
402 occurred. Nevertheless, the amount of methane was still negligible.

403

404 *3.3. Effect of storage time on methane potentials*

405 In this study, the low sugar content of the sugar beet waste at the start of the
406 experiment was not sufficient to produce enough lactic acid during ensiling, to drop the

407 pH down 4.5 and to inhibit microbial activity. As a consequence, succession of other
408 pathways such as butyric or acetic acid fermentation were observed, that could be
409 detrimental for the storage quality (Pahlow et al., 2003). According to Bureenok et al.
410 (2016), high quality silage should fulfill the above criteria : pH<4.5, lactic acid content
411 > 30 g.kg_{TS}⁻¹, butyric acid < 10% of total_{VFA} and TAN <100 g_N.kg_{Ntotal}⁻¹. Thus, the
412 present silage could not be considered as well preserved silage: pH of 5.18±0.16, lactic
413 acid content of 3.57±2.45 g.kg_{TS}⁻¹, butyric acid content of 37.2% of total_{VFA} and TAN
414 of 142.9±2.8 g_N.kg_{Ntotal}⁻¹. However, it is important to make a distinction between silage
415 applicable for animal feeding and for biogas production. Indeed, for biogas production,
416 the main objective is to preserve (or even increase) the methane potential (Teixeira
417 Franco et al., 2016 ; Egg, 1993). Therefore, contrary to silage for animal feeding,
418 palatability and protein digestibility are disregarded. In a context of an industrial
419 application of this storage for biogas production, the biogas produced during ensiling
420 cannot be recovered and must be considered as potential energy loss. In this way,
421 undesirable fermentations (e.g., *Prevotella* and *Clostridium* based fermentations) could
422 induce a loss of energy by the release of H₂ and consequently a decrease of the methane
423 potential (BMP) (Teixeira Franco et al., 2016). The BMP monitoring as well as the
424 theoretical maximum weight loss and the theoretical maximum loss of methane
425 potential during the experiments are reported on Table 3.

426 After day 2, the BMP value expressed in mLCH₄ per g_{VS} in the silage, slightly
427 increased according to the time of storage (increase of the BMP of about 4.0% at the
428 end). However, to evaluate if co-ensiling had a positive effect on the methane potential
429 of the initial substrates, a mass balance was required to account for the organic matter
430 degraded into biogas during the storage. As reported on table 3, the theoretical

431 maximum weight losses gradually increased to reach a maximum of 6.9% of VS at the
432 end of the experiment, suggesting a potential overestimation of the BMP value with the
433 storage time. In fact, only hydrogen or methane productions can lead to loss of energetic
434 potential. In this study, only traces of methane (less than 0.1%) were measured at the
435 end of the storage time. In addition, the theoretical maximum production of hydrogen
436 (table 3) indicated a maximum energetic loss lower than 4% of the BMP. As a
437 consequence, loss of methane potential throughout the storage was considered as minor
438 or within the range of BMP accuracy. Thus, it was concluded that the methane potential
439 was well preserved all along the storage period. In addition, during co-ensiling, due to
440 the succession of metabolic pathways, easily degradable intermediates for
441 methanogenesis were produced (e.g., lactic acid, acetic acid, butyric acid, ethanol)
442 which could improve methane production kinetics in digesters.

443 This work suggests that, silage process implemented for higher initial sugars
444 content of such co-ensiling (via homolactic fermentation) could enhance energetic
445 potential by avoiding hydrogen loss while damaging the lignocellulosic structure.
446 Indeed, according to Herrmann et al. (2011), extended hydrolysis throughout long-term
447 storage, by organic acids in low pH, could induced a degradation of complex sugars
448 usually inaccessible during anaerobic digestion. Interestingly, and despite a succession
449 of metabolic pathways, the energetic potential of the co-ensiling was preserved during 6
450 months. As a consequence, even in poor silage quality, ensiling sugar beet waste with
451 wheat straw can represent an interesting solution for long-term storage of fresh agro-
452 industrial organic wastes and by extension of non-storable waste. In addition, the
453 production of intermediates of methanogenesis could be interesting to improve
454 productions kinetics of methane in such co-silage. Moreover, according to Ambye-

455 Jensen (2014), high final acetic acid concentration could also confer to silage a good
456 resistance against spoilage in aerobic conditions and thus provides aerobic stability
457 during the feed-out step.

