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1 **Methane production and active microbial communities during anaerobic digestion of three**
2 **commercial biodegradable coffee capsules under mesophilic and thermophilic conditions**

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10
11 **1) INTRODUCTION:**

12 Due to inadequate end-of-life management, conventional plastics have become one of the
13 most abundant sources of environmental pollution. Geyer et al. (2017) estimated that almost
14 60% of all plastics ever produced, corresponding to 5 billion tons, have been discarded in the
15 environment (natural or landfills). These plastics have accumulated in the world's oceans;
16 where large quantities of debris represent a threat to marine species by entanglement,
17 suffocation, or ingestion (Compa et al., 2019; Gregory, 2009). Microplastics are a threat to
18 both marine life and human beings (Ajith et al., 2020; Sarker et al., 2020). The use of
19 biodegradable plastics, will be promoted by the European directive 2018/581, which plans for
20 the generalization of separation at source and treatment of organic wastes. A biodegradable
21 plastic can be defined as a plastic that undergoes a significant change in its chemical structure
22 under specific environmental conditions, resulting in the loss in some of its properties by the
23 action of naturally occurring microorganisms in a given period of time. This is measured by
24 standard test methods appropriate to the plastic and to the application (ISO 472, 2013). The
25 biodegradation of a biodegradable plastic depends greatly on the environment in which it
26 takes places. This is why biodegradable plastics should not be discarded directly in the
27 environment but should be preferentially treated in a controlled recycling environment
28 (composting or anaerobic digestion).

29 Anaerobic digestion (AD) is a biological process by which organic matter is converted, in an
30 oxygen-free environment, into biogas (mainly composed of carbon dioxide and methane). The
31 process of AD has proven to be a promising method for the valorisation of organic materials
32 such as agricultural wastes (manure, crop residues, and winery waste products), food wastes,
33 and sewage sludge (Da Ros et al., 2017; Hanum et al., 2019; Monlau et al., 2013; Moretti et

34 al., 2020). The literature recommends balancing the carbon to nitrogen (C/N) ratio of
35 feedstocks to between 20 and 30 to prevent both nutrient limitation and ammonia toxicity
36 (Esposito et al., 2012; Hawkes, 1980). Some feedstocks that are usually processed in
37 anaerobic digesters, such as food waste or sewage sludge, have a low C/N ratio (Esposito et
38 al., 2012). Plastics, on the other hand, contain very low levels of nitrogen. Co-digestion of
39 biodegradable plastics with other types of waste can help adjust the carbon-nitrogen ratio to
40 the recommended values (Benn and Zitomer, 2018). Anaerobic digesters are typically
41 operated at mesophilic (30-40 °C) or thermophilic (around 55 °C) temperatures in wet or dry
42 processes (Brown et al., 2012). The process of AD can be divided into four main steps:
43 hydrolysis, acidogenesis, acetogenesis and methanogenesis through acetotrophic,
44 hydrogenotrophic or methylotrophic pathway (Demirel and Scherer, 2008; Evans et al., 2019).

45 The microbiome involved in the process of AD has undergone intensive study in recent years
46 (Azizi et al., 2016; Castellano-Hinojosa et al., 2018; De Vrieze et al., 2018; Guo et al., 2015;
47 Levén et al., 2007; Li et al., 2015; Liu et al., 2018; Sundberg et al., 2013). However, little is
48 known about the microorganisms involved in the AD of biodegradable plastics. In the reviews
49 by Shah et al. (2014, 2008) and Emadian et al. (2017), several microorganisms were reported
50 to decompose biodegradable plastics, but most of them did not come from anaerobic media.
51 Yagi and co-workers have published several studies aimed at the detection of microorganisms
52 that participate in anaerobic digestion of biodegradable plastic. They performed denaturing
53 gradient gel electrophoresis of the 16S rRNA amplicons (RT-PCR-DGGE). In their first two
54 papers they were not successful at identifying the microorganisms responsible for the
55 thermophilic digestion of poly(lactic acid) (PLA) (Yagi et al., 2011, 2010). However, they
56 highlighted that some of the microorganisms participating in the anaerobic biodegradation of
57 cellulose and PLA at 55°C differ. Subsequently, Yagi and co-workers succeeded in
58 identifying some microorganisms that participated in the degradation of PLA, poly(ϵ -
59 caprolactone) (PCL), and poly(3-hydroxybutyrate) (PHB) under mesophilic and thermophilic
60 conditions (Yagi et al., 2014, 2013). Venkiteshwaran et al.(2019) have examined the
61 microbial community shift during anaerobic co-digestion of PHB and synthetic primary
62 sludge (dog food and basal nutrients) by Illumina sequencing. No previously known PHB
63 degraders were observed in the co-digesters. Finally, Tseng et al. (2019) examined the
64 microbial populations involved in anaerobic digestion of PLA under thermophilic conditions
65 by PCR-DGGE. They pointed out the importance of the genus *Tepidimicrobium*, which is

66 thought to comprise key bacteria that decompose lactic acid and that supply CO₂ and H₂ to
67 hydrogenotrophic methanogens.

68 The aim of this study was to evaluate the methane potential of three biodegradable plastics
69 under mesophilic and thermophilic conditions using Biochemical Methane Potential (BMP)
70 testing and to analyze the active microbial communities (16S rRNA) during the process.
71 These plastics were commercially available in the form of coffee capsules and were certified
72 as being biodegradable under industrial composting conditions according to the EN 13432
73 standard. The EN 13432 standard defines the requirements for packaging recoverable through
74 composting and biodegradation, with test scheme and evaluation criteria for the final
75 acceptance of packaging.

76

77 **2) MATERIALS AND METHODS**

78 **2.1) Biodegradable plastic supports and inocula**

79 Biodegradable plastics were purchased in the form of coffee capsules (Launay[®], Beanarella[®],
80 and Tintoretto[®]) certified as being biodegradable under industrial composting conditions (EN
81 13432). They were made of Vegemat[®] from Vegeplast (France), Ecovio[®] from BASF
82 (Germany), or Mater-Bi[®] from Novamont (Italy). Mater-Bi[®] is a class of compounds based on
83 thermoplastic starch (TPS) and other polymers such as cellulose acetate, poly(vinyl alcohol),
84 PCL, and PBAT (Aldas et al., 2020). Ecovio[®] (BASF) is a mixture of PLA and a fossil-based and
85 biodegradable polymer commercialised as Ecoflex[®] (BASF) (Ecovio[®] BASF website,2020).
86 Vegemat[®] (Vegeplast) is a mixture of biobased and biodegradable polymers obtained
87 through the processing of agropolymers and polyesters (Pluquet et al., 2016). The capsules
88 were separated from the coffee, and the opercula and were ground using a cutting mill (SM
89 100 Retsch, Haan, Germany) and a centrifugal mill (ZM 100, Retsch, Haan, Germany) to a
90 particle size of 1 mm. In order to produce the spent coffee and to simulate a passage
91 through a coffee maker, the ground coffee (Grand'Mère[®], France) was mixed with water
92 using a magnetic stirrer (200 rpm, 15 min). After a centrifugation step (5 000 x g, 5 min), the
93 solid fraction was collected and dried at 60 °C until a constant mass was obtained.

