Bioresource Technology 390 (2023) 129889

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Microbial community acclimatization enhances bioplastics biodegradation and biogas production under thermophilic anaerobic digestion

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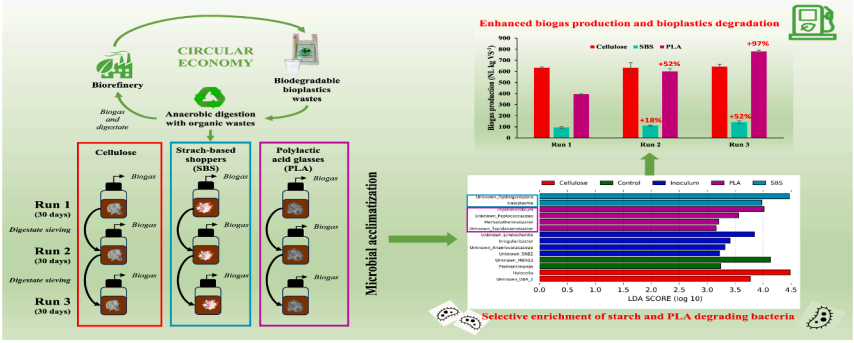
HIGHLIGHTS

- First study of microbial acclimation effects on bioplastics anaerobic digestion.
- Inoculum acclimation to the substrate improves anaerobic digestion of bioplastics.
- Enhanced growth of starch and PLA degraders increases bioplastic degradation.
- Acclimation increased biogas production of starch-based bioplastic by 52%.
- Acclimation increased biogas production of PLA-based bioplastic by 97%.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Anaerobic digestion
Biogas
Starch-based bioplastic
Polylactic acid-based bioplastic
Microbial acclimatization

ABSTRACT

This paper reports the results of a novel study of microbial acclimatization for bioplastics anaerobic degradation and conversion into biogas. Three sequential anaerobic digestion (AD) runs were carried out to favour microbial acclimatization to two different bioplastics, starch-based (SBS) and polyactic-acid (PLA).

AD of SBS and PLA bioplastics was favoured by the acclimatization of the inoculum to the substrate after each run of AD. SBS conversion into biogas increased by 52 % (from 94 to 143 NL $kgVS^{-1}$) and it was correlated with the enhanced growth of starch degrading bacteria such as *Hydrogenispora*, *Halocella* and *Haloplasma*. PLA anaerobic degradation increased by 97 % (from 395 to 779 NL_{biogas} $kgVS^{-1}$) and it was related to the acclimatization of known PLA-degraders such as *Tepidimicrobium*, *Methanothermobacter* and *Tepidanaerobacter*. Microbial acclimatization appears a suitable and low-cost strategy to enhance bioplastics circularity by promoting their anaerobic biodegradation and conversion into biogas.

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https://doi.org/10.1016/j.biortech.2023.129889
Received 4 September 2023; Received in revised form 9 October 2023; Accepted 16 October 2023
Available online 20 October 2023

1. Introduction

The incorrect management of plastic wastes and their resistance to degradation has led to contamination and the accumulation of plastics in all environments, including the terrestrial poles and ocean depths (Bergmann et al., 2022). The harmful effects of microplastics and nanoplastics formed by the fragmentation of plastics on environmental and human health is now widely accepted. The combination of bioaccumulation of microplastics and nanoplastics into the food chain and of the plastics' ability to transport organic and inorganic contaminants has led to attempts to remediate and limit plastic pollution (MacLeod et al., 2021). The use of bioplastics, introduced a few decades ago to replace petroleum-based plastics, can help in reducing plastic pollution when coupled to the good practices of reduction, reuse and recycling of plastic, such as those prescribed by the European Waste Directive (Directive 2008/98EC, 2008). Bioplastics are a wide category of materials. The most promising are those biobased and biodegradable (e.g. starch-based bioplastics, polylactic acid (PLA) and polyhydroxyalkanoates) (Rosenboom et al., 2022). The production of bioplastics from renewable raw materials and their biodegradability greatly reduces the environmental impacts of these materials when compared to plastics of fossil origin (Rosenboom et al., 2022). A plastic fragment accidentally released into the soil or sea may take hundreds or thousands of years to degrade, while a bioplastic fragment degrades in a few months or years (Cucina et al., 2021a).