458 **Conclusions**

459 Based on a long-term experimental work, this study shows that a succession of
460 metabolic pathways occurred and depended on the biological conditions, and the initial
461 sugars/metabolites content in the silage. Low initial sugar content was not sufficient to
462 lower the pH below 4.5 and avoid undesirable fermentation. Nonetheless, methane
463 potential was nearly not impacted during the storage. Even though no improvement of
464 the biodegradability of wheat straw was observed, mixing wheat straw with sugar beet
465 waste was a successful storage strategy which could be a promising and relevant
466 solution to store fresh biodegradable waste for long-term prior to AD.

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623	Abbreviations
624	AD: Anaerobic Digestion
625	BLAST: Basic Local Alignment Search Tool
626	BMP: Biomethane Chemical Potential
627	DNA: Deoxyribonucleic Acid
628	FM: Fresh Matter
629	NCBI: National Center for biotechnology Information
630	NIRS: Near Infrared Spectroscopy
631	OTU: Operational Taxonomic Unit
632	PCA: Principal Component Analysis
633	PCR: Polymerase Chain Reaction
634	qPCR: Quantitative Polymerase Chain Reaction
635	RNA: Ribonucleic Acid
636	TAN: Total Ammonia Nitrogen
637	TKN: Total Kjeldahl Nitrogen
638	TS: Total Solids
639	VFA: Volatile Fatty Acid
640	VS: Volatile Solids
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647 **Table captions**

648 **Table 1:** Physicochemical characteristics of initial substrates on Fresh Matter
649 (FM), Total Solid (TS) or Volatile Solid (VS) basis.

650 **Table 2:** Main anaerobic reactions according to Cavalcante et al. (2016),
651 Teixeira Franco et al. (2016), Rooke and Hatfield (2003), Piltz and Kaiser (2004) and
652 Motte et al. (2013).

653 **Table 3:** Evolution of BioMethane Potential (BMP, in $\text{mL}_{\text{CH}_4} \cdot \text{g VS}^{-1}$), theoretical
654 maximum weight loss (% of TS) and theoretical loss of potential (mL_{CH_4} for 1 g_{VS}) all
655 along the experiment. Theoretical maximum weight loss was estimated taking into
656 account the maximum gases productions. In the same way, the theoretical maximum
657 loss of methane potential was based on the theoretical maximum hydrogen production.
658 This hydrogen production was calculated according to associated metabolites
659 productions (Table 2).

660

Table 1

Physicochemical characteristics of initial substrates on Fresh Matter (FM), Total Solid (TS) and Volatile Solid (VS) basis.

Parameters	Units	Substrate	
		Sugar beet waste	Wheat Straw
TS	% of FM	17.6 ± 0.9	89.0 ± 0.1
VS	% of FM	12.4 ± 0.9	85.2 ± 0.2
Ash	% of FM	5.1 ± 0.3	3.9 ± 0.2
Total soluble	% of TS	46.7 ± 1.3	17.07 ± 0.0
Cellulose	% of TS	17.1 ± 0.0	45.15 ± 0.0
Hemicellulose	% of TS	17.6 ± 0.7	29.29 ± 0.0
Lignin	% of TS	5.7 ± 0.4	7.98 ± 0.1
FlashBMP	ml _{CH₄} .g ⁻¹ _{VS}	254.7 ± 1.7	258.1 ± 3.0
pH*	Unit pH	5.47 ± 0.57	7.25 ± 0.01
Total Nitrogen	g _N .kg ⁻¹ _{TS}	10.00 ± 0.63	ND
NH ₄ ⁺ NH ₃ *	g _N .kg ⁻¹ _{TS}	0.45 ± 0.22	ND
	g _N .kg ⁻¹ _{Ntotal}	45.2 ± 25.1	ND
Lactic acid*	g.kg ⁻¹ _{TS}	42.1 ± 9.7	< 0.7
Acetic acid*	g.kg ⁻¹ _{TS}	28.9 ± 4.0	1.85 ± 1.09
Butyric acid*	g.kg ⁻¹ _{TS}	< 4.3	< 0.7
Ethanol*	g.kg ⁻¹ _{TS}	< 18.6	< LOD
Fructose*	g.kg ⁻¹ _{TS}	25.2	< LOD
Glucose*	g.kg ⁻¹ _{TS}	24.3	0.78 ± 0.32
Xylose*	g.kg ⁻¹ _{TS}	5.5	0.94 ± 0.39
Arabinose*	g.kg ⁻¹ _{TS}	< 8.7	< 1.4

ND: Not determined

LOD: Limit Of Detection

*: Concentration measured in the soluble phase.

