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Marie-Lou Hillion, Roman Moscoviz, Eric Trably, Yoann Leblanc, Nicolas Bernet, et al.. Co-ensiling as a new technique for long-term storage of agro-industrial waste with low sugar content prior to anaerobic digestion. Waste Management, 2018, 71, pp.147-155. 10.1016/j.wasman.2017.10.024 . hal-02624481

HAL Id: hal-02624481
https://hal.inrae.fr/hal-02624481

Submitted on 4 Aug 2023

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

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Abstract

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

Keywords

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

1. Introduction

Over the past decades, human population growth has led to increasing quantities of waste and a stronger demand in energy and more especially in fossil fuels. The total energy consumption is expected to increase by 48% from 2012 to 2040 (U.S. Energy Information Administration, 2016). As a consequence, the production of renewable energy from waste represents a sustainable alternative to fossil fuel consumption with concomitant waste treatment. Among the other renewable technologies, anaerobic digestion (AD) has recently received an increasing amount of attention worldwide. AD consists in the biological conversion of organic matter into a methane-rich biogas that can further be used for energetic purposes (i.e., heat, steam, electricity, biofuel). A digestate that can be further used as organic fertilizer in agriculture is also generated. Anaerobic digestion presents the advantage to treat various kinds of organic wastes, such as co-products generated from industrial or agricultural productions. Among them, waste issued from the agri-food industry presents a high potential through high availability, large quantities and high performances in anaerobic digestion processes, with an average biochemical methane potential (BMP) of about 400 $NL_{CH4}.kg_{VS}^{-1}$ (Fisgativa et al., 2017). However, agro-industrial waste is most often produced seasonally. Considering that an anaerobic digester must be continuously fed throughout the year, such seasonal waste has to be stored prior to use. This type of feedstock is characterized by a high moisture content (77.2 ± 10.0 %) and a high amount of carbohydrates and proteins (36.4 ± 20.8%$_{VS}$ and 21.0 ±13.0%$_{VS}$, respectively) (Fisgativa et al., 2016), resulting in a high degradability and low stability at ambient temperature. As a consequence, controlling long-term storage is crucial to prevent biological degradation and avoid losses in methane potential.

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

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

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

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

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

2. Materials and Methods

2.1. Substrate origin

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

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

2.2. Experimental set-up

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

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

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

2.3. Silage characterization

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

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

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

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

Fibre distributions of soluble, hemicellulose, cellulose and lignin were 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, CO_2 was separated from other gases on a capillary
157 column R-Q-bond (30 m length for 0.32 mm of internal diameter). Meanwhile H_2, O_2,
158 N_2 and CH_4 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 H_2 and CO_2 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 NH_4^+, NO_3^- and NO_2^- 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 H_2SO_4 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 H_2SO_4 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., CO_2 and
193 H_2) 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):

196 $$CO_2(g_{loss}.g_{TS}^{-1}) = [Acetic\ acid\ (mol.kg_{TS}^{-1}) + Ethanol\ (mol.kg_{TS}^{-1}) +$$

197 $$Propionic\ acid(mol.kg_{TS}^{-1}) + 2\ Butyric\ acid(mol.kg_{TS}^{-1}) +$$

198 $$Isobutyric\ acid(mol.kg_{TS}^{-1}) + Valeric\ acid(mol.kg_{TS}^{-1}) +$$

199 $$Isovaleric\ acid(mol.kg_{TS}^{-1}) + Caproic\ acid(mol.kg_{TS}^{-1})] \times \mathrm{CO_2\ Molar\ Mass}/1000$$

200 (Eq. 2)

201 $$H_2(g_{loss}.g_{TS}^{-1}) = 2 \times [Acetic\ acid\ (mol.kg_{TS}^{-1}) + Butyric\ acid(mol.kg_{TS}^{-1}) +$$

202 $$Isobutyric\ acid\ (mol.kg_{TS}^{-1}) + Valeric\ acid\ (mol.kg_{TS}^{-1}) +$$

203 $$Isovaleric\ acid\ (mol.kg_{TS}^{-1})] \times \mathrm{H_2\ Molar\ mass}/1000$$ (Eq. 3)

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).

