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

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## **1) INTRODUCTION:**

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

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

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

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

thought to comprise key bacteria that decompose lactic acid and that supply CO<sub>2</sub> and H<sub>2</sub> to hydrogenotrophic methanogens.

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

## **2) MATERIALS AND METHODS**

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

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

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

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

$$\text{Theoretical methane production (NL(CH}_4\text{).g}^{-1}\text{(C}_x\text{H}_y\text{O}_z\text{N}_n\text{S}_s\text{))} = \frac{22,4 \times \left(\frac{x}{2} + \frac{y}{8} - \frac{z}{4} - \frac{3n}{8} - \frac{s}{4}\right)}{12x + y + 16z + 14n + 32s}$$

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

## 2.2) Biochemical Methane Potential (BMP) tests

Ground biodegradable plastic samples, spent coffee, and cellulose (positive control purchased from Tembec, France) were digested in batch bottles under mesophilic (38 °C) and thermophilic (58 °C) conditions according to an experimental protocol adapted from the recommendations of the European Inter-Laboratory studies in which APESA is involved (Hafner et al., 2020). The BMP bottles were filled with 300 mL of inoculum, water, and test material mixture at an inoculum to substrate ratio (ISR) of 2.7 g(VS).g(VS)<sup>-1</sup>. The organic loading of the test corresponded to 9 g(VS).L<sup>-1</sup>. Previous research work has suggested an ISR ≥ 2 in order to obtain reproducible constant kinetics (Chynoweth et al., 1993; Raposo et al., 2011, 2008). A blank control without test material was also carried out. Before placing an airtight seal on the bottle, the gas phase was flushed out with nitrogen (Alphagaz™ Smartop, Air Liquide, France). The daily biogas production was estimated by the increase in pressure using a manometer (2023P, Digitron, Croatia). The biogas composition was determined using a gas chromatograph (Micro GC 490, Agilent, USA) equipped with two columns. The first column (M5SA 10m, Agilent, USA) was used at 80 °C and 200 kPa to separate O<sub>2</sub>, N<sub>2</sub>, and CH<sub>4</sub> using Argon as the carrier phase; the second column (PPU 10m BF) was used at 80 °C and 150 kPa to separate the CO<sub>2</sub> from the other gases using Helium as the carrier phase. The

injector temperature was 110 °C. The detection of gaseous compounds was achieved using a thermal conductivity detector. The biogas production of the negative control, endogenous to the inoculum, was subtracted from the production of the other bottles. The BMP tests were performed in triplicate. The calibration was carried out with two standard gases composed of 9.5% CO<sub>2</sub>, 0.5% O<sub>2</sub>, 81% N<sub>2</sub> and 10% CH<sub>4</sub> and 35% CO<sub>2</sub>, 5% O<sub>2</sub>, 20% N<sub>2</sub>, and 40% CH<sub>4</sub> (special gas, Air Liquide®, France). All of the results are presented for normalized conditions of temperature and pressure (Patm, 0 °C). The degradation yield was estimated by comparing the observed methane production to the theoretical methane production (Table 1).

## **2.3) Microbial analysis**

### **2.3.1) Sampling**

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

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

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

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

### **2.3.2) Sequencing and bioinformatics analyses**

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

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

## **3) RESULTS AND DISCUSSION**

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

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

The methane potential of cellulose (positive control) under mesophilic and thermophilic conditions was  $315 \pm 5$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) and  $337 \pm 3$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), respectively. Similar values and kinetics have been reported elsewhere for cellulose controls (Hansen et al., 2004; Raposo et al., 2011), thus indicating that the inocula were well-suited to anaerobic digestion under both the thermophilic and the mesophilic conditions.

The spent coffee was readily biodegradable, under both the mesophilic and the thermophilic conditions, with almost no lag phase (< 1 day) and a high level of methane production over a period of 15 to 20 days. A similar degradation behaviour was noted under the mesophilic and the thermophilic conditions; the methane potentials were  $294 \pm 8$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) and  $301 \pm 3$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), respectively, corresponding to a conversion into methane of between 56 and 57% of the spent coffee. The methane production and kinetics from spent coffee were comparable to those of cellulose. The methane potential of spent coffee has been previously described, ranging from 240 to 340 NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) (Lane, 1983; Neves et al., 2006).