Table 2

Main anaerobic reactions according to Cavalcante et al. (2016), Teixeira Franco et al. (2016), Rooke and Hatfield (2003), Piltz and Kaiser (2004) and Motte et al. (2013).

Common reaction name	Substrate	Product
Glucose fermentation	Glucose + H₂O	→ Acetic acid + Ethanol + 2 CO ₂ + 2H ₂
	2 Glucose + H₂O	→ 2 Lactic acid + Acetic acid + Ethanol + 2 CO ₂ + 2H ₂
Succinic fermentation	Glucose + 2 CO₂	→ 2 Succinic acid + O ₂
Homolactic fermentation	Glucose / Fructose	→ 2 Lactic acid
	Glucose / Fructose + H₂O	→ Lactic acid + Acetic acid + CO ₂ + 2H ₂
Heterolactic fermentation	Glucose	→ Lactic acid + Ethanol + CO ₂
	Arabinose / Xylose	→ Lactic acid + Acetic acid
Ethanol fermentation	Glucose	→ 2 Ethanol + 2 CO ₂
Acetic fermentation	Glucose	→ 3 Acetic acid
	Glucose + 2 H₂O	→ 2 Acetic acid + 2 CO ₂ + 4 H ₂
	Propionic acid + 2 H₂O	→ Acetic acid + CO ₂ + 3 H ₂
	Butyric acid + 2 H₂O	→ 2 Acetic acid + 2 H ₂
	Ethanol + 2 H₂O	→ Acetic acid + 2 H ₂
	Lactic acid + H₂O	→ Acetic acid + CO ₂ + 2 H ₂
Homoacetogenesis	4 H₂ + 2 CO₂	→ Acetic acid + 2 H ₂ O
Butyric fermentation	Glucose	→ Butyric acid + 2 CO ₂ + 2 H ₂
	2 Lactic acid	→ Butyric acid + 2 CO ₂ + 2 H ₂
Propionic fermentation	3 Glucose	→ 4 Propionic acid + 2 Acetic acid + 2CO ₂ + 2 H ₂ O
	3 Lactic acid	→ 2 Propionic acid + Acetic acid + CO ₂ + H ₂ O
	Succinic acid	→ Propionic acid + CO ₂
Carboxylic chain elongation process	Acetic acid + Ethanol	→ Butyric acid + H ₂ O
	Propionic acid + Ethanol	→ Valeric acid + H ₂ O
	Butyric acid + Ethanol	→ Caproic acid + H ₂ O
	Acetic acid + Lactic acid	→ Butyric acid + CO ₂ + H ₂ O
Deamination	Butyric acid + Lactic acid	→ Caproic acid + CO ₂ + H ₂ O
	Lysine + 2 H₂O	→ Acetic acid + Butyric acid + 2 NH ₃
	Alanine + 2 H₂O	→ Acetic acid + NH ₃ + CO ₂
	Leucine + 2 H₂O	→ Isovaleric acid + NH ₃ + CO ₂ + 2 H ₂
	Isoleucine + 2 H₂O	→ Valeric acid + NH ₃ + CO ₂ + 2 H ₂
Methanogenesis	Valine + 2 H₂O	→ Isobutyric acid + NH ₃ + CO ₂ + 2 H ₂
	Acetic acid	→ CO ₂ + CH ₄
	CO₂ + 4H₂	→ CH ₄ + 2 H ₂ O

Table 3

Evolution of BioMethane Potential (BMP, in $\text{mL}_{\text{CH}_4} \cdot \text{g}^{-1} \text{VS}$), theoretical maximum weight loss (% of TS) and theoretical loss of potential (mL_{CH_4} for 1 gVS) all along the experiment. Theoretical maximum weight loss was estimated taking into account the maximum gas productions. In the same way, the theoretical maximum loss of methane potential was based on the theoretical maximum hydrogen production. This hydrogen production was calculated according to associated metabolite productions (Table 2).