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

2.8. Statistical data analysis

Statistical analysis were performed on major Operational Taxonomic Units (OTUs) having a relative abundance higher than 1.5% in at least one condition. Raw abundancies were transformed using Hellinger transformation corresponding to the ‘‘decostand ()” function from the vegan R package (Oksanen et al., 2016). Using these transformed data, a principal component analysis (PCA) was carried out using the “prcomp()” function from the built-in R stats package (R Core Team, 2014). The package ‘‘ggbiplot” (Vu, 2011) was used for graphical representation. Correlations between principal components and produced metabolites were assessed using the ‘‘envfit()” function of the vegan R package. Moreover, correlations between individual OTU abundancies and metabolite concentrations were calculated with the “rcorr” 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 $_{\text{Total soluble sugar}}.kg_{TS}^{-1}$), 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 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

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

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

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

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

When anaerobic conditions were reached, a lactic fermentation pathway was favored as commonly reported during ensiling. Lactic fermentation consists in converting monosaccharides (e.g., glucose, fructose, xylose) and disaccharides (e.g., sucrose, lactose), into lactic acid as well as ethanol, acetic acid and carbon dioxide depending on the fermentation type (Table 2). During this second step lasting 14 days, most of the soluble sugars were rapidly consumed. This degradation was concomitant 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 10^8 and +31.6 10^8 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, Pvalue<0.01). In the same way, the presence of OTU5 affiliated to
327 *Dysgonomonas sp.*, correlated with lactic acid (r=0.78, Pvalue<0.01) and succinic acid
328 (r=0.73, Pvalue<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 $g_{Total\ Soluble\ Sugar}.kg_{TS}^{-1}$
340 (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 $g.kg_{TS}^{-}$

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 10^{8} 16S rRNA copy
363 number.g $_{FM}^{-1}$) and OTU41 (+0.9 10^{8} 16S rRNA copy number.g $_{FM}^{-1}$) affiliated to
364 *Prevotella sp.*, and to OTU36 (+0.7 10^{8} 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

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

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

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

3.3. Effect of storage time on methane potentials

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

pH down 4.5 and to inhibit microbial activity. As a consequence, succession of other pathways such as butyric or acetic acid fermentation were observed, that could be detrimental for the storage quality (Pahlow et al., 2003). According to Bureenok et al. (2016), high quality silage should fulfill the above criteria : pH<4.5, lactic acid content > 30 $g.kg_{TS}^{-1}$, butyric acid < 10% of $total_{VFA}$ and TAN <100 $g_N.kg_{Ntotal}^{-1}$. Thus, the present silage could not be considered as well preserved silage: pH of 5.18±0.16, lactic acid content of 3.57±2.45 $g.kg_{TS}^{-1}$, butyric acid content of 37.2% of total $_{VFA}$ and TAN of 142.9±2.8 $g_N.kg_{Ntotal}^{-1}$. However, it is important to make a distinction between silage applicable for animal feeding and for biogas production. Indeed, for biogas production, the main objective is to preserve (or even increase) the methane potential (Teixeira Franco et al., 2016 ; Egg, 1993). Therefore, contrary to silage for animal feeding, palatability and protein digestibility are disregarded. In a context of an industrial application of this storage for biogas production, the biogas produced during ensiling cannot be recovered and must be considered as potential energy loss. In this way, undesirable fermentations (e.g., *Prevotella* and *Clostridium* based fermentations) could induce a loss of energy by the release of H_2 and consequently a decrease of the methane potential (BMP) (Teixeira Franco et al., 2016). The BMP monitoring as well as the theoretical maximum weight loss and the theoretical maximum loss of methane potential during the experiments are reported on Table 3.