Despite all the potential benefits of bioplastics introduction and diffusion, some drawbacks and limitations of bioplastics have emerged from scientific literature in recent years. This has led to doubts about bioplastics' effectiveness in fighting plastic pollution (Cucina, 2023). Currently, the reportedly low sustainability of bioplastics in terms of their relatively slow degradability and high carbon footprint have emerged as some of the main drawbacks of bioplastics. In the context of the circular economy, *end of life* scenarios of bioplastics represent a crucial aspect to further reduce the carbon footprint of bioplastics in the move towards sustainability (Van Roijen and Miller, 2022). Composting bioplastics with organic waste is a widely and successfully used practice to degrade bioplastics: however, the energy requirements to support the process and the dissipation of energy contained in bioplastics by aerobic metabolism, suggests the need for new solutions to achieve a more sustainable bioplastic circularity (Zhao et al., 2020). Anaerobic digestion (AD) of bioplastics with organic waste is a better end-of-life strategy than composting, as previously reported (Cucina et al., 2022). Two notable advantages of the AD process are: (i) the production of renewable energy (biogas) and (ii) the production of an organic fertilizer (digestate) that can be used to replace synthetic fertilizers.

Anaerobic biodegradability of bioplastics depends upon the type of bioplastic and on the operating conditions of the process (i.e., temperature, hydraulic retention time (HRT), total solids content). Temperature represents the main factor regulating the degradation of bioplastics, since thermophilic temperatures are necessary to reach the glass transition of many bioplastics and allow the transition from their crystalline structure to the more degradable amorphous one (Vardar et al., 2022). Even though some bioplastics are certified as compostable or degradable in an anaerobic environment, anaerobic biodegradation of bioplastics is found to be only partial in real processes operated on a large scale and under real operating conditions; this fact limits bioplastics valorisation through biogas production and favours the permanence of bioplastic residues in the digestate (Cucina et al., 2022). This latter factor may lead to technical difficulties in AD plants (clogging of pipes and pumps) and also impede the use of digestate in agriculture (bioplastic residues in Europe are currently not distinguished from other plastic residues under the legislation on organic fertilizers) (Cucina, 2023; EU Regulation 2019/1009).

Multiple technologies have been explored to enhance the degradation of bioplastics in AD, such as, 1. pretreatment technologies (mechanical, thermal, chemical, and biological), 2. the addition of additives into bioplastic composition to enhance biodegradability, and 3. bioaugmentation (addition of specific microbial strains) (Cazaudehore et al., 2022a). To date, the development of new more degradable bioplastics and the use of bioplastic pre-treatments before AD have been the most studied approaches to improve the anaerobic degradability of bioplastics (Cazaudehore et al., 2022a; Nanda et al., 2022). Despite promising results, some limitations remain in the development of new highly degradable biocomposites (i.e., difficulties in obtaining the necessary mechanical characteristics) or in the application of pretreatments (i.e., high energy costs) (Cucina et al., 2023; Güler and Bağcı, 2020). In this context, a further strategy to improve the anaerobic biodegradation of bioplastics, and not yet explored (Cazaudehore et al., 2022a), could be to favour the development of microbial consortia adapted to the degradation of bioplastics. This approach has been extensively studied to promote the degradation of recalcitrant organic matter and organic contaminants within wastewater and sewage sludge treatment plants, with interesting results. For example, Kurade et al. (2020) described the positive effect of sewage sludge acclimatization on the biological degradation of fats in a sewage treatment plant, resulting in increased biomethane production. The acclimatization of microorganisms is one of the main factors necessary for the degradation of some organic contaminants which are resistant to biodegradation (e.g. polycyclic aromatic hydrocarbons and bisphenols) (Premnath et al., 2021; Peng et al., 2017). The acclimatization of microorganisms before bioaugmentation procedures can further promote the biodegradation of microplastics in wastewater treatment plants (Tang, 2023). Although the potential of acclimatization of the microbial community for the anaerobic degradation of bioplastics appears obvious, to date there is only one study in this regard. Venkiteshwaran et al. (2019) studied the effect of polyhydroxybutyrates co-digestion on the microbial community, and highlighted that a high biodegradability of these materials was associated with changes in the bacterial community, but not in the archaeal community. They also pointed out that the results were probably related to the bioplastics under study being highly biodegradable in anaerobic environments due to their composition. There is evidently a research gap concerning the possible acclimatization of the microbial community to the anaerobic degradation of the most widespread types of biobased bioplastics on the market today (i.e., starch and PLA-based bioplastics), and how it may affect bioplastics conversion into biogas.

In this context, the objectives of this study were (i) to achieve acclimatization to PLA and starch-based bioplastics of the microbial communities under thermophilic anaerobic mono-digestion through a series of three batch cycles, (ii) to investigate the effect of acclimatization on bioplastics biodegradation rate and biomethane production, and (iii) to characterise the microbial community structure and highlight the bacterial drivers of acclimatization.