The digestion of the biodegradable plastics exhibited very different behaviors under the mesophilic versus the thermophilic conditions. Under the mesophilic conditions, the AD of the three plastics exhibited a long lag phase (almost 25 days) and slow kinetics (Figure 1A), suggesting that the hydrolysis step is limiting and underperforming. Finally, the conversion of the plastics into biogas did not reach completion within the 100-day test period. The methane potential of the Beanarella®, Launay®, and Tintoretto® samples at the end of the test was  $127 \pm 1$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS),  $92 \pm 0$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), and  $67 \pm 3$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), respectively. The biodegradability corresponded to  $24 \pm 0\%$ ,  $18 \pm 0\%$ , and  $12 \pm 1\%$  for the Beanarella®, Launay®, and Tintoretto® samples, respectively. The thermophilic anaerobic digestion seems to be more suitable to the treatment of the three biodegradable plastics selected (Figure 1B). The biodegradation kinetics were better, with a shorter lag phase (almost 10 days) and an greater rate of methane production. The methane potentials were reached during the 100-day test period for the three plastics samples. The methane potential of the Beanarella®, Launay®, and Tintoretto® samples was  $308 \pm 7$  NL (CH<sub>4</sub>) kg<sup>-1</sup>



(VS),  $355 \pm 7$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), and  $257 \pm 14$  NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), respectively. Digestion of the Beanarella®, Launay®, and Tintoretto® samples exhibited  $58 \pm 1\%$ ,  $69 \pm 1\%$ , and  $47 \pm 1\%$  conversion of plastic to methane, respectively. Mater-Bi®, the main constituent of the Tintoretto® capsule, has been digested previously in other studies (Calabro et al., 2019; Puechner et al., 1995; Vasmara and Marchetti, 2016). In a similar period (90 days), the degradation of Mater-Bi® resulted in 33 L (CH<sub>4</sub>) kg<sup>-1</sup> (VS) at 35 °C, while it resulted in 267 L (CH<sub>4</sub>) kg<sup>-1</sup> (VS) at 55 °C in the study by Vasmara and Marchetti (2016); similar methane productions were observed for Tintoretto® in our study. No data regarding anaerobic digestion of the two other materials (Vegemat® and Ecovio®) are available in the literature. Most polymers, such as PLA, PCL, poly(butylene adipate-co-terephthalate) (PBAT), and poly(butylene succinate) (PBS), are biodegraded slowly by AD under mesophilic conditions (Abou-Zeid et al., 2004; Day et al., 1994; Nunziato et al., 2018; Vargas et al., 2009; Vasmara and Marchetti, 2016; Yagi et al., 2014). For example, in the study by Yagi et al., (2014), only between 29 and 49% of the PLA was degraded within 277 days in mesophilic digesters, and the degradation did not reach completion. However, some polymers are biodegraded rapidly even under mesophilic condition, such as PHB, poly(3-hydroxybutyrate) (PHBV), and TPS (Abou-Zeid et al., 2001; Benn and Zitomer, 2018; Greene, 2018; Ryan et al., 2017; Soda et al., 2016). PHB was reported to degrade between 50 and 80% within 40 days depending on the grade used (Benn and Zitomer, 2018). A difference in efficiency between mesophilic and thermophilic digestion of plastics has already been shown elsewhere (Nunziato et al., 2018; Vargas et al., 2009; Vasmara and Marchetti, 2016; Yagi et al., 2014, 2013).

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

During the mesophilic and the thermophilic anaerobic digestion of spent coffee and coffee capsules, the digestates were sampled at two different times for microbial analysis, as described in Figure 1. For the thermophilic condition, the sampling was carried out in two distinct phases. The first sampling took place during a high methane production phase; the methane production rate was between 10.8 and 12.5 NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) day<sup>-1</sup> for the coffee capsules. At the second sampling time, the methane production rate was much lower (between 1.2 and 3.3 NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) day<sup>-1</sup>). Due to the low efficacy of mesophilic anaerobic digestion of plastics, both of the samplings took place during a low methane production phase. The methane production rate was between 0.8 and 2.1 NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) day<sup>-1</sup> for the various plastics.