After day 2, the BMP value expressed in $mLCH_4$ per g_{VS} in the silage, slightly increased according to the time of storage (increase of the BMP of about 4.0% at the end). However, to evaluate if co-ensiling had a positive effect on the methane potential of the initial substrates, a mass balance was required to account for the organic matter degraded into biogas during the storage. As reported on table 3, the theoretical

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

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

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

Conclusions

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

Acknowledgements

The authors thank Benjamin Louchard and Gaëlle Santa-Catalina for given support during this experimental study. The authors also wish to express their gratitude to “Saint Louis Sucre” company for providing agro-industrial waste. The laboratory EBInnov® in Cergy, France, lent the necessary materials to realize silage bags. Microbial analysis was performed by the Genome & transcriptome core facilities of the “Génopole” in Toulouse, France.

This work was supported by “Vol-B Biomasse” company in Rouen, France, under CIFRE convention N°2013/1289

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Abbreviations

AD: Anaerobic Digestion

BLAST: Basic Local Alignment Search Tool

BMP: Biomethane Chemical Potential

DNA: Deoxyribonucleic Acid

FM: Fresh Matter

NCBI: National Center for biotechnology Information

NIRS: Near Infrared Spectroscopy

OTU: Operational Taxonomic Unit

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

TAN: Total Ammonia Nitrogen

TKN: Total Kjeldahl Nitrogen

TS: Total Solids

VFA: Volatile Fatty Acid

VS: Volatile Solids

Table captions

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

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).

Table 3: Evolution of BioMethane Potential (BMP, in $mL_{CH4}.g_{VS}^{-1}$), theoretical maximum weight loss (% of TS) and theoretical loss of potential (mL_{CH4} for 1 g_{VS}) all along the experiment. Theoretical maximum weight loss was estimated taking into account the maximum gases 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 metabolites productions (Table 2).

Table 1

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

		Substrate	
Parameters	Units	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+ H_2O**	→ Acetic acid + Ethanol + 2 CO_2 + 2H_2
	2 Glucose + H_2O	→ 2 Lactic acid + Acetic acid + Ethanol + 2 CO_2 + 2H_2
Succinic fermentation	**Glucose + 2 CO_2**	→ 2 Succinic acid + O_2
Homolactic fermentation	**Glucose / Fructose**	→ 2 Lactic acid
Heterolactic fermentation	**Glucose / Fructose + H_2O**	→ Lactic acid + Acetic acid + CO_2 + 2H_2
	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 H_2O	→ 2 Acetic acid + 2 CO_2 + 4 H_2
	Propionic acid + 2 H_2O	→ Acetic acid + CO_2 + 3 H_2
	Butyric acid + 2 H_2O	→ 2 Acetic acid + 2 H_2
	Ethanol + 2 H_2O	→ Acetic acid + 2 H_2
	Lactic acid + H_2O	→ Acetic acid + CO_2 + 2 H_2
Homoacetogenesis	**4 H_2 + 2 CO_2**	→ Acetic acid + 2 H_2O
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_2O
	3 Lactic acid	→ 2 Propionic acid + Acetic acid + CO_2 + H_2O
	Succinic acid	→ Propionic acid + CO_2
Carboxylic chain elongation process	**Acetic acid + Ethanol**	→ Butyric acid + H_2O
	Propionic acid + Ethanol	→ Valeric acid + H_2O
	Butyric acid + Ethanol	→ Caproic acid + H_2O
	Acetic acid + Lactic acid	→ Butyric acid + CO_2 + H_2O
	Butyric acid + Lactic acid	→ Caproic acid + CO_2 + H_2O
Deamination	**Lysine + 2 H_2O**	→ Acetic acid + Butyric acid + 2 NH_3
	Alanine + 2 H_2O	→ Acetic acid + NH_3 + CO_2
	Leucine + 2 H_2O	→ Isovaleric acid + NH_3 + CO_2 + 2 H_2
	Isoleucine + 2 H_2O	→ Valeric acid + NH_3 + CO_2 + 2 H_2
	Valine + 2 H_2O	→ Isobutyric acid + NH_3 + CO_2 + 2 H_2
Methanogenesis	**Acetic acid**	→ CO_2 + CH_4
	CO_2 + 4H_2	→ CH_4 + 2 H_2O