Storage time (days)	BMP ($\text{mL}_{\text{CH}_4} \cdot \text{g}^{-1} \text{VS}$)	Estimated maximum	Theoretical maximum loss of	
		weight loss (% of VS)	potential ($\text{mL}_{\text{CH}_4} \cdot \text{g}^{-1} \text{VS}$)	(% of the BMP)
0	233.9 ± 0.5	0.0%	0.0	0.0%
2	231.1 ± 1.7	0.2%	0.0	0.0%
7	238.8 ± 6.6	0.7%	1.1	0.5%
14	239.5 ± 3.3	1.3%	2.1	0.9%
21	243.7 ± 5.9	1.8%	2.5	1.0%
30	237.8 ± 2.7	2.6%	3.6	1.5%
60	249.2 ± 1.8	4.2%	5.8	2.3%
120	240.4 ± 2.4	6.2%	8.0	3.3%
180	243.2 ± 4.7	6.9%	9.4	3.9%

1 **Figure captions**

2 **Figure 1:** Evolution of major OTUs (relative abundance > 1.5% for all storage
3 times) abundance expressed in **16S rRNA gene copy numbers.g_{FM}⁻¹** along the
4 experiment, sorted by category: a) bacteria affiliated to aerobic orders, b) bacteria
5 affiliated to Lactobacillales order, c) bacteria affiliated to Enterobacteriales,
6 Bacteroidales and Clostridiales order, d) others bacteria with relative abundance <1.5%
7 for all storage times.

8 **Figure 2:** Dynamics of a) simple soluble sugars concentrations (g.kg_{TS}⁻¹), b)
9 metabolites concentrations (g.kg_{TS}⁻¹), and c) delta production of metabolites between
10 two storage times (Δ g.kg_{TS}⁻¹) during the 180 days of the ensiling. Symbols on figure a)
11 correspond to sugars: Glucose (□), Xylose (★), Arabinose (◈) and Fructose (▲).
12 Symbols on figure b) and c) correspond to metabolites: Lactate (▼), Acetate (⊕),
13 Butyrate (●), Propionate (■), Succinate (◆), Ethanol (⊙), Valerate (▲). Dotted Lines
14 indicate phase separation.

15 **Figure3:** Principal component analysis (PCA) ordination plot of major OTUs
16 after Hellinger transformation. Significant correlations between metabolites and
17 principal components are represented as arrows which lengths are proportional to R².
18 Circle lines correspond to a R² values of 0.5 (inner circle) and 1(outer circle). Symbols
19 correspond to: storage time (●) and Operation Taxonomic Unit (OTU) (+).

20 **Figure 4:**Dynamics evolution of a) pH value and b) Total Ammonia Nitrogen
21 (TAN) expressed as g_N.kg_{Ntotal}⁻¹. Total Kjeldahl Nitrogen (TKN) corresponds directly to
22 total nitrogen since no nitrate and nitrite were found by chromatography in ours
23 samples. Dotted Lines indicate phase separation.