94 The total solids (TS) and the volatile solids (VS) were determined using APHA standard
95 methods (APHA, 2005). Elemental analysis (Carbon, Hydrogen, Nitrogen, and Sulphur) was

96 performed on the different samples using an Elemental Vario Macro Cube analyser
97 (Elementar, Germany). The oxygen content was estimated by the difference between the VS,
98 carbon, hydrogen, nitrogen, and sulphur content. The theoretical methane production was
99 estimated from the elemental composition using the following equation (Achinas and
100 Euverink, 2016; Boyle, 1977; Buswell and Mueller, 1952):

101 Theoretical methane production (NL(CH₄).g⁻¹(C_xH_yO_zN_nS_s)) =
$$\frac{22,4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4} \right)}{12x + y + 16z + 14n + 32s}$$

102 Two different inocula were used in this study. They were prepared from wastewater
103 treatment plant (WWTP) sludge and acclimated for two months for anaerobic digestion at
104 38 °C (mesophilic inoculum) or 58 °C (thermophilic inoculum). The mesophilic inoculum was
105 fed with grass and WWTP sludge; the thermophilic inoculum with grass, wheat waste, and
106 WWTP sludge. The chemico-physical characteristics of the substrates and inocula used here
107 are presented in Table 1.

108 **2.2) Biochemical Methane Potential (BMP) tests**

109 Ground biodegradable plastic samples, spent coffee, and cellulose (positive control
110 purchased from Tembec, France) were digested in batch bottles under mesophilic (38 °C)
111 and thermophilic (58 °C) conditions according to an experimental protocol adapted from the
112 recommendations of the European Inter-Laboratory studies in which APESA is involved
113 (Hafner et al., 2020). The BMP bottles were filled with 300 mL of inoculum, water, and test
114 material mixture at an inoculum to substrate ratio (ISR) of 2.7 g(VS).g(VS)⁻¹. The organic
115 loading of the test corresponded to 9 g(VS).L⁻¹. Previous research work has suggested an ISR
116 ≥ 2 in order to obtain reproducible constant kinetics (Chynoweth et al., 1993; Raposo et al.,
117 2011, 2008). A blank control without test material was also carried out. Before placing an
118 airtight seal on the bottle, the gas phase was flushed out with nitrogen (Alphagaz™ Smartop,
119 Air Liquide, France). The daily biogas production was estimated by the increase in pressure
120 using a manometer (2023P, Digitron, Croatia). The biogas composition was determined using
121 a gas chromatograph (Micro GC 490, Agilent, USA) equipped with two columns. The first
122 column (M5SA 10m, Agilent, USA) was used at 80 °C and 200 kPa to separate O₂, N₂, and CH₄
123 using Argon as the carrier phase; the second column (PPU 10m BF) was used at 80 °C and
124 150 kPa to separate the CO₂ from the other gases using Helium as the carrier phase. The

125 injector temperature was 110 °C. The detection of gaseous compounds was achieved using a
126 thermal conductivity detector. The biogas production of the negative control, endogenous to
127 the inoculum, was subtracted from the production of the other bottles. The BMP tests were
128 performed in triplicate. The calibration was carried out with two standard gases composed
129 of 9.5% CO₂, 0.5% O₂, 81% N₂ and 10% CH₄ and 35% CO₂, 5% O₂, 20% N₂, and 40% CH₄
130 (special gas, Air Liquide[®], France). All of the results are presented for normalized conditions
131 of temperature and pressure (Patm, 0 °C). The degradation yield was estimated by
132 comparing the observed methane production to the theoretical methane production (Table
133 1).

134 **2.3) Microbial analysis**

135 **2.3.1) Sampling**

136 Digestate samples were collected, in triplicate, at the start of the BMP tests (T0) in order to
137 characterise the initial state of the microbial populations, as well as at two different times
138 (T1 and T2) during the anaerobic digestion process. When feasible, the sampling dates were
139 adjusted to occur during either a phase of high methane production in the case of the first
140 sampling (T1) or later during a phase of lower methane production in the case of the second
141 sampling time (T2). The specific days of the sampling are indicated in Figure 1. The digestate
142 samples were mixed with two volumes of RNAProtect Bacteria Reagent[®] (Qiagen, USA),
143 incubated for 5 min at room temperature, and then centrifuged (5 000 x g, 5 min). The
144 supernatants were discarded and the pellets were stored at -20 °C for less than two weeks
145 before their transfer to – 80 °C.

146 **2.3.2) Nucleic acid extraction, reverse transcription of the RNA, and PCR** 147 **amplification**

148 Nucleic acid extractions were performed using a Fast RNA[®] Pro Soil Direct kit (Qiagen, USA),
149 according to the manufacturer's instructions to collect a mixture of RNA and DNA. The DNA
150 and the RNA were then separated using an AllPrep DNA/RNA kit (Qiagen, USA). The absence
151 of DNA in the RNA extracts was verified by PCR followed by 1% agarose gel electrophoresis.
152 RNA reverse transcription was carried out using the instructions provided with the M-MLV
153 reverse transcriptase (Invitrogen[™], USA).

154 PCR of the V4-V5 region (nucleotides 515-928) of the 16S rRNA gene (from cDNA and DNA)
155 was performed using AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, USA) with the
156 following reaction mix: AmpliTaq 1X, 515F primer 0.6 μM (GTGYCAGCMGCCGCGGTA, Wang
157 and Qian, 2009), 928R primer 0.6 μM (ACTYAAAKGAATTGRCGGGG, Wang and Qian, 2009),
158 and cDNA 10 μL or DNA 1 μL. The amplification was performed with an initial denaturation
159 step at 95 °C for 10 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 65 °C, and 40 s at 72
160 °C. The amplification reaction ended with a 7-min extension step at 72 °C. The amplification
161 was confirmed by analysis of the reaction mix by 1% agarose gel electrophoresis.

162 **2.3.2) Sequencing and bioinformatics analyses**

163 Amplicons were sequenced by the Get-PlaGe sequencing service (INRA, Toulouse, France)
164 using Illumina MiSeq 250 bp paired-end technology. Bioinformatics processing of the data
165 was performed using the method described by Escudié et al., 2018 on the Galaxy FROGS
166 pipeline (Afgan et al., 2018). After a pre-processing step (merging, denoising, and
167 dereplications of the reads), the sequences were clustered into Operational Taxonomic Units
168 with an aggregation distance of three bases. OTUs containing less than 0.0005% of the total
169 sequences were deleted, as were chimeric OTUs. Taxonomic assignments were performed
170 using the Silva database v.128 (Pruesse et al., 2007). The number of sequences per sample
171 was normalized based on the minimum number of sequences per sample found (7 744
172 sequences). The sequence data have been deposited in GenBank under accession number
173 PRJNA648017.

174 The statistical analyses were performed using R Studio version 3.2.3 software ([http://www.r-](http://www.r-project.org)
175 [project.org](http://www.r-project.org)). Diversity indices (Richness, Chao, Exponential of Shannon, and Inverse of
176 Simpson) were calculated using Vegan and Bat packages. The differences in the diversity
177 index between the different samples and conditions were tested using the Kruskal-Wallis
178 test. Non-metric distance scaling (NMDS) plots of the community data were generated using
179 the Bray-Curtis distance measures with the Phyloseq and ggplots2 packages. Heatmaps and
180 Venn diagrams were produced using gplots package.

181 **3) RESULTS AND DISCUSSION**

182 **3.1) Performances of the anaerobic digestion processes**

183 The methane productions during the anaerobic digestion of the three coffee capsules
184 (Launay[®], Beanarella[®], and Tintoretto[®]), spent coffee and cellulose, under the mesophilic
185 (38 °C) and the thermophilic (58 °C) conditions, are represented in Figure 1.