2. Materials and methods

2.1. Digestate sampling and bioplastics preparation

An anaerobic digestion experiment, consisting of three AD runs of 30 days, was set up and carried out to evaluate the potential effects of digestate enrichment and acclimatization of bioplastics-degrading microorganisms on biogas production and bioplastics degradation. The length of each run (30 days) was selected since it was considered a time length representative of HRT of AD plant operating with organic wastes.

Anaerobic digestate (inoculum) was sampled before each AD run from an AD full-scale plant operating under thermophilic conditions (55 ± 2 °C) and used to treat the organic fraction of municipal solid wastes (OFMSW) in the province of Lodi (Lombardy Region, Italy). Digestate samples were firstly heated in an oven at 55 ± 2 °C until no biogas production was evident. Digestate was characterized prior to each AD run (Table 1).

Two bioplastics type were selected and used as substrates: 1) starch-based shoppers, composed of ~ 30–40 % starch or derivatives and of ~

60–70 % by synthetic, hydrophilic and biodegradable polyesters and additives (SBS) and 2) PLA-based glasses (PLA) available at Italian supermarkets. Prior to the experiments, bioplastics were characterised by FT-IR analysis to define their composition (see e-supplementary materials). Bioplastics were certified as compostable by TÜV Austria (Austria) and the Italian Consortium of Composters (CIC) (Italy), according to EN13432 (2002) standard. For the experiments, bioplastic samples were cut into squares of 2.5 × 2.5 cm according to ISO14853 (2018).

2.2. Experimental design

The three AD runs were carried out under thermophilic conditions (55 ± 2 °C) using glass bottles (500 mL volume) which were hermetically closed after flushing the headspace with N_2. Each run duration was 30 days, for a total incubation of 90 days (3runs × 30 days = 90 days).

For the first AD run, three bottles were filled with 300 mL of digestate and 3 g of SBS, three bottles were filled with 300 mL of digestate and 3 g of PLA, three bottles were filled with 300 mL of digestate and 3 g cellulose (positive control) and three bottles were filled with 300 mL of digestate (blank control). After 30 days all the bottles were opened. The content of the bottles with SBS and PLA samples was sieved at 2 mm and the digestate was collected for sampling and the subsequent AD run. Digestate from positive and blank bottles was also sampled and collected for the subsequent AD trials. Bioplastics residues (bioplastics fragments under 2 mm are considered degraded by international standards) were, removed from the AD in each bottle, rinsed with water and dried at 40 °C until constant weight. Bioplastics residues were then weighed and the bioplastics degradation (%) was determined according to the equation:

$$Degradation(\%) = \left(\frac{BPt0 - BPt}{BPt0}\right)x100$$

where BPt_0 is the mass of bioplastics at the beginning of AD and $BP\ t$ is the mass of bioplastics recovered after AD.

For the second AD run, three bottles were filled with: 150 mL of fresh digestate, 150 mL of digestate from the first AD run of SBS and 3 g of SBS. Another three bottles were filled with: 150 mL of fresh digestate, 150 mL of digestate from the first AD run of PLA and 3 g of PLA; three bottles were filled with: 150 mL of fresh digestate, 150 mL of digestate from the first AD run of cellulose and 3 g of cellulose (positive control); and three bottles were filled with: 150 mL of fresh digestate and 150 mL of digestate from the first blank AD run. The mixing of fresh digestate with acclimatized digestate was carried out to simulate real AD plants, where digestate is commonly recirculated with fresh feedstock to reduce water consumption, improve fermentation efficiency and system stability (Zheng et al., 2020). To avoid a possible overestimation of biogas production due to the presence of solubilized bioplastics and/or bioplastics fragments < 2 mm in the digestate of the first run, in the second run of AD, the SBS and PLA biogas produced was subtracted from the biogas quota produced by blanks bottles prepared with an equal volume of fresh digestate and digestate coming from the first AD run of SBS and PLA, respectively. After 30 days of the second run of AD, the same procedure described for the first AD run, was followed to collect samples of digestate and bioplastics.

Table 1
Inocula characterization (Av. ± St. Dev., n = 3).