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

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

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

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

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

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

### 3.3) Distribution of the main phylum

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

#### 3.3.1) Archaea

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

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

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

### **3.3.2) Bacteria:**

#### **a) Mesophilic condition**

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

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

#### b) Thermophilic condition

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

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

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

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

spent coffee or biodegradable plastics (Figure 6). For the digesters fed with spent coffee, most of the Firmicutes sequences belonged to the genus *Ruminiclostridium* (14% of sample sequences) and *Coprothermobacter* (15% of the sample sequences). Species of the *Ruminiclostridium* genus are often found in rumen or other anaerobic media and they exhibit lignocellulolytic activity (Ravachol et al., 2015; Zhang et al., 2018). Members of the *Coprothermobacter* genus are known to be anaerobic and thermophilic microorganisms with a proteolytic activity (Pavan et al., 2018). For the digesters fed with plastics, most of the Firmicutes sequences belonged to the genus *Tepidimicrobium* (42% of the sample sequences). Figure 6, representing the 20 most abundant OTU at the genus level observed in the thermophilic digesters fed with plastics, highlights the high abundance of *Tepidimicrobium* at the first sampling time (when the biodegradation of the plastic was active). By contrast, the *Tepidimicrobium* abundance was very low at the second sampling time (when the biodegradation of the plastic was low). This suggests that *Tepidimicrobium* sp. may have a significant role during the degradation of biodegradable plastics. Only two species of this genus have been described, *Tepidimicrobium ferriphilum* (Slobodkin, 2006) and *Tepidimicrobium xylanilyticum* (Niu et al., 2009). The first one was isolated from a freshwater hot spring and the second one from a thermophilic anaerobic digester treating municipal solid waste and sewage. The *Tepidimicrobium xylanilyticum* isolated from an anaerobic digester was able to grow on a variety of carbohydrates (xylan, xylose, glucose, cellobiose, etc.) and on a number of proteinaceous compounds. It has been reported that *Tepidimicrobium xylanilyticum* cannot use starch as a substrate (Niu et al., 2009), which we believe is one of the components of Launay® and Tintoretto® coffee capsules. Members of the *Tepidimicrobium* genus found in the reactors fed with plastic may be involved in the degradation of polyesters, such as PLA, PBAT, or PCL. Moreover, *Tepidimicrobium xylanilyticum* was identified by Tseng et al. (2019) in an anaerobic digester treating PLA or lactic acid under thermophilic conditions. Subsequently, Tseng et al. (2020) isolated and characterized one strain of *T. xylanilyticum* from this digester. Contrary to the type strain of *T. xylanilyticum*, that strain was able to consume lactate and could produce H<sub>2</sub>, CO<sub>2</sub> and acetate. They also found out that the accumulation of lactate inhibited the physicochemical depolymerisation of PLA in lactate. More studies on *Tepidimicrobium* is crucial for gaining a better understanding of their involvement in the biodegradation of other biodegradable polymers.



#### **4) CONCLUSION:**

The digestion of the three biodegradable coffee capsules was much more efficient under the thermophilic than under the mesophilic conditions. The methane potential at 100 days, at the mesophilic temperature, ranged between 67 and 127 NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS), while it ranged between 257 and 294 NL (CH<sub>4</sub>) kg<sup>-1</sup> (VS) at the thermophilic temperature. Hydrogenotrophic methanogens were predominant in the plastic-fed reactors, at both operating temperatures. The microbial populations from the reactors fed with plastics versus spent coffee grounds were significantly different, under both the mesophilic and the thermophilic conditions. At the level of the main OTUs, at the same sampling time and at the same operational temperature, the populations coming from the reactors fed with the different plastics were only slightly different. Most of the differences between these populations were due to rare OTUs. The *Tepidimicrobium* genus was found to be dominant in the thermophilic digesters fed with biodegradable plastics during the high methane production phase. This genus may have a significant role during the biodegradation of the biodegradable plastics under thermophilic conditions. To extend the findings of this study, the microbial community composition and the dynamics of continuous anaerobic digestion pilot-fed with plastics and other organic wastes will be assessed in order to determine the performance and stability of the process.

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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 (CH<sub>4</sub>) kg<sup>-1</sup> (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.