186 The methane potential of cellulose (positive control) under mesophilic and thermophilic
187 conditions was 315 ± 5 NL (CH₄) kg⁻¹ (VS) and 337 ± 3 NL (CH₄) kg⁻¹ (VS), respectively. Similar
188 values and kinetics have been reported elsewhere for cellulose controls (Hansen et al., 2004;
189 Raposo et al., 2011), thus indicating that the inocula were well-suited to anaerobic digestion
190 under both the thermophilic and the mesophilic conditions.

191 The spent coffee was readily biodegradable, under both the mesophilic and the thermophilic
192 conditions, with almost no lag phase (< 1 day) and a high level of methane production over a
193 period of 15 to 20 days. A similar degradation behaviour was noted under the mesophilic
194 and the thermophilic conditions; the methane potentials were 294 ± 8 NL (CH₄) kg⁻¹ (VS) and
195 301 ± 3 NL (CH₄) kg⁻¹ (VS), respectively, corresponding to a conversion into methane of
196 between 56 and 57% of the spent coffee. The methane production and kinetics from spent
197 coffee were comparable to those of cellulose. The methane potential of spent coffee has
198 been previously described, ranging from 240 to 340 NL (CH₄) kg⁻¹ (VS) (Lane, 1983; Neves et
199 al., 2006).

200 The digestion of the biodegradable plastics exhibited very different behaviors under the
201 mesophilic versus the thermophilic conditions. Under the mesophilic conditions, the AD of
202 the three plastics exhibited a long lag phase (almost 25 days) and slow kinetics (Figure 1A),
203 suggesting that the hydrolysis step is limiting and underperforming. Finally, the conversion
204 of the plastics into biogas did not reach completion within the 100-day test period. The
205 methane potential of the Beanarella[®], Launay[®], and Tintoretto[®] samples at the end of the
206 test was 127 ± 1 NL (CH₄) kg⁻¹ (VS), 92 ± 0 NL (CH₄) kg⁻¹ (VS), and 67 ± 3 NL (CH₄) kg⁻¹ (VS),
207 respectively. The biodegradability corresponded to $24 \pm 0\%$, $18 \pm 0\%$, and $12 \pm 1\%$ for the
208 Beanarella[®], Launay[®], and Tintoretto[®] samples, respectively. The thermophilic anaerobic
209 digestion seems to be more suitable to the treatment of the three biodegradable plastics
210 selected (Figure 1B). The biodegradation kinetics were better, with a shorter lag phase
211 (almost 10 days) and an greater rate of methane production. The methane potentials were
212 reached during the 100-day test period for the three plastics samples. The methane
213 potential of the Beanarella[®], Launay[®], and Tintoretto[®] samples was 308 ± 7 NL (CH₄) kg⁻¹

214 (VS), 355 ± 7 NL (CH₄) kg⁻¹ (VS), and 257 ± 14 NL (CH₄) kg⁻¹ (VS), respectively. Digestion of the
215 Beanarella[®], Launay[®], and Tintoretto[®] samples exhibited $58 \pm 1\%$, $69 \pm 1\%$, and $47 \pm 1\%$
216 conversion of plastic to methane, respectively. Mater-Bi[®], the main constituent of the
217 Tintoretto[®] capsule, has been digested previously in other studies (Calabro et al., 2019;
218 Puechner et al., 1995; Vasmara and Marchetti, 2016). In a similar period (90 days), the
219 degradation of Mater-Bi[®] resulted in 33 L (CH₄) kg⁻¹ (VS) at 35 °C, while it resulted in 267 L
220 (CH₄) kg⁻¹ (VS) at 55 °C in the study by Vasmara and Marchetti (2016); similar methane
221 productions were observed for Tintoretto[®] in our study. No data regarding anaerobic
222 digestion of the two other materials (Vegemat[®] and Ecovio[®]) are available in the literature.
223 Most polymers, such as PLA, PCL, poly(butylene adipate-co-terephthalate) (PBAT), and
224 poly(butylene succinate) (PBS), are biodegraded slowly by AD under mesophilic conditions
225 (Abou-Zeid et al., 2004; Day et al., 1994; Nunziato et al., 2018; Vargas et al., 2009; Vasmara
226 and Marchetti, 2016; Yagi et al., 2014). For example, in the study by Yagi et al., (2014), only
227 between 29 and 49% of the PLA was degraded within 277 days in mesophilic digesters, and
228 the degradation did not reach completion. However, some polymers are biodegraded rapidly
229 even under mesophilic condition, such as PHB, poly(3-hydroxybutyrate) (PHBV), and TPS
230 (Abou-Zeid et al., 2001; Benn and Zitomer, 2018; Greene, 2018; Ryan et al., 2017; Soda et al.,
231 2016). PHB was reported to degrade between 50 and 80% within 40 days depending on the
232 grade used (Benn and Zitomer, 2018). A difference in efficiency between mesophilic and
233 thermophilic digestion of plastics has already been shown elsewhere (Nunziato et al., 2018;
234 Vargas et al., 2009; Vasmara and Marchetti, 2016; Yagi et al., 2014, 2013).

235 Higher level of plastic conversion in methane were observed in thermophilic condition
236 (between 47 and 69%) than in mesophilic condition (between 12 and 24%) in 100 days.
237 There are two possible explanation for this. Firstly, the thermophilic condition (58°C)
238 provides more favorable environmental conditions for the degradation of plastics than the
239 mesophilic condition (38 °C). When the temperature rises and approaches the glass
240 transition temperature of a polymer, this polymer becomes more accessible to
241 microorganisms and makes it easier to degrade. (Shi and Palfery, 2010). Secondly, the
242 microbial composition of thermophilic and mesophilic inoculum is different. The
243 microorganisms present in thermophilic reactors could be more efficient to degrade
244 biodegradable plastics than mesophilic ones.

245 During the mesophilic and the thermophilic anaerobic digestion of spent coffee and coffee
246 capsules, the digestates were sampled at two different times for microbial analysis, as
247 described in Figure 1. For the thermophilic condition, the sampling was carried out in two
248 distinct phases. The first sampling took place during a high methane production phase; the
249 methane production rate was between 10.8 and 12.5 NL (CH₄) kg⁻¹ (VS) day⁻¹ for the coffee
250 capsules. At the second sampling time, the methane production rate was much lower
251 (between 1.2 and 3.3 NL (CH₄) kg⁻¹ (VS) day⁻¹). Due to the low efficacy of mesophilic
252 anaerobic digestion of plastics, both of the samplings took place during a low methane
253 production phase. The methane production rate was between 0.8 and 2.1 NL (CH₄) kg⁻¹ (VS)
254 day⁻¹ for the various plastics.

255

256 **3.2) Sequence data analysis, community diversity indices, and beta diversity**

257 The microbial community composition was evaluated by amplicon sequencing of the 16
258 rRNA transcripts and the 16S rRNA gene in order to compare the active and the total
259 microbial community. Samples collected in triplicate at the different sampling times (T0, T1,
260 and T2) and under both the mesophilic and the thermophilic conditions were analyzed by
261 amplicon sequencing of the 16S rRNA transcripts. The digestates sampled at the initial and
262 the final sampling time (T0 and T2) were analyzed by amplicon sequencing of the 16S rRNA
263 gene only. A total of 650 496 sequences were collected after normalization (7 744 sequences
264 per sample). Almost 96.3% of the total sequences were assigned to the bacterial domain,
265 while the remaining 3.7% could be assigned to the archaeal domain. Similar
266 Bacterial/Archaea ratios have been reported previously in the literature (Guo et al., 2015;
267 Moset et al., 2015; Sundberg et al., 2013; Yang et al., 2014). For instance, Sundberg et al.,
268 2013 reported an average bacterial abundance of 95% of the total sequences during the
269 examination of the microbial community composition of 21 full-scale anaerobic digesters.