Parameter	Unit	Run 1	Run 2	Run 3
Total solids	%	4.6 ± 0.1	3.9 ± 0.4	3.5 ± 0.2
Volatile solids	% d.m.[b]	57 ± 1	59 ± 2	62 ± 2
Total organic C	% d.m.	33 ± 1	28 ± 1	33 ± 0
pH	pH unit	8.4 ± 0	8.3 ± 0	8.4 ± 0
Titrable acidity	$gCH_3COOH\ L^{-1}$	2.2 ± 0.3	1.7 ± 0.2	2.2 ± 0.1
Total alkalinity	$gCaCO_3\ L^{-1}$	49 ± 3	28 ± 1	40 ± 1
FOS/TAC	–	0.21 ± 0.03	0.29 ± 0.06	0.26 ± 0.02
Ammonium-N	$gN\text{-}NH_3\ L^{-1}$	1.6 ± 0.1	1.5 ± 0.1	2.2 ± 0.2

The third AD run was set up similarly to the second one, using 150 mL of fresh digestate and 150 mL of digestate from the second AD run, adding 3 g of SBS or 3 g of PLA or 3 g of cellulose (positive control). Blank controls were set up with digestate only. Again, SBS and PLA biogas productions were subtracted of the biogas quota produced by blanks bottles prepared with an equal volume of fresh digestate and digestate coming from the second AD run of SBS and PLA, respectively. After 30 days, the experiment was terminated, and bottles were opened to recover bioplastics residues and collect digestate samples.

During the whole experimental period, bottles were periodically analysed for both quantitative and qualitative determination of biogas production. Quantitative biogas production was estimated by withdrawing extra-pressure gas with a 100-mL syringe. Biogas production of blank control bottles was subtracted from biogas production of every sample. Qualitative characterization of biogas was performed by a gas chromatograph (Carlo Erba Megaseries 5300, capillary column 25-m × 0.32-mm diameter and flame ionization detector – FID) to determine CH_4:CO_2 ratio in the biogas. The carrier gas was nitrogen at 20 kPa pressure and temperatures of injector and FID were 130 and 150 °C, respectively. Comparison of obtained peak areas was carried out with a standard gas mixture of 30:70 CH_4:CO_2.

2.3. Chemical and spectroscopic analyses

Chemical analyses on digestate fresh samples were carried out following standard procedures. Total solids (TS) and volatile solids (VS) were determined according to standard procedures of the American Public Health Association (APHA, 2017). Total organic carbon (TOC) determination was carried out by the wet acid oxidation method following standard methods (APHA, 2017). pH was determined in aqueous solution using a 1:2.5 sample/water ratio and a pH probe (US Department of Agriculture – US Composting Council, 2002). Total N and ammonium-N were determined according to the analytical method for wastewater sludges (APHA, 2017). Titrable acidity and total volatile fatty acids concentration (mg/l) to the alkalinity buffer capacity (eq. mg/l CaCO3) ratio (i.e. FOS/TAC) were determined following the method described by Di Maria et al. (2014).

Bioplastics were characterized by spectroscopic investigation, using the Fourier Transform InfraRed (FT-IR) spectra, which were collected in total reflectance mode (ATR) with a Shimadzu IRAffinity-1S equipped with a Miracle Pike ATR device (Shimadzu Italia srl, Milano, Italy). The investigated wavenumber range was of 4,000–500 cm^{-1} and the resolution was of 2 cm^{-1}. Bioplastic samples were dried, cleaned and gently brushed with a tooth-brush to remove all the deposits formed on their surfaces before spectroscopic analysis. Peak areas were determined using Shimadzu LabSolutions IR software (Shimadzu Italia srl, Milano, Italy).

2.4. 16S rRNA next generation sequencing

Samples for microbial analyses were collected before (Inoculum) and after (Control, Cellulose, PLA and SBS) each run. To recover the microbial communities, digestates were pelleted by centrifugation at 13,000 rpm for 20 min at 4 °C. All samples were stored at −80℃ prior to DNA extraction. Pellets (~0.060 g) were extracted using a DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to manufacturer's guidance. The yield and purity (A260/A280 and A260/A230) of the extracted DNA was quantified on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) while eventual fragmentation was determined through gel electrophoresis 1 % (w/v) 1 × TAE agarose gels. Replicates (n = 3) were pulled together to minimize extraction variability. DNA was stored at − 80 °C until analysis.

Illumina sequencing was performed on all samples for prokaryotic communities at Stab Vida Lda (Lisbon, Portugal). For bacteria, the 16S rRNA gene was selected and amplified using primers 341F (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAAT

CC). The generated DNA libraries were sequenced with MiSeq Reagent Kit Nano in the Illumina MiSeq platform, using 300 bp paired-end sequencing reads. The nucleotide sequences generated and analysed are available at the NCBI SRA repository (Accession number: PRJNA1010170). The sequences resulting from the NGS were quality checked through the FastQC software and analysed using DADA2 for R (Callahan et al., 2016). Reads were truncated at 280 (forward) and 220 (reverse) in order to remove the low-quality section of the reads. The adapter sequence was further removed with the trimLeft function set at the length of the primers for both forward and reverse reads. For taxonomic assignment, the SILVA database was used as reference.