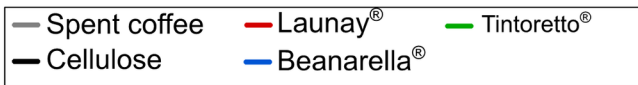
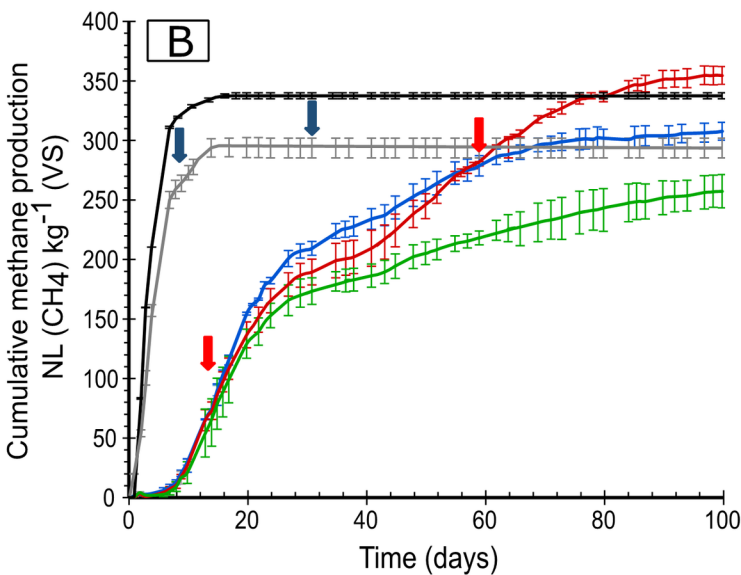
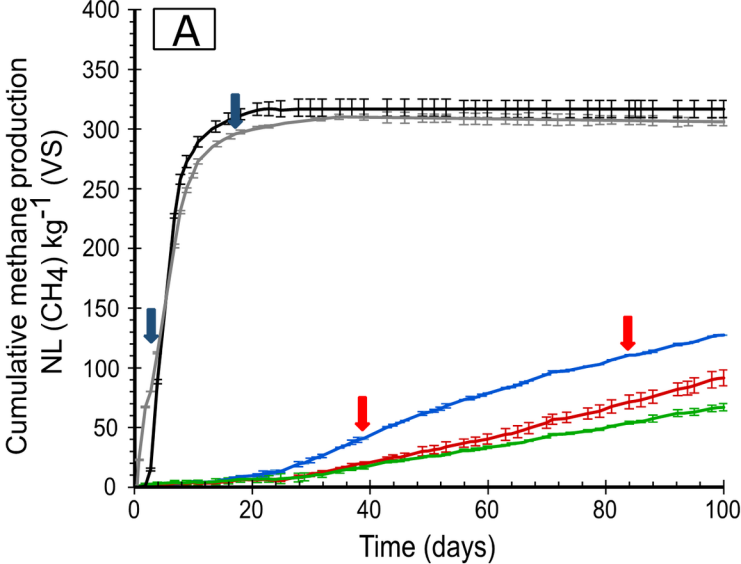
**Figure 4:** Heatmap of the 20 most abundant genera observed in the mesophilic digesters. The legend shows the relative abundances. The sample clusterization is on the top of the heatmap and the evolutionary dendrogram of the OTU at the right of the heatmap.

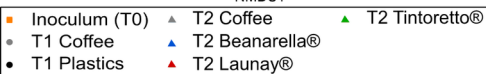
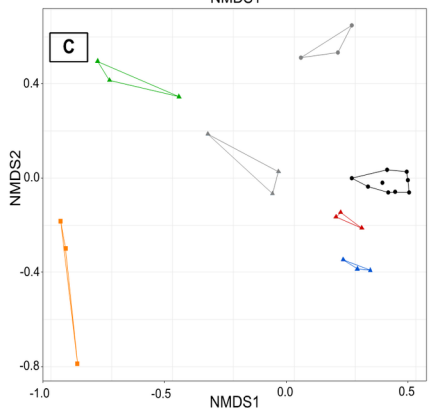
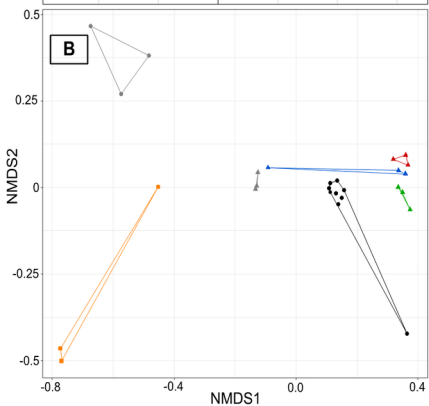
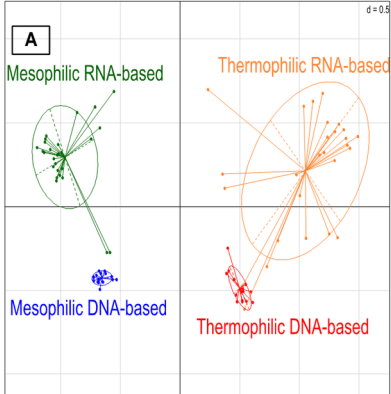
**Figure 5:** The main bacterial and archaeal phyla (representing more than 1% of the total sequences in at least one sample) observed in the thermophilic digesters. Phyla with an abundance of less than 5% are not labelled.