270 A comparison of the alpha diversity indices from the analysis of the 16S rRNA gene and
271 transcripts is shown in Table 2. The first analysis focused on the total microbial population
272 (DNA-based analysis) while the second one shows the active microbial community (RNA-
273 based analysis). Lower diversity was observed in the RNA-based analysis compared to the
274 DNA-based analysis, as expected. Similarly, lower diversity indices were observed for the

275 thermophilic samples than for the mesophilic ones, as reported previously (Azizi et al., 2016;
276 Levén et al., 2007; Li et al., 2015; Sekiguchi et al., 1998; Sundberg et al., 2013). The low
277 values of the composite indices (exponential of Shannon and inverse of Simpson) compared
278 to the richness indices (number of OTU and Chao1) indicate a highly uneven distribution of
279 the populations in the different samples. This highlights a paucity of dominant OTUs (with a
280 high number of sequences) and a high number of rare OTUs (with a low number of
281 sequences).

282 Non-metric distance scaling based on the Bray-Curtis index was performed to examine the
283 beta diversity of all of the samples collected from both the mesophilic and the thermophilic
284 digesters over time, and for the digestion of the different substrates (Figure 2). The first
285 NMDS (Figure 2A), on the entire data set, distinguished microbial communities from the
286 mesophilic and the thermophilic reactors. The temperature was been shown to be one of
287 the most important parameters driving the microbial community composition of anaerobic
288 digesters (Azizi et al., 2016; Sundberg et al., 2013). In the same way, the microbial
289 communities from the DNA-based and the RNA-based analysis were clearly separated. The
290 analysis of the 16s rRNA transcripts revealed a different microbial community structure than
291 that of the 16S rRNA gene, thus showing the relevance of studying RNA (De Vrieze et al.,
292 2018). For this reason, the RNA-based analysis will be discussed in more detail below. The
293 other two NMDS (Figure 2 B and C) focused on the thermophilic and the mesophilic samples
294 based on RNA analysis. With the thermophilic condition, the microbial communities of the
295 reactors digesting plastics (Beanarella[®], Launay[®], and Tintoretto[®]) were clustered together
296 at the first sampling time, and they shared a similar microbial composition structure. On the
297 other hand, at the second sampling time, a number of big differences were noted between
298 the communities from the reactors digesting the different plastic samples. In particular, the
299 communities of the Beanarella[®] reactors were very different from those of the Launay[®] and
300 the Tintoretto[®] reactors. The difference in activity between T1 and T2 could explain this
301 shift. At the first sampling time, the measured methane production was high, while it was
302 quite low at the second sampling time (see 3.1). Thus, one could expect to find more
303 bacteria involved in the hydrolysis and fermentation of the substrate being active at the first
304 sampling time than at the second. Similarly, different community compositions were
305 observed for reactors with spent coffee grounds at the first and the second sampling time,

306 under either the mesophilic or the thermophilic condition. However, the digestion of the
307 plastic samples under mesophilic conditions was inefficient and no differences in methane
308 production were observed between the first and the second sampling time. This led to very
309 similar active microbial populations at the two sampling times from the mesophilic reactors
310 digesting the different plastic samples.

311 **3.3) Distribution of the main phylum**

312 Twelve major phyla (represented by more than 1% of the total sequences each in at least
313 one sample) were observed in the mesophilic reactors (Figure 3). As described above, the
314 thermophilic reactors were less diverse; sequences could only be assigned to eight major
315 phyla (Figure 5).

316 **3.3.1) Archaea**

317 Methanogenesis, the final step of the anaerobic digestion resulting in the production of
318 methane, is exclusively due to the *Archaea*. *Euryarchaeota* was the major phylum of the
319 archaeal domain, representing 99.5 and 100% of the total archaea sequences in the
320 mesophilic and the thermophilic digesters, respectively. *Bathyarchaeota* represented 0.5%
321 of the archaeal sequences found in the mesophilic digesters. The abundance of the
322 *Euryarchaeota* phylum in the inocula was quite low: 0.4% and 0.6% of the total sequences, in
323 the mesophilic and thermophilic inocula, respectively. The low abundance of active archaea
324 at the beginning of the BMP test (T0) could be explained by the fact that the inocula were
325 exhausted before their use (they were not fed) in order to lower the endogenous methane
326 production (Figures 3 and 5). The active *Euryarchaeota* increased during the digestion of the
327 compounds (spent coffee or plastics) to between 1% and 11% of the total sequences (Figures
328 3 and 5). At the genus level, *Methanothermobacter* and *Methanoculleus* dominated the
329 thermophilic digesters, representing 67.5 and 27.6% of the total archaeal sequences,
330 respectively. *Methanothermobacter* was the most abundant archaea in the plastic-fed
331 digesters at 58 °C, while it was *Methanoculleus* in the coffee-fed reactors. Similarly,
332 *Methanoculleus* and *Methanosaeta* were dominant in the mesophilic condition, at 55.7 and
333 29.4% of the total archaeal sequences, respectively. *Methanosaeta* were predominant in the
334 digesters fed with spent coffee, and *Methanoculleus* in the plastic-fed reactors, at 38 °C.

335 Except for members of the *Methanosarcina* genus, which are able to perform all three
336 pathways of methane production (De Vrieze et al., 2012); all the archaeal genera found here
337 could be assigned to a specific methane production pathway. Most of the archaeal
338 sequences could be attributed to hydrogenotrophic methanogens genera. Under the
339 thermophilic conditions, hydrogenotrophic methanogens (*Methanoculleus*,
340 *Methanothermobacter*, and *Methanobacterium*) represented 96.7% of the archaeal
341 sequences. Acetoclastic methanogens represented 0.1% (*Methanosaeta*) and
342 methylotrophic methanogens 1.6% (*Methanosillicoccus*). *Methanosarcina* accounted for
343 1.6% of all of the archaeal sequences. In the mesophilic reactors, the dominance of
344 hydrogenotrophic methanogens (*Methanoculleus*, *Methanospirillum*, *Methanobacterium*,
345 and *Methanothermobacter*) was less pronounced, at 69.4% of all of the archaeal sequences.
346 Acetoclastic methanogens (*Methanosaeta*) accounted for 29.4% of the archaeal sequences.
347 Similarly, methylotrophic methanogens (*Methanomasillicoccus*) accounted for 1.1% of under
348 the mesophilic conditions.

349 A dominance of hydrogenotrophic methanogens is common in many thermophilic anaerobic
350 digesters (Goberna et al., 2009; Hori et al., 2006; Krakat et al., 2010; Sasaki et al., 2011;
351 Sundberg et al., 2013). A number of recent studies have shown that an increase in
352 temperature correlated with a growing abundance of hydrogenotrophic methanogens (Liu et
353 al., 2018; Pap et al., 2015). Hydrogenotrophic dominance is less common in mesophilic
354 digesters, although it has been reported in the literature (Sundberg et al., 2013; Wirth et al.,
355 2012; Zhu et al., 2011). Several parameters such as temperature, a change in substrate
356 composition, and a high concentration of ammonia or volatile fatty acids have been shown
357 to favor the development of hydrogenotrophic microorganisms (De Vrieze et al., 2012;
358 Sasaki et al., 2011; Schnürer and Nordberg, 2008). This situation was supported by the high
359 abundance of acetate-oxidizing bacteria able to engage in a syntrophic partnership with
360 hydrogenotrophic methanogens, such as *Tepidanaerobacter*, *Thermacetogenium*, and
361 *Tepidanaerobacter* (Hattori et al., 2000; Westerholm et al., 2011).