Table 3

Evolution of BioMethane Potential (BMP, in $mL_{CH4}.g^{-1}_{VS}$), theoretical maximum weight loss (% of TS) and theoretical loss of potential (mL_{CH4} for 1 g_{VS}) 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	**BMP**	**Estimated maximum weight loss**	**Theoretical maximum loss of potential**	
(days)	($mL_{CH4}.g^{-1}_{VS}$)	(% of VS)	($mL_{CH4}.g^{-1}_{VS}$)	(% 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%

Figure captions

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

Figure 2: Dynamics of a) simple soluble sugars concentrations ($g.kg_{TS}^{-1}$), b) metabolites concentrations ($g.kg_{TS}^{-1}$), and c) delta production of metabolites between two storage times ($\Delta\ g.kg_{TS}^{-1}$) during the 180 days of the ensiling. Symbols on figure a) correspond to sugars: Glucose (), Xylose ( ), Arabinose () and Fructose (). Symbols on figure b) and c) correspond to metabolites: Lactate (), Acetate (), Butyrate (), Propionate (), Succinate (), Ethanol (), Valerate (). Dotted Lines indicate phase separation.

Figure3: Principal component analysis (PCA) ordination plot of major OTUs after Hellinger transformation. Significant correlations between metabolites and principal components are represented as arrows which lengths are proportional to R^2. Circle lines correspond to a R^2 values of 0.5 (inner circle) and 1(outer circle). Symbols correspond to: storage time () and Operation Taxonomic Unit (OTU) ( ).

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

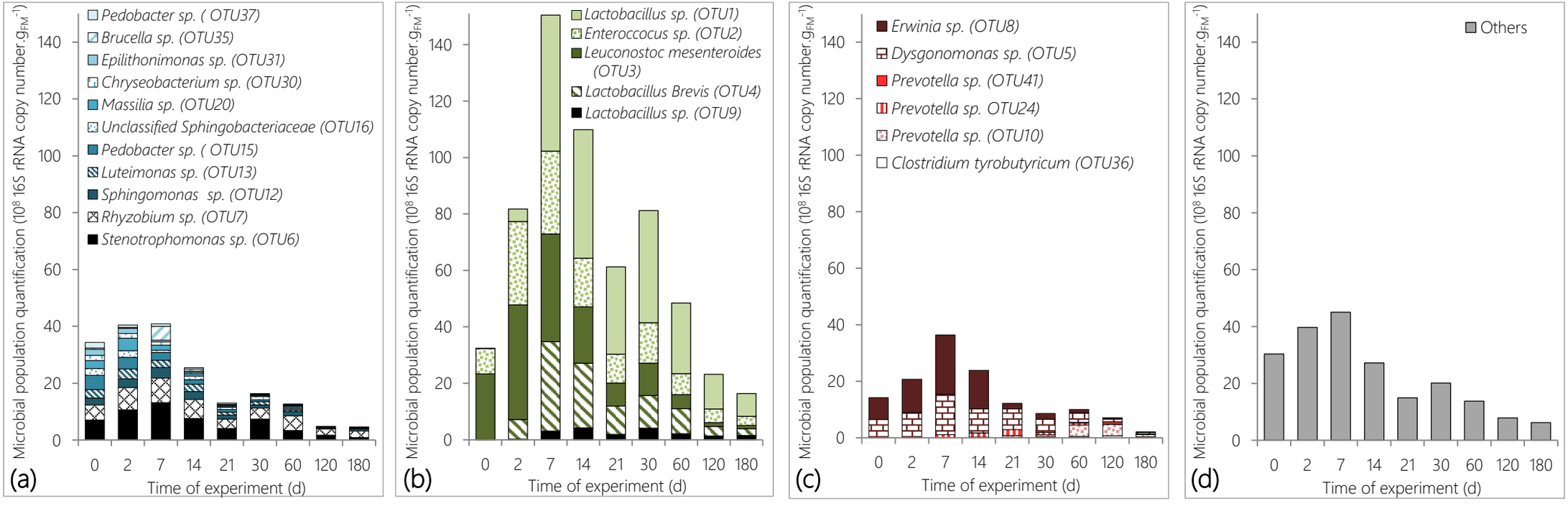
Microbial population quantification (10^8 16S rRNA copy number.g_{FM}^{-1})
0
20
40
60
80
100
120
140
0 2 7 14 21 30 60 120 180
Time of experiment (d)
(a)
Pedobacter sp. (OTU37)
Brucella sp. (OTU35)
Epilithonimonas sp. (OTU31)
Chryseobacterium sp. (OTU30)
Massilia sp. (OTU20)
Unclassified Sphingobacteriaceae (OTU16)
Pedobacter sp. (OTU15)
Luteimonas sp. (OTU13)
Sphingomonas sp. (OTU12)
Rhyzobium sp. (OTU7)
Stenotrophomonas sp. (OTU6)
(b)
Lactobacillus sp. (OTU1)
Enteroccocus sp. (OTU2)
Leuconostoc mesenteroides (OTU3)
Lactobacillus Brevis (OTU4)
Lactobacillus sp. (OTU9)
(c)
Erwinia sp. (OTU8)
Dysgonomonas sp. (OTU5)
Prevotella sp. (OTU41)
Prevotella sp. OTU24)
Prevotella sp. (OTU10)
Clostridium tyrobutyricum (OTU36)
(d)
Others