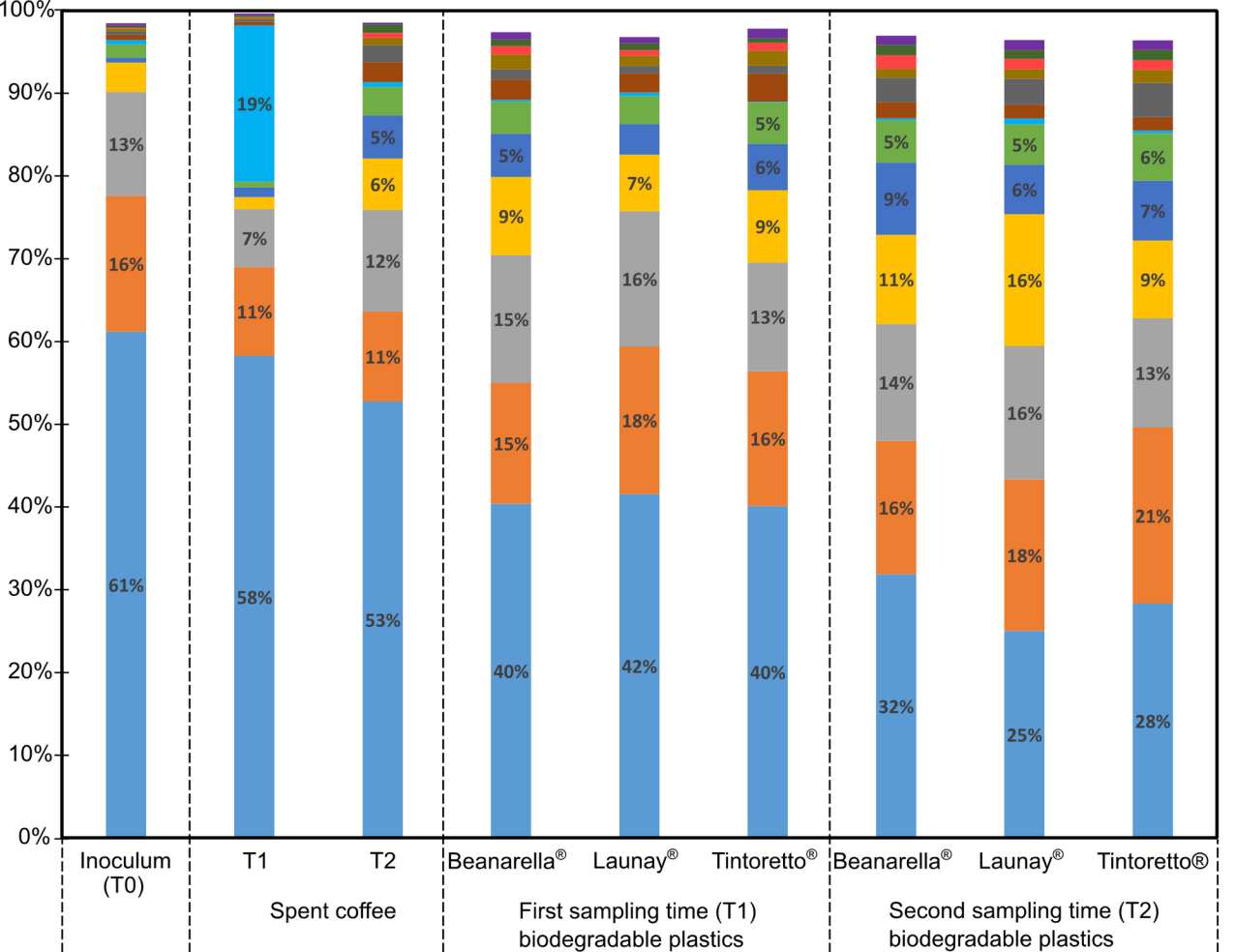
**Figure 6:** Heatmap of the 20 most abundant genera observed in the thermophilic digesters. The legend shows the relative abundances. The sample clusterization is on the top of the heatmap and the evolutionary dendrogram of the OTU at the right of the heatmap.