362 **3.3.2) Bacteria:**

363 a) Mesophilic condition

364 The *Bacteroidetes* phylum was predominant in the initial inoculum, representing 61% of the
365 sample sequences, with *Firmicutes* (16%) and *Chloroflexi* (13%) being the other two major
366 phyla. The *Bacteroidetes* phylum decreased during the anaerobic digestion, although it
367 remained the most abundant phylum in each reactor (between 25% and 58% of the
368 sequences of the samples). The digestion of the compounds (spent coffee or plastics) also
369 pointed to an increase in abundance of the *Proteobacteria* and *Synergistetes*. Interestingly,
370 the *Sprichaetae* phylum was only abundant in the reactor with spent coffee grounds at the
371 first sampling time (19%), while it was found in low abundance at the second sampling time
372 and in the reactors with plastics (between 0.1 and 0.7%). Most of these OTU (97%) were
373 attributed to the genus *Treponema*. Some *Treponema* species have been reported to be
374 homoacetogens (Graber et al., 2004; Zhang et al., 2009) while others are hydrolytic bacteria,
375 such as *Treponema amylovorum*, which, for example, are able to degrade starch (Wyss et al.,
376 1997).

377 As seen in the NMDS representation (Figure 2), the microbial community composition of the
378 plastic-fed reactors was very close for the same sampling time. At the phylum level, a similar
379 profile was observed (Figure 3), with a dominance of *Bacteroidetes*, *Firmicutes*, *Chloroflexi*,
380 and *Proteobacteria*. The methane production resulting from the digestion of the
381 biodegradable plastic was not significantly different between the first and the second
382 sampling time, which may explain the low variation of the phyla profile. As a result, the
383 microbial community compositions of T1 and T2 were very similar, with the exception of a
384 decrease in *Bacteroidetes* and a slight increase in *Proteobacteria* and other minor phyla (as
385 *Fibrobacterales* and *Synergistetes*). Similarly, no notable differences were seen in the
386 distribution of the 20 most abundant genera (Figure 4). Thus, these analyses did reveal OTUs
387 specific for the degradation of plastic in anaerobic media, which may be due to the lack of
388 efficacy of the mesophilic process. No previously known taxa participating in the anaerobic
389 digestion of biodegradable plastic were found in the mesophilic reactors (Venkiteshwaran et
390 al., 2019; Yagi et al., 2014)

391 b) Thermophilic condition

392 *Bacteroidetes* was the predominant phylum initially (accounting for 49% of the sample
393 sequences) in the inoculum, followed by *Thermotogae* (27%) and *Firmicutes* (22%). However,
394 during the digestion (of the various plastics and the spent coffee), *Firmicutes* became

395 predominant, representing between 55% and 84% of the sequences. The digestion was also
396 marked by a pronounced decrease in the abundance of *Bacteroidetes*, dropping from 49% of
397 the sequences in the inoculum to less than 8% in the reactors treating the various
398 substrates. By contrast, *Synergistetes* increased from 1% in the inoculum to between 2 and
399 19% in the reactors. Interestingly, a high abundance of *Thermotogae* (37%) was found at the
400 first sampling time for the reactor with spent coffee (Figure 5), while they were found at low
401 abundance (between 2 and 9%) elsewhere (in other reactors and at the second sampling
402 time for the reactor with coffee). All of the sequences belonging to the phylum *Thermotogae*
403 could be attributed to the genus *Defluviitoga* (Figure 6). *Defluviitoga* are well-known
404 bacteria involved in thermophilic hydrolysis and fermentation of a large diversity of
405 monosaccharides, disaccharides, and polysaccharides including cellulose and xylan (Ben
406 Hania et al., 2012; Li et al., 2019; Maus et al., 2016).

407 As described above, at the first sampling time, the microbial community composition of the
408 thermophilic reactors treating the various plastic samples was very similar (Figure 5).
409 *Firmicutes* (77-84%) and *Synergistetes* (5-9%) dominated the microbial community of those
410 reactors. However, a number of differences were observed at the second sampling time on
411 the NMDS and on the repartition at the phylum level (Figures 5 and 6). The digestion of
412 Beanarella® and Tintoretto® resulted in a quite high amount of *Bacteroidetes* (8% and 5%,
413 respectively), while digestion of Launay® capsules led to a low degree of development of this
414 phylum (0.4%). Similarly, the digestion of Tintoretto® was marked by a high abundance of
415 sequences attributed to *Proteobacteria* (6.5%), while they were very low for the other two
416 plastics (0.3-0.7%). These *Proteobacteria* were mostly attributed to *Alpha-proteobacteria*,
417 and more specifically to the *Phyllobacteriaceae* family. Most of the time, species of
418 *Phyllobacteriaceae* are found in aerobic plant-associated environments, although they have
419 also been identified in anaerobic digestion media (Guo et al., 2015; Willems, 2014).

420 At the first sampling time, the measured methane production was high, while it was quite
421 low at the second sampling time (see 3.1). Thus, one could expect to find more bacteria
422 involved in the hydrolysis of the substrate and its transformation to methane at the first
423 sampling time than at the second sampling time. At this time point, the phylum *Firmicutes*
424 dominated both reactors fed with plastics and spent coffee grounds. These *Firmicutes*
425 corresponded to different genera depending on whether they came from reactors with

426 spent coffee or biodegradable plastics (Figure 6). For the digesters fed with spent coffee,
427 most of the Firmicutes sequences belonged to the genus *Ruminiclostridium* (14% of sample
428 sequences) and *Coprothermobacter* (15% of the sample sequences). Species of the
429 *Ruminiclostridium* genus are often found in rumen or other anaerobic media and they
430 exhibit lignocellulolytic activity (Ravachol et al., 2015; Zhang et al., 2018). Members of the
431 *Coprothermobacter* genus are known to be anaerobic and thermophilic microorganisms with
432 a proteolytic activity (Pavan et al., 2018). For the digesters fed with plastics, most of the
433 Firmicutes sequences belonged to the genus *Tepidimicrobium* (42% of the sample
434 sequences). Figure 6, representing the 20 most abundant OTU at the genus level observed in
435 the thermophilic digesters fed with plastics, highlights the high abundance of
436 *Tepidimicrobium* at the first sampling time (when the biodegradation of the plastic was
437 active). By contrast, the *Tepidimicrobium* abundance was very low at the second sampling
438 time (when the biodegradation of the plastic was low). This suggests that *Tepidimicrobium*
439 *sp.* may have a significant role during the degradation of biodegradable plastics. Only two
440 species of this genus have been described, *Tepidimicrobium ferriphilum* (Slobodkin, 2006)
441 and *Tepidimicrobium xylanilyticum* (Niu et al., 2009). The first one was isolated from a
442 freshwater hot spring and the second one from a thermophilic anaerobic digester treating
443 municipal solid waste and sewage. The *Tepidimicrobium xylanilyticum* isolated from an
444 anaerobic digester was able to grow on a variety of carbohydrates (xylan, xylose, glucose,
445 cellobiose, etc.) and on a number of proteinaceous compounds. It has been reported that
446 *Tepidimicrobium xylanilyticum* cannot use starch as a substrate (Niu et al., 2009), which we
447 believe is one of the components of Launay® and Tintoretto® coffee capsules. Members of
448 the *Tepidimicrobium* genus found in the reactors fed with plastic may be involved in the
449 degradation of polyesters, such as PLA, PBAT, or PCL. Moreover, *Tepidimicrobium*
450 *xylanilyticum* was identified by Tseng et al. (2019) in an anaerobic digester treating PLA or
451 lactic acid under thermophilic conditions. Subsequently, Tseng et al. (2020) isolated and
452 characterized one strain of *T. xylanilyticum* from this digester. Contrary to the type strain of
453 *T. xylanilyticum*, that strain was able to consume lactate and could produce H₂, CO₂ and
454 acetate. They also found out that the accumulation of lactate inhibited the physicochemical
455 depolymerisation of PLA in lactate. More studies on *Tepidimicrobium* is crucial for gaining a
456 better understanding of their involvement in the biodegradation of other biodegradable
457 polymers.