Fig 1.

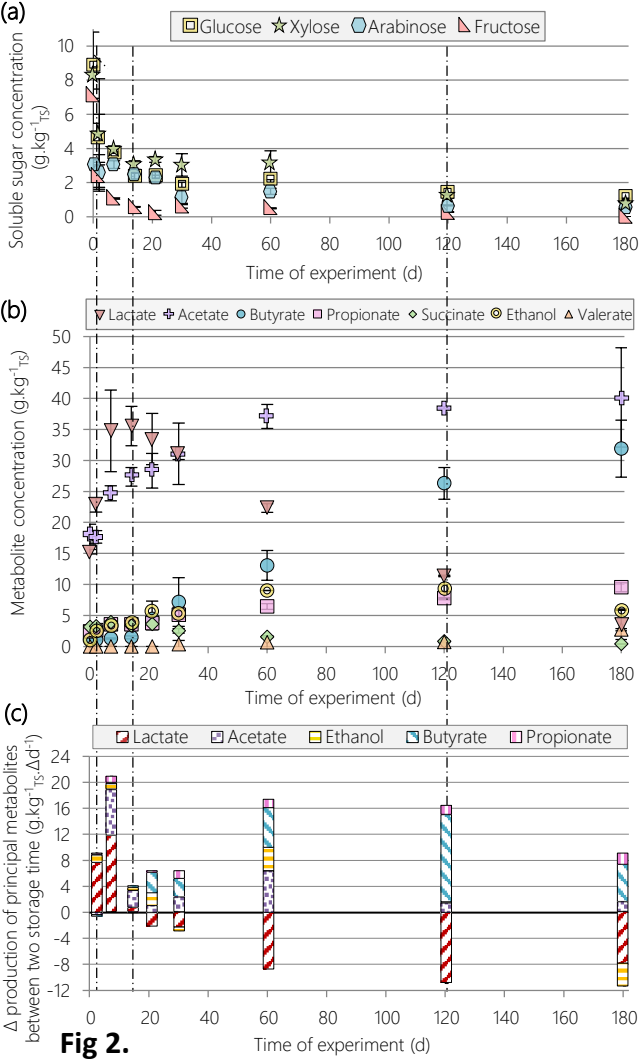
(a)
Glucose
Xylose
Arabinose
Fructose
Soluble sugar concentration (g.kg-1TS)
Time of experiment (d)
(b)
Lactate
Acetate
Butyrate
Propionate
Succinate
Ethanol
Valerate
Metabolite concentration (g.kg-1TS)
Time of experiment (d)
(c)
Lactate
Acetate
Ethanol
Butyrate
Propionate
Δ production of principal metabolites between two storage time (g.kg-1TS.Δd-1)
Time of experiment (d)

Fig 2.