24

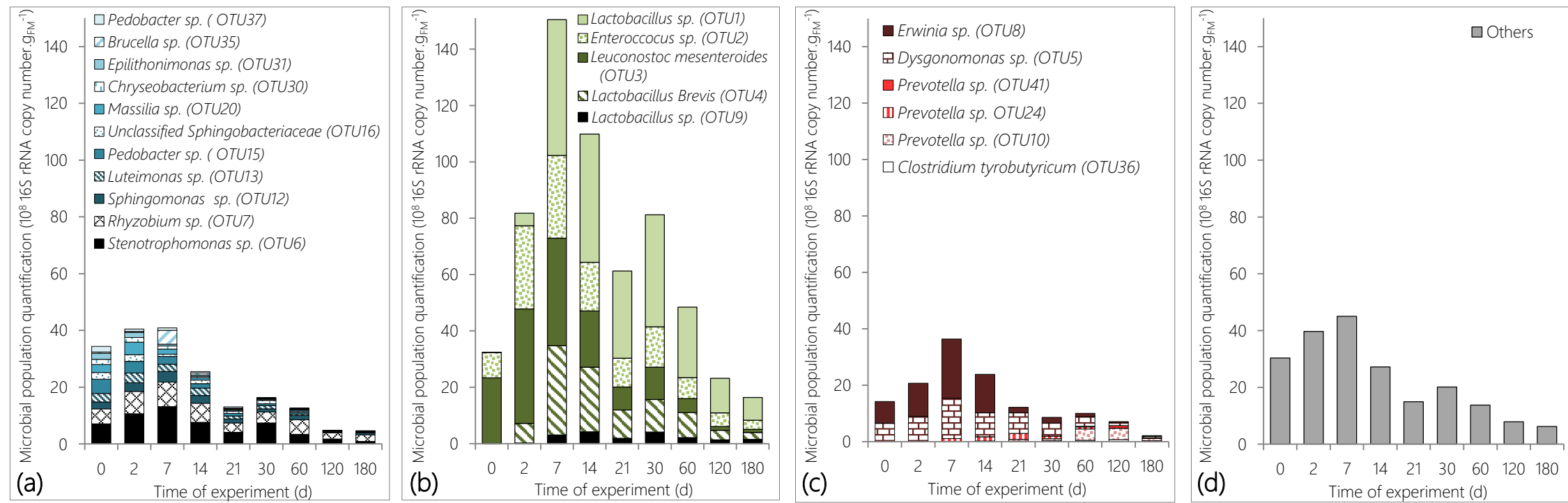


Fig 1.

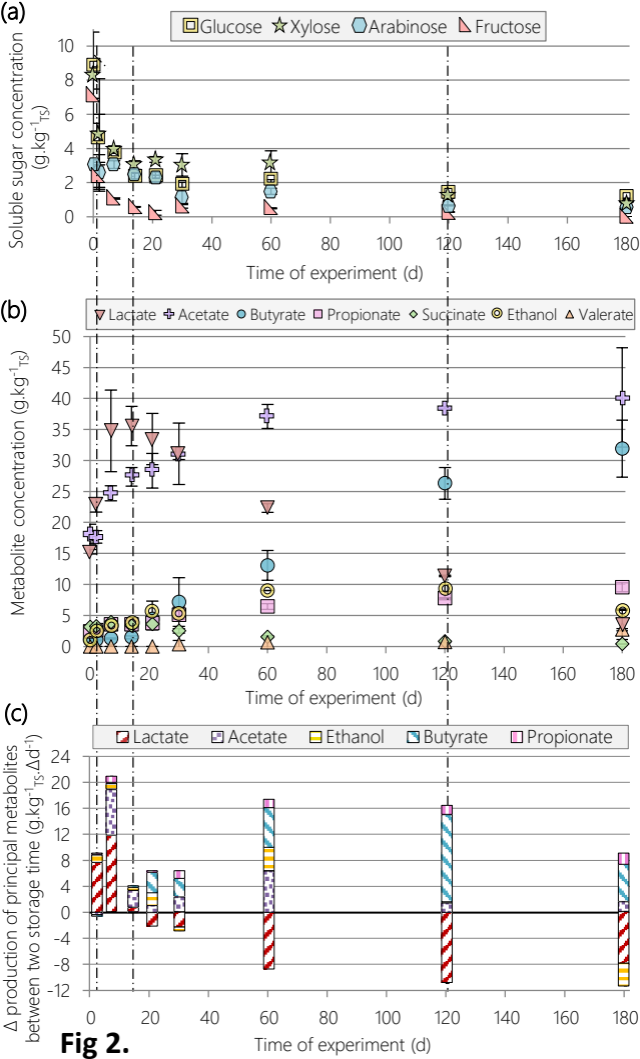


Fig 2.