458

459

460 **4) CONCLUSION:**

461 The digestion of the three biodegradable coffee capsules was much more efficient under the
462 thermophilic than under the mesophilic conditions. The methane potential at 100 days, at
463 the mesophilic temperature, ranged between 67 and 127 NL (CH₄) kg⁻¹ (VS), while it ranged
464 between 257 and 294 NL (CH₄) kg⁻¹ (VS) at the thermophilic temperature.
465 Hydrogenotrophic methanogens were predominant in the plastic-fed reactors, at both
466 operating temperatures. The microbial populations from the reactors fed with plastics
467 versus spent coffee grounds were significantly different, under both the mesophilic and the
468 thermophilic conditions. At the level of the main OTUs, at the same sampling time and at the
469 same operational temperature, the populations coming from the reactors fed with the
470 different plastics were only slightly different. Most of the differences between these
471 populations were due to rare OTUs. The *Tepidimicrobium* genus was found to be dominant
472 in the thermophilic digesters fed with biodegradable plastics during the high methane
473 production phase. This genus may have a significant role during the biodegradation of the
474 biodegradable plastics under thermophilic conditions. To extend the findings of this study,
475 the microbial community composition and the dynamics of continuous anaerobic digestion
476 pilot-fed with plastics and other organic wastes will be assessed in order to determine the
477 performance and stability of the process.

478

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TABLE and FIGURE CAPTIONS

Table 1: The main characteristics of the samples (inocula and substrates) used in this study.

Figure 1: The mean cumulative methane production (NL (CH₄) kg⁻¹ (VS)) in the mesophilic digesters (38 °C, **A**) and the thermophilic digesters (58 °C, **B**). The error bars represent the standard deviations of the biological replicates. The arrows indicate the times at which the samplings for the microbial community analyses were conducted. The colour of the arrows indicates whether the sampling was done on plastic-fed digesters (red arrows) or on digesters fed with spent coffee grounds (blue arrows).

Table 2: Alpha diversity indices of the mesophilic and the thermophilic samples from amplicon sequencing of the 16S rRNA gene (DNA-based) and transcripts (RNA-based) analyses (Hill numbers).

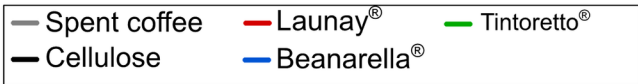
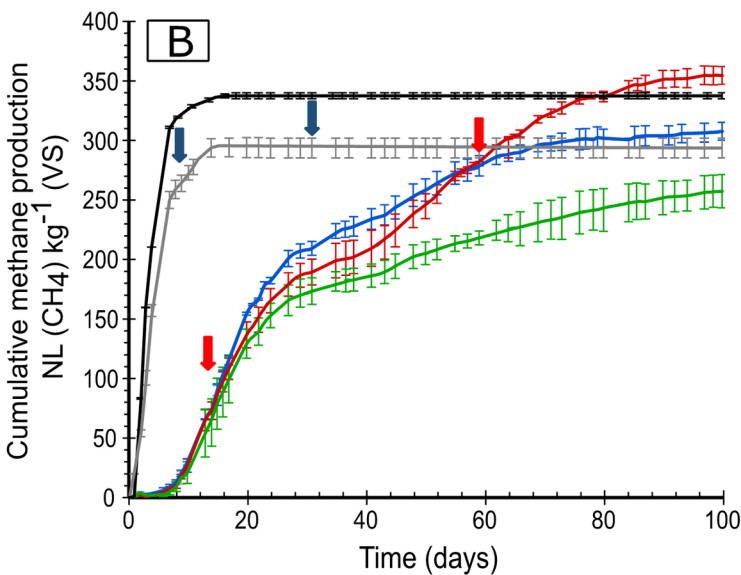
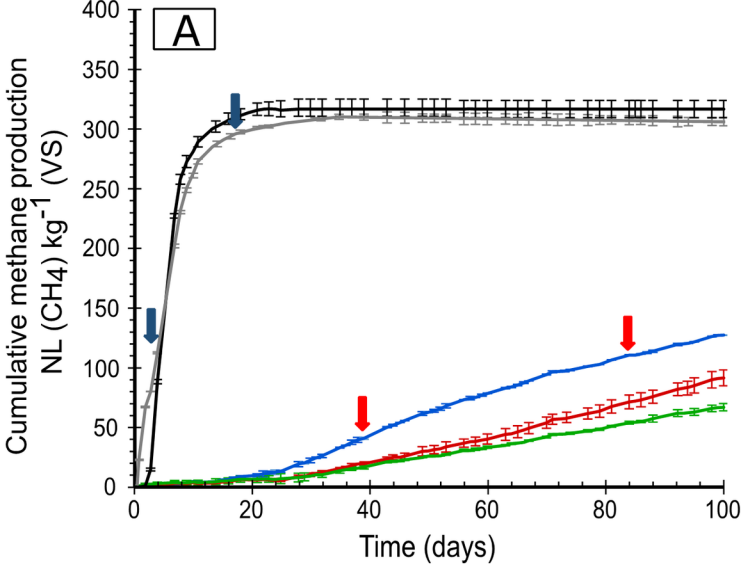
Figure 2: Non-metric distance scaling (NMDS) analysis of the Bray-Curtis distance indices for the microbial communities at the OTU level of the entire data set (**A**), of RNA from the mesophilic digesters (**B**) and the RNA from the thermophilic digesters (**C**). The inocula (T0) are represented as squares, the first session of sampling (T1) as dots, and the second session (T2) as triangles. The different plastics at the first session of sampling are grouped as T1 plastics because their microbial populations were very close.

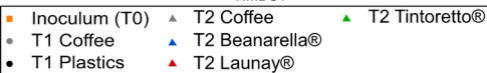
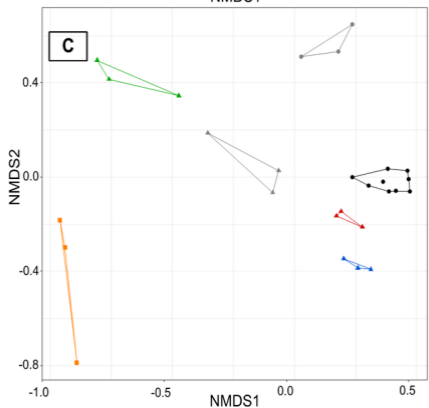
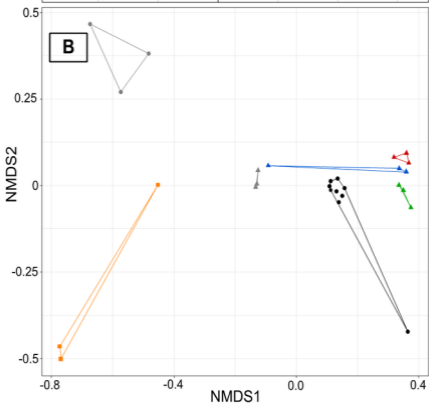
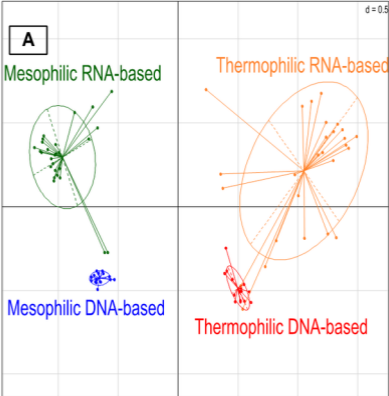
Figure 3: The main bacterial and archaeal phyla (representing more than 1% of the total sequences in at least one sample) observed in the mesophilic digesters. Phyla with an abundance of less than 5% are not labelled.