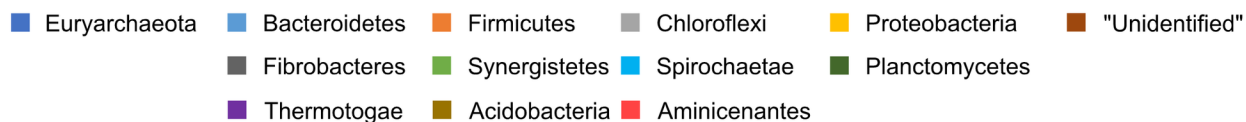


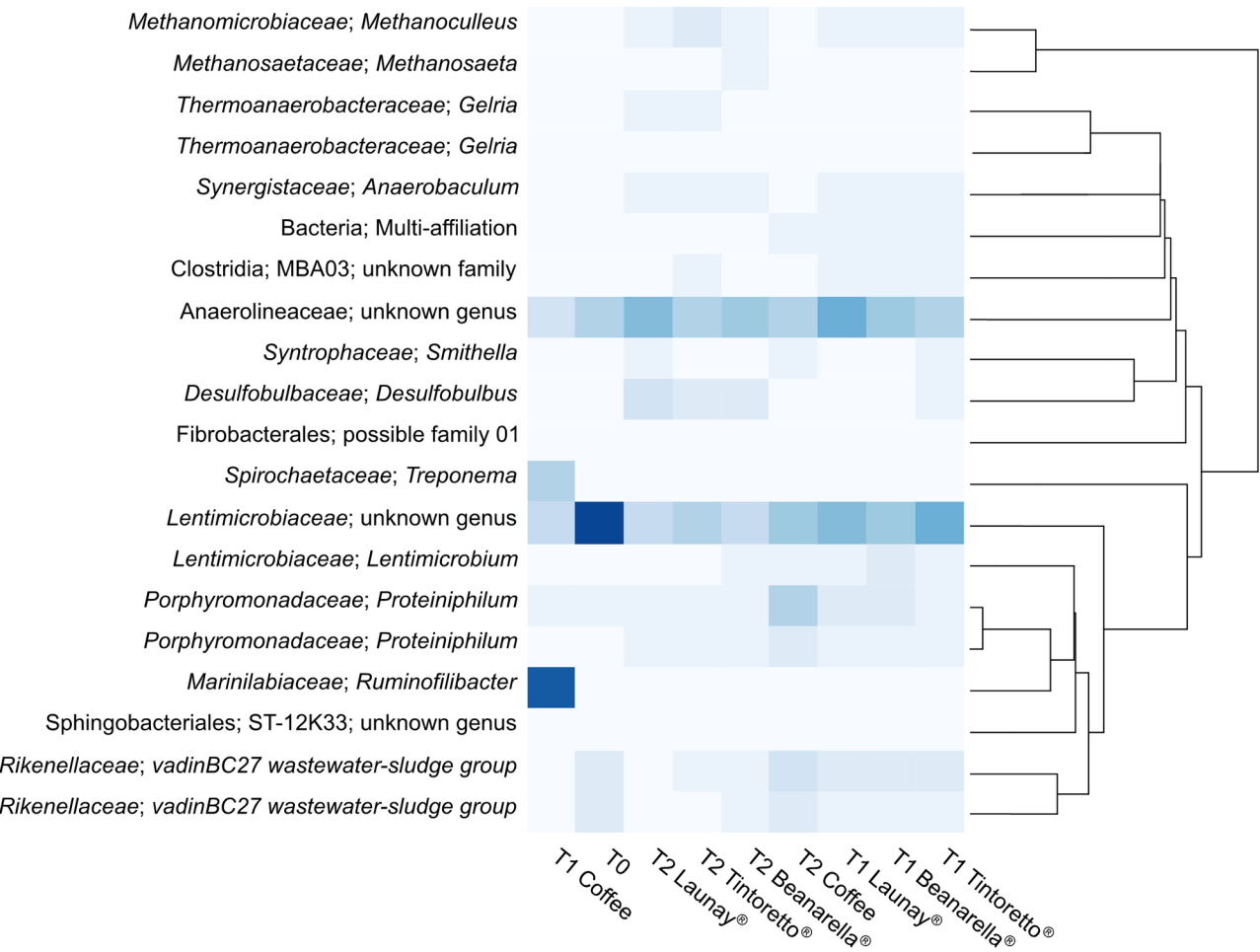
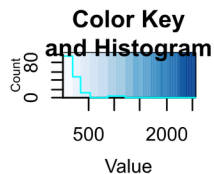