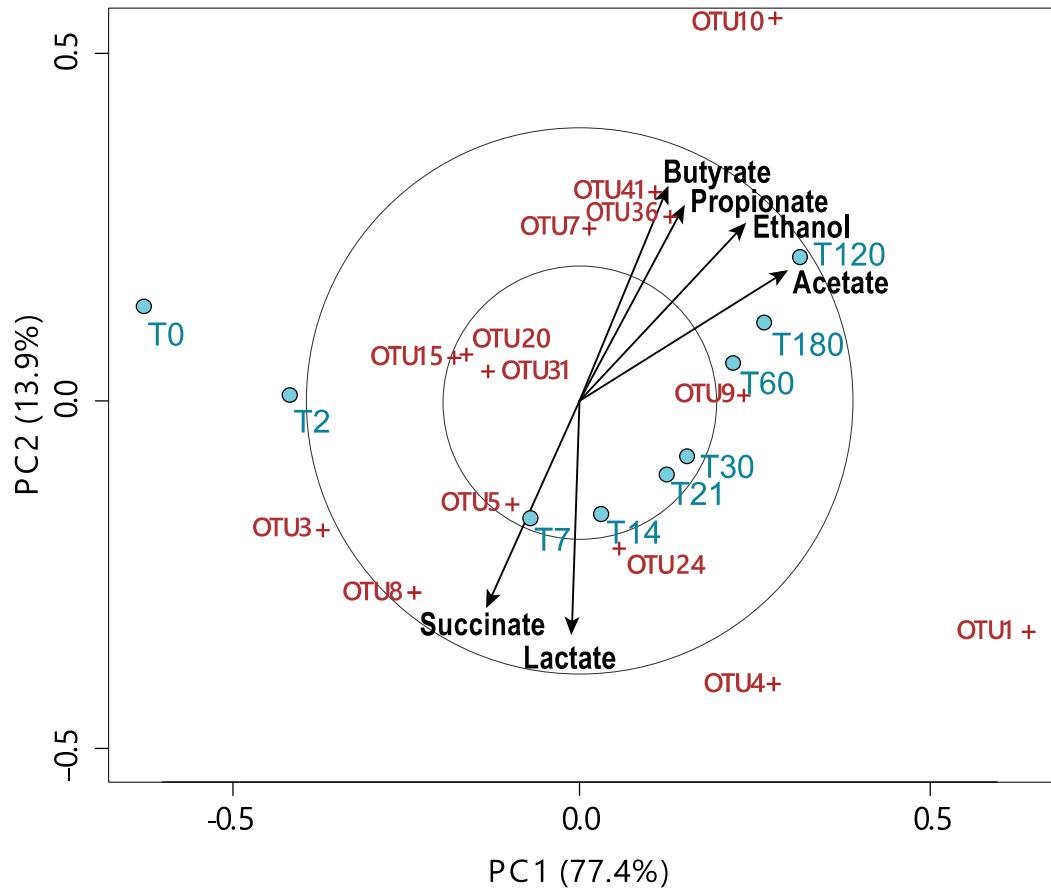


Fig 3.

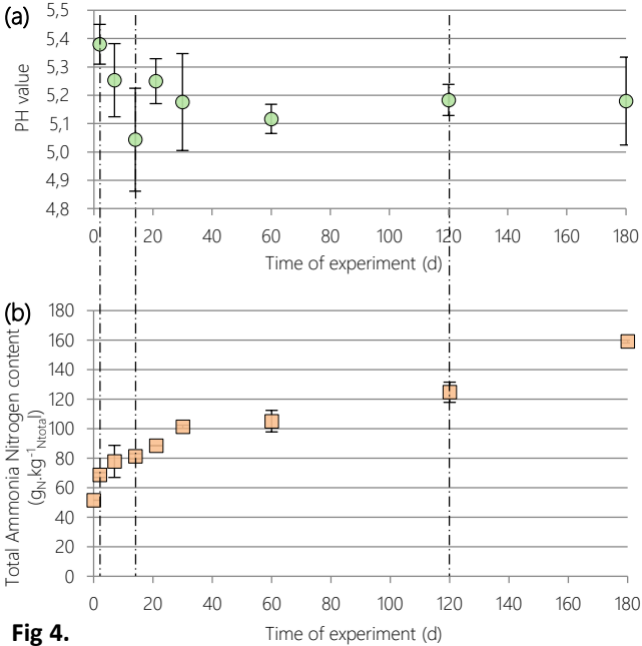


Fig 4.