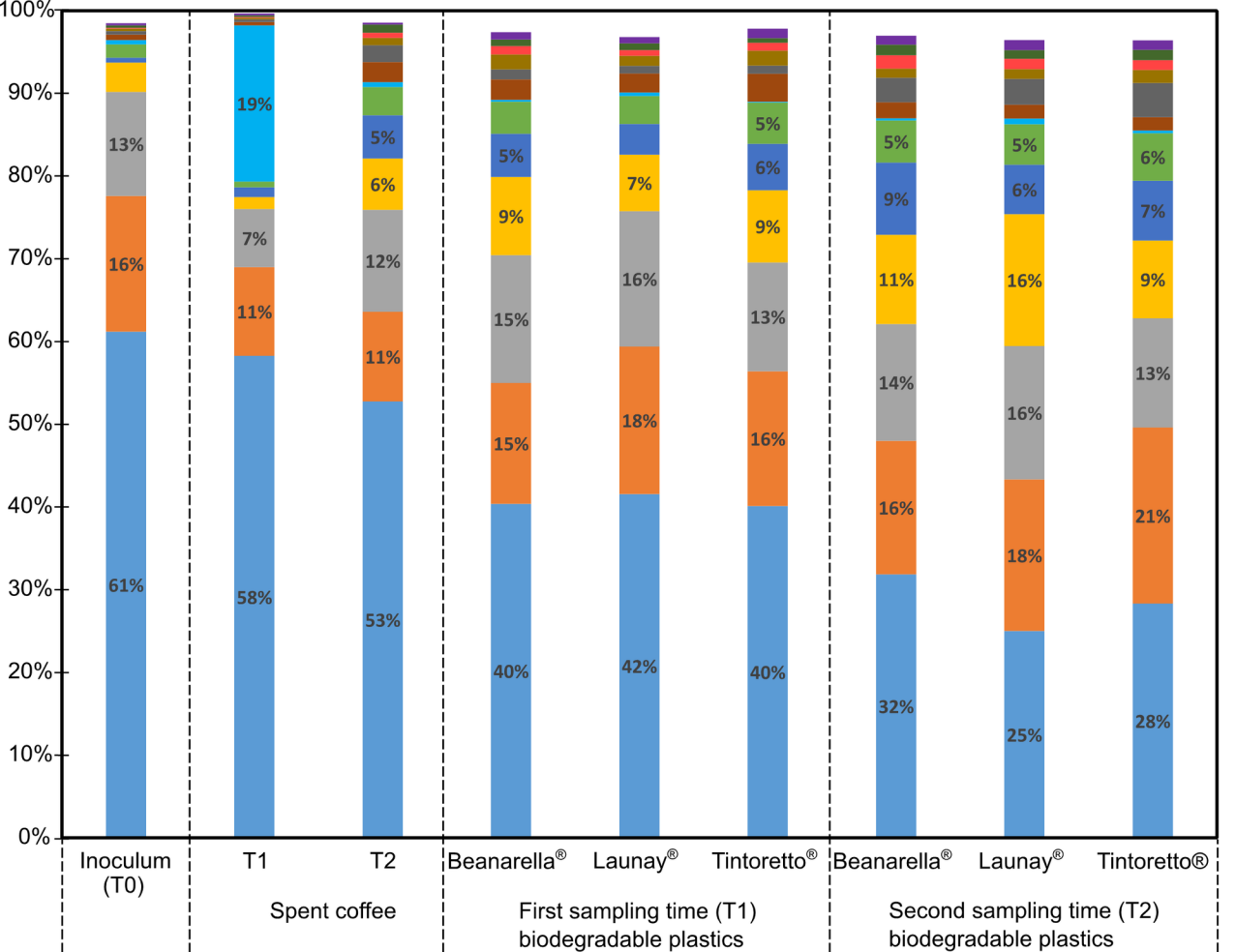
Figure 4: Heatmap of the 20 most abundant genera observed in the mesophilic digesters. The legend shows the relative abundances. The sample clusterization is on the top of the heatmap and the evolutionary dendrogram of the OTU at the right of the heatmap.

Figure 5: The main bacterial and archaeal phyla (representing more than 1% of the total sequences in at least one sample) observed in the thermophilic digesters. Phyla with an abundance of less than 5% are not labelled.

Figure 6: Heatmap of the 20 most abundant genera observed in the thermophilic digesters. The legend shows the relative abundances. The sample clusterization is on the top of the heatmap and the evolutionary dendrogram of the OTU at the right of the heatmap.







Archeae:

Euryarchaeota

Bacteria:

Bacteroidetes

Firmicutes

Chloroflexi

Proteobacteria

"Unidentified"

Fibrobacteres

Synergistetes

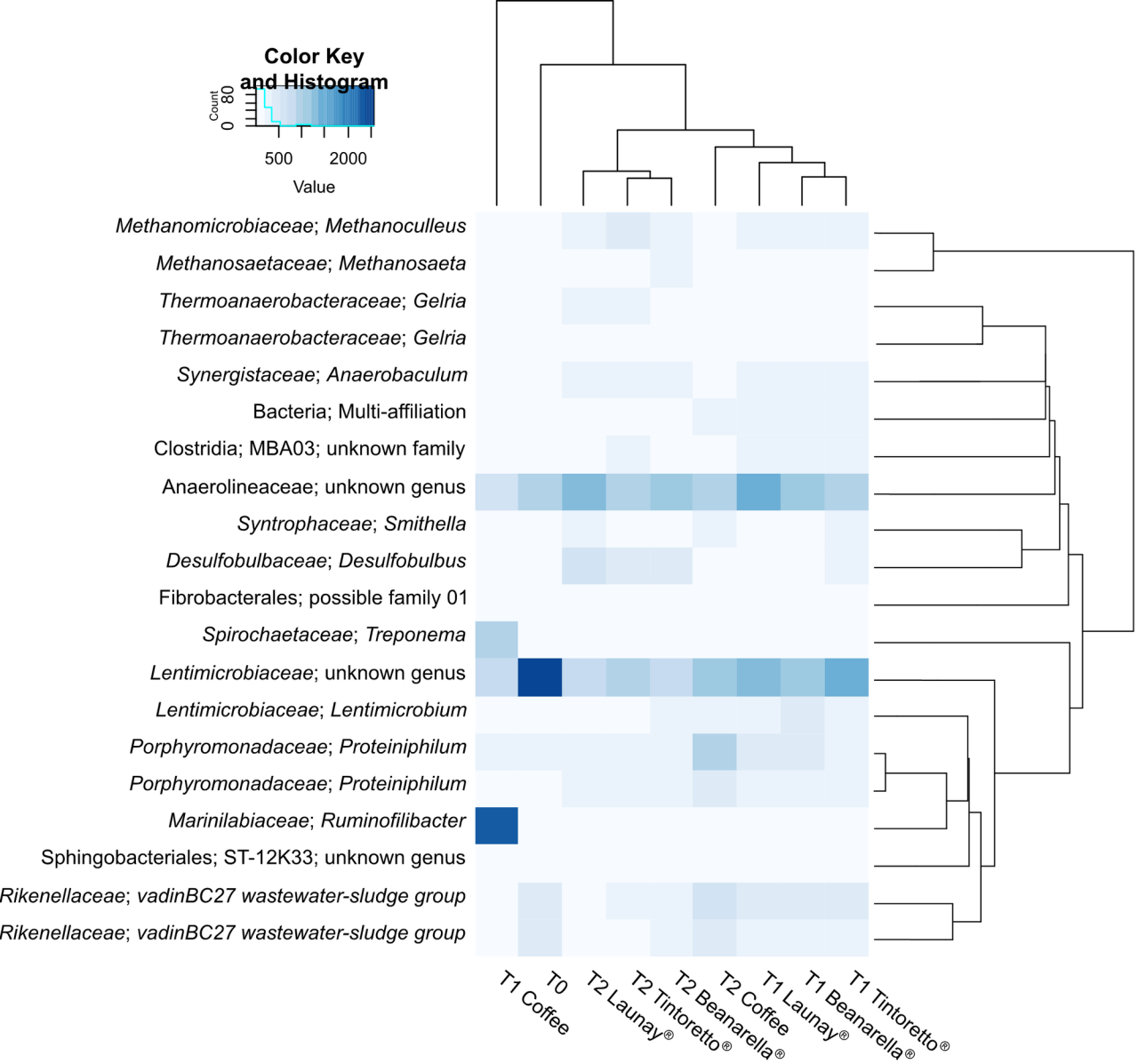
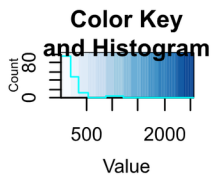
Spirochaetae