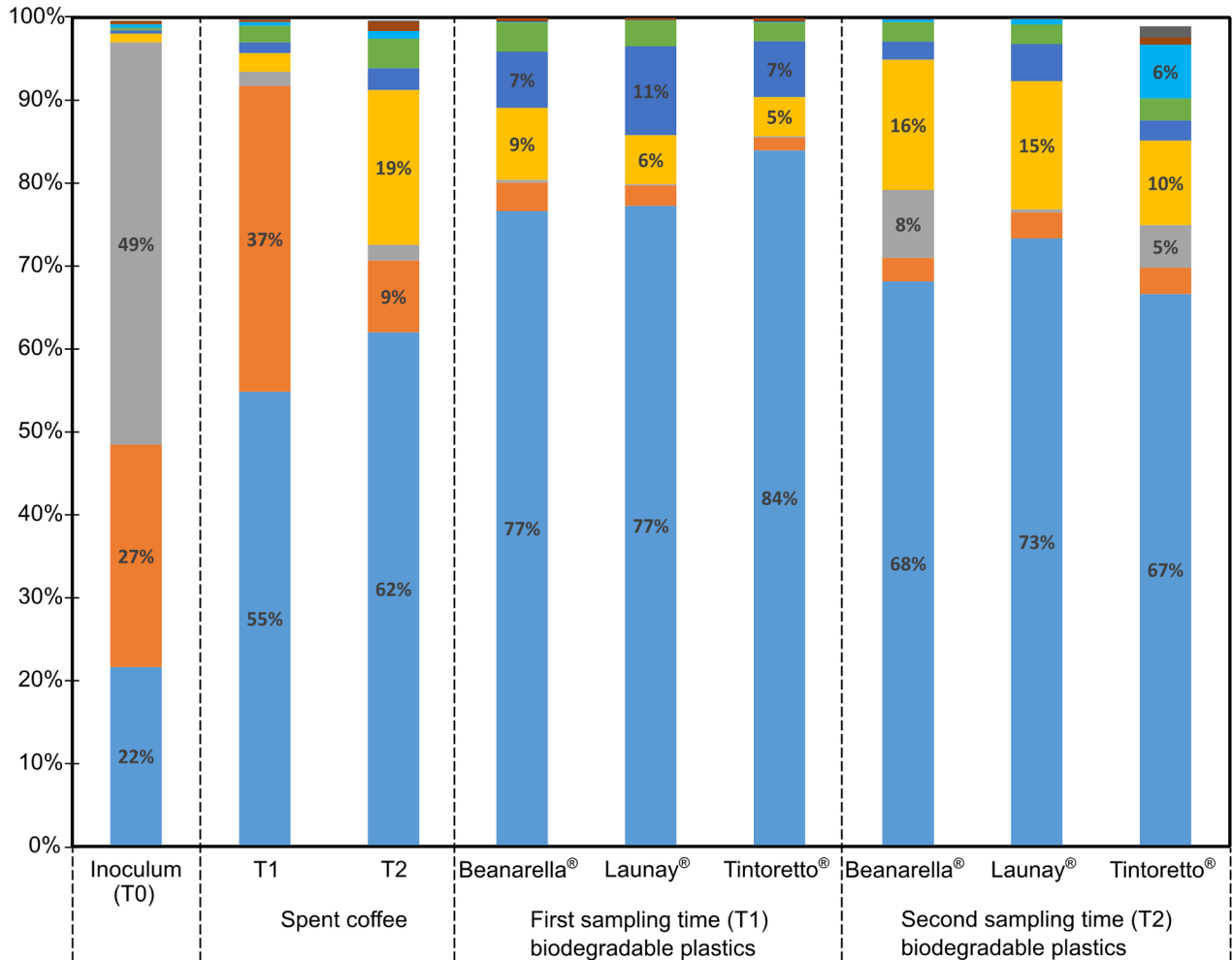


Archeae:

Bacteria:







Archeae:

Bacteria:

Euryarchaeota

Firmicutes

Thermotogae

Actinobacteria

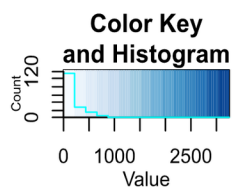
"Unidentified"