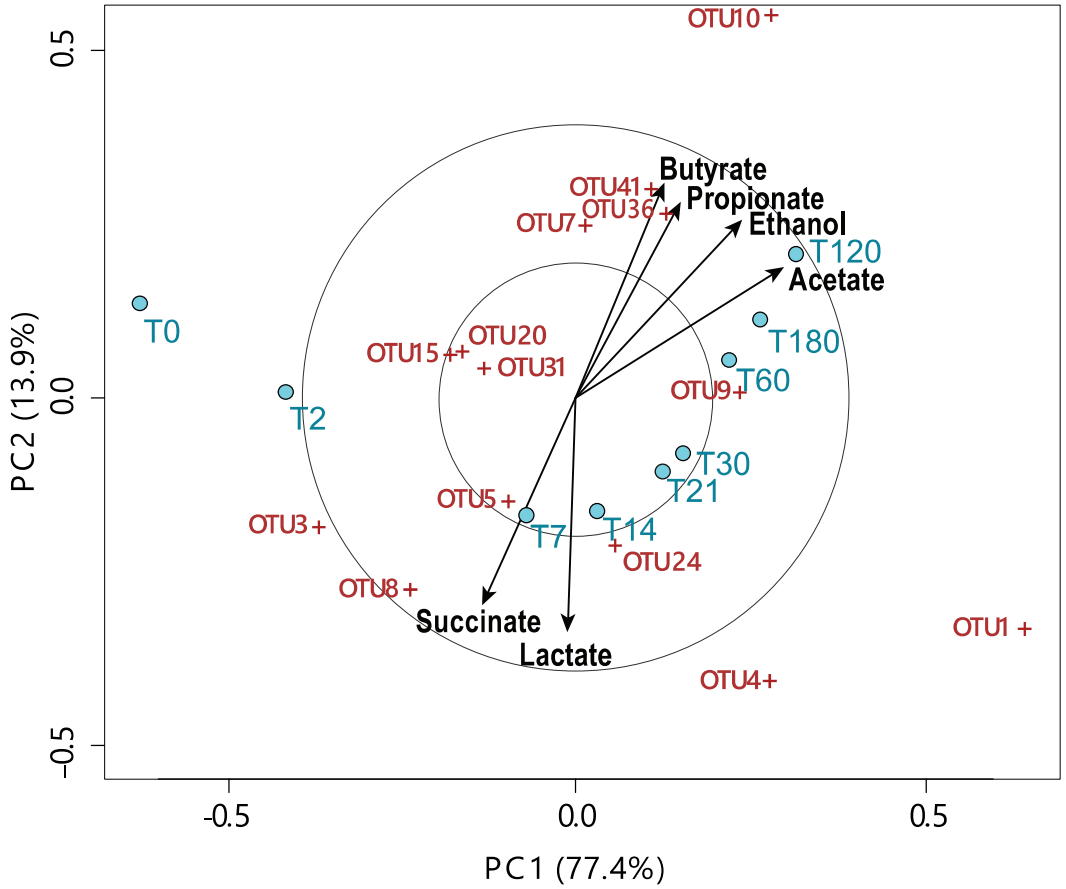
OTU10
Butyrate
OTU41
Propionate
OTU36
OTU7
Ethanol
T120
Acetate
T0
T180
OTU15
OTU20
+ OTU31
T60
OTU9
T2
T30
T21
OTU5
T7
T14
OTU3
OTU24
OTU8
Succinate
Lactate
OTU1
OTU4
0.5
0.0
-0.5
PC2 (13.9%)
-0.5
0.0
0.5
PC1 (77.4%)

Fig 3.