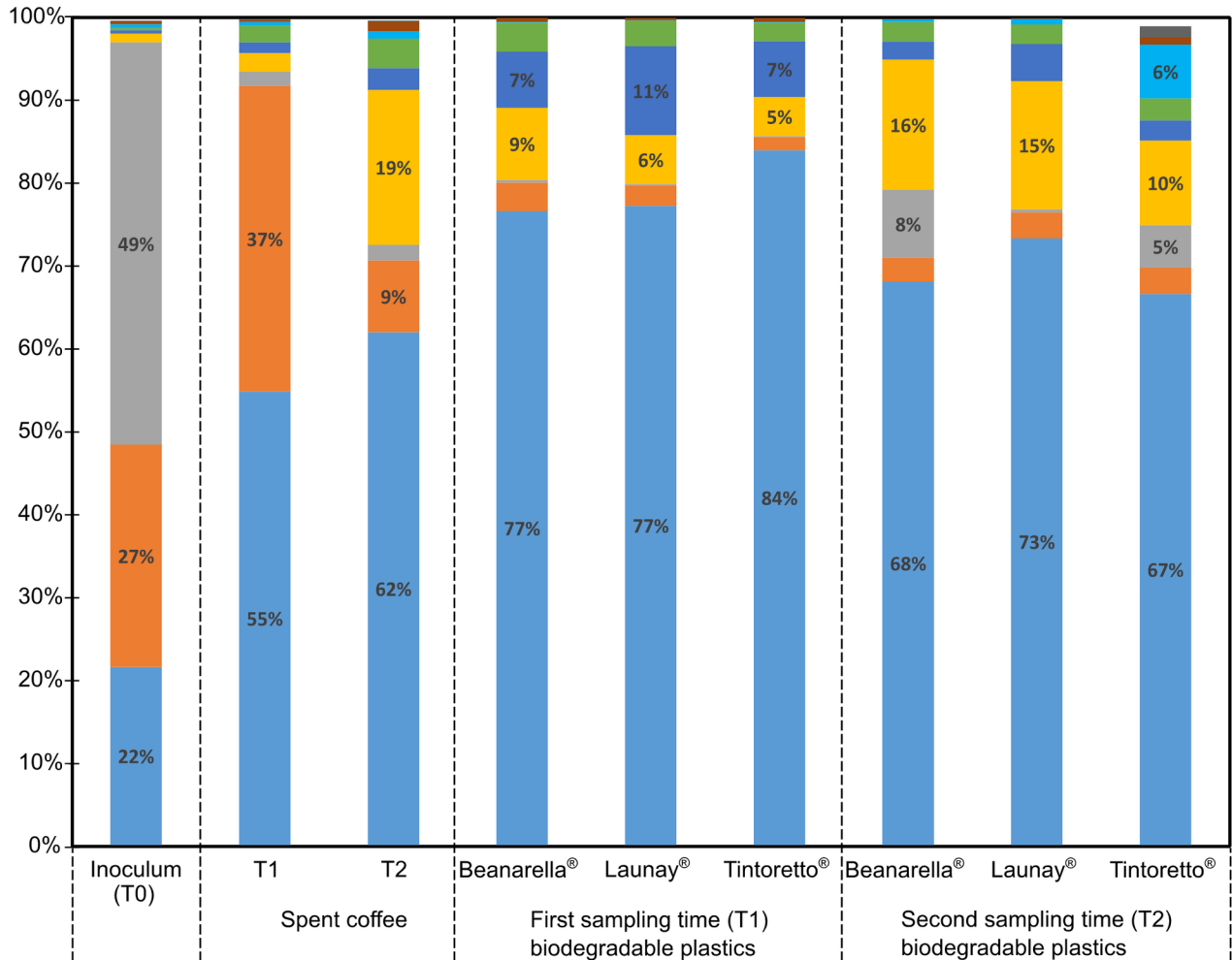
Planctomycetes

Thermotogae

Acidobacteria

Aminicenantes





Archaeae:

Bacteria:

■ Euryarchaeota

■ Firmicutes

■ Thermotogae

■ Actinobacteria

■ "Unidentified"

■ Synergistetes

■ Bacteroidetes

■ Proteobacteria

■ Atribacteria

Samples	TS (% raw mass)	VS (% raw mass)	Ash (% raw mass)	pH	C (% dry mass)	H (% dry mass)	N (% dry mass)	S (% dry mass)	O (% dry mass)	Theoretical methane potential (NL (CH ₄) kg ⁻¹ (VS))
Beanarella®	99.8 ± 0	70.6 ± 0.1	29.2 ± 0.1	-	37.7 ± 0	4.4 ± 0.3	0 ± 0	0 ± 0	28.6	531
Launay®	99.7 ± 0	94.1 ± 0	5.6 ± 0	-	49.7 ± 0	5.8 ± 0	0 ± 0	0.4 ± 0.2	38.5	518
Tintoretto®	99.8 ± 0	98.4 ± 0	1.4 ± 0	-	54 ± 0	6.1 ± 0	0 ± 0	0.2 ± 0.0	38.4	546
Spent coffee	95.3 ± 0.1	90.9 ± 0.1	4.4 ± 0.1	-	48.9 ± 0.3	6.7 ± 0.1	2.8 ± 0.1	0.3 ± 0.01	36.5	524
Mesophilic inoculum	3.9 ± 0	2.6 ± 0.2	1.3 ± 0.2	7.59	-	-	-	-	-	-
Thermophilic inoculum	3.7 ± 0.1	2.5 ± 0.3	1.3 ± 0.3	8.02	-	-	-	-	-	-

Methane production and active microbial communities during anaerobic digestion of three commercial biodegradable coffee capsules under mesophilic and thermophilic conditions

G. Cazaudehore, F. Monlau, C. Gassie, A. Lallement and R. Guyoneaud

Graphical abstract