Synergistetes

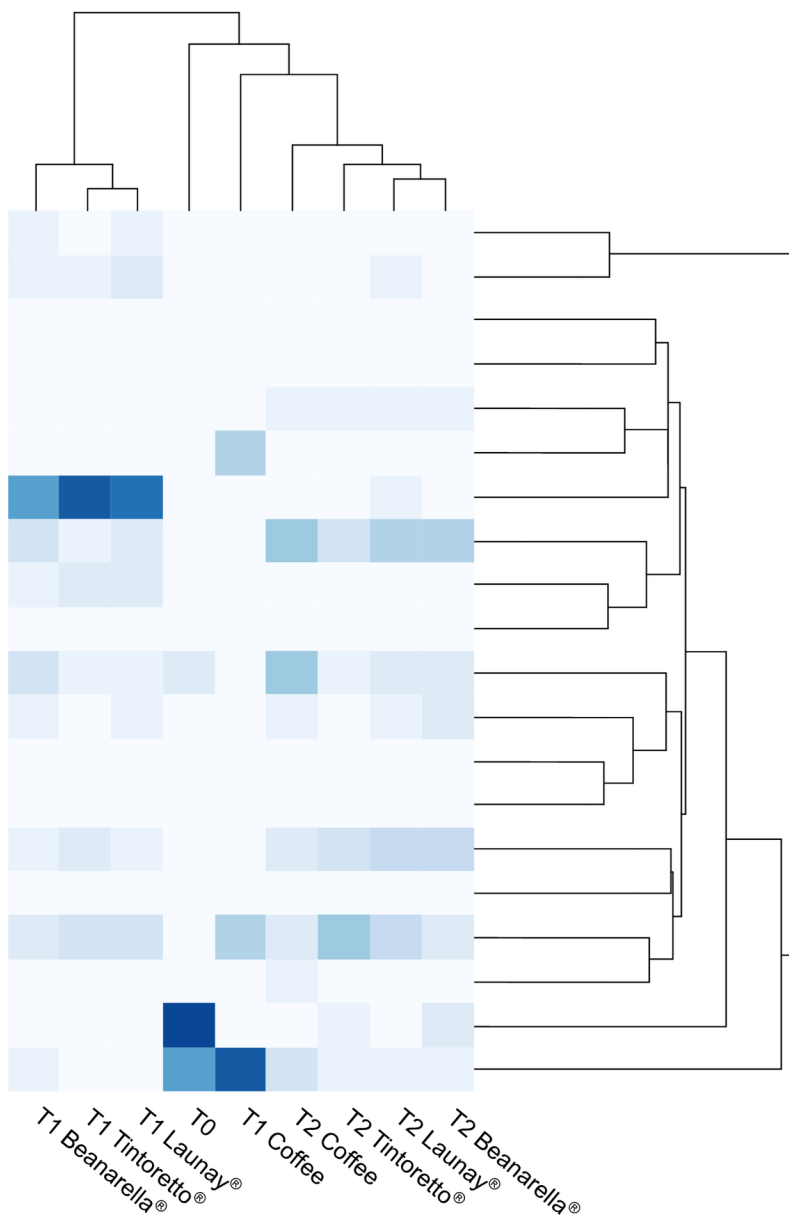
Bacteroidetes

Proteobacteria

Atribacteria



*Methanomicrobiaceae; Methanoculleus*  
*Methanobacteriaceae; Methanothermobacter*  
Firmicutes; BSA1B-03; unknown order  
*Peptococcaceae; unknown genus*  
Clostridia; unknown order  
*Ruminococcaceae; Ruminiclostridium*  
Clostridiales; *Family XI; Tepidimicrobium*  
*Synergistaceae; Anaerobaculum*  
*Thermoanaerobacteraceae; Syntrophaceticus*  
*Thermoanaerobacteraceae; Syntrophaceticus*  
Clostridia; MBA03; unknown family  
*Limnochordaceae; unknown genus*  
*Limnochordaceae; unknown genus*  
Limnochordales; unknown family  
Clostridia; D8A-2; unknown family  
*Heliobacteriaceae; Hydrogenispora*  
*Thermodesulfobiaceae; Coprothermobacter*  
Atribacteria Incertae Sedis; unknown order  
*Lentimicrobiaceae; unknown genus*  
*Petrotogaceae; Defluviitoga*



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 <sub>4</sub> ) kg <sup>-1</sup> (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