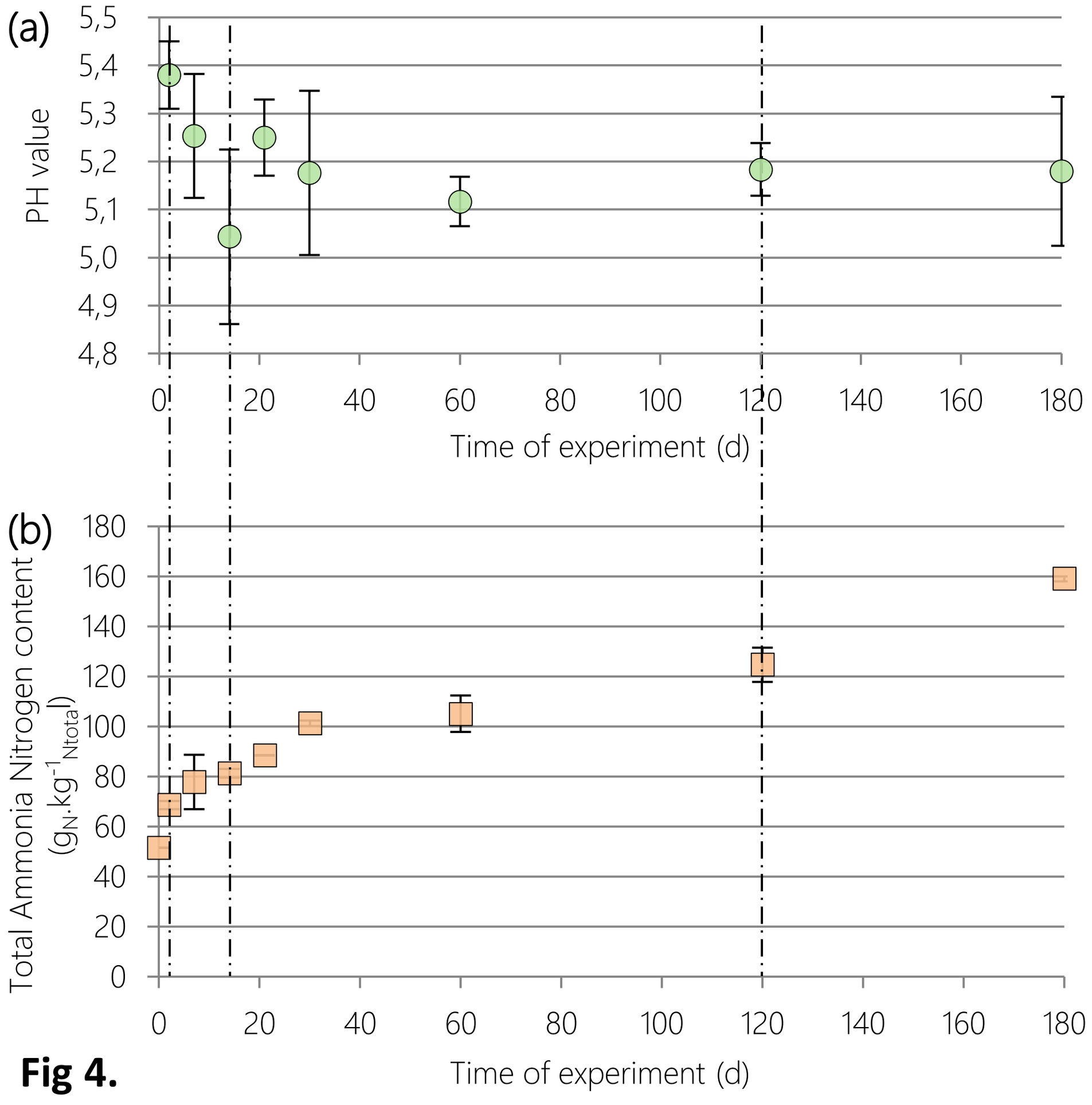
(a)
PH value
5,5
5,4
5,3
5,2
5,1
5,0
4,9
4,8
0
20
40
60
80
100
120
140
160
180
Time of experiment (d)
(b)
Total Ammonia Nitrogen content
($g_N.kg^{-1}_{Ntotal}$)
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
Time of experiment (d)

Fig 4.