2.5. Statistics and data analysis

All experiments and analyses were replicated three times. Mean and standard deviation (SD) values were calculated according to standard procedures (Microsoft Excel Software). Determination of significant differences among the parameters analysed at a level of significance of $P < 0.05$ was carried out by analysis of variance (ANOVA) and Tukey's test.

A first-order kinetic model was applied to evaluate the anaerobic digestion rates, as suggested by Fernández-Cegrí et al. (2012):

$$B = B_0 \times [1 - e^{(-k \times t)}]$$

where B is the cumulative biomethane yield at time t, B_0 is the ultimate biomethane yield of the substrate, k (d^{-1}) is the apparent kinetic constant and t (d) is the time. K was calculated by adjusting experimental data (B, t) and using non-linear regression (Microsoft Excel Software).

All statistical analyses on the microbiological data were performed on R studio (version 4.2.3) as by Clagnan et al. (2022). Linear Discriminant Analysis Effect Size (LEfSe) were executed as per https://huttenhower.sph.harvard.edu/galaxy/, enzymatic profiles were created as per Nagpal et al. (2019) while Venn diagrams were created at http://bioinformatics.psb.ugent.be/webtools/Venn/.

3. Results and discussion

3.1. Effects of microbial acclimatization on starch-based bioplastics degradation and biogas production

SBS digestion during Run 1 resulted in the production of 94 ± 8 NL $kgVS^{-1}$ of biogas (56 $NLCH_4$ $kgVS^{-1}$) in about 30 days, which was lower than data reported in literature (Table 2). Productions of 186 and 142 $NLCH_4$ $kgVS^{-1}$ in about 30 days of thermophilic digestion of starch-based shoppers were reported by Calabro' et al. (2020) and Cucina et al. (2022), respectively. This difference probably is the result of two main factors, (i) the different origin of the inoculum used and (ii) the different composition of the bioplastics used in previous tests. Indeed, in the present study a thermophilic inoculum made of OFMSW digestate was used, whereas other studies used manure and agricultural wastes digestate (Calabro' et al., 2020) and sewage sludge digestate (Cucina et al., 2022), that may be characterized by a higher efficiency for bioplastics anaerobic biodegradation. Furthermore, Calabro' et al. (2020) tested starch-based shoppers composed of at least 60 % starch, whereas in the present study the SBS contained about 20–40 % of starch (Cucina et al., 2022).

After Run 2, the biogas production from SBS increased significantly ($p < 0.05$) to 111 ± 5 NL $kgVS^{-1}$ (+18 %), and the increase was even more ($p < 0.05$) after Run 3, i.e. 143 ± 11 NL $kgVS^{-1}$ of biogas (+52 % with respect to Run 1). Considering the biogas production obtained after 30 days of AD of SBS in Run 3 (143 NL $kgVS^{-1}$), some considerations arise. First of all, it is relevant that the result obtained at the end of Run 3 was comparable to the results obtained after chemical and thermo-chemical pre-treatment of SBS (Cucina et al., 2023; Calabro' et al., 2020) or even higher than those obtained after mechanical pre-treatments of SBS (Calabro' et al., 2020). Microbial acclimatization to enhance SBS biogas production therefore represents an interesting and more sustainable strategy than bioplastic pre-treatments which require energy and chemicals to be carried out.

Table 2
Biogas production and composition during the three anaerobic digestion runs (Av. ± St. Dev., n = 3). Means followed by the same letters are not statistically different according to Tukey test ($P \leq 0.05$).

	Parameter	Run 1	Run 2	Run 3
Cellulose	Biogas (NL $kgVS^{-1}$)	633 ± 8	633 ± 46	642 ± 23
	Biomethane (% v/v)	66 ± 9	67 ± 9	63 ± 6
	Biomethane (NL $kgVS^{-1}$)	418	424	404
SBS	Biogas (NL $kgVS^{-1}$)	94 ± 8a	111 ± 5b	143 ± 11c
	Biomethane (% v/v)	60 ± 5	63 ± 5	64 ± 5
	Biomethane (NL $kgVS^{-1}$)	56	70	92
PLA	Biogas (NL $kgVS^{-1}$)	395 ± 3a	600 ± 25b	779 ± 13c
	Biomethane (% v/v)	59 ± 2a	68 ± 2b	63 ± 2a
	Biomethane (NL $kgVS^{-1}$)	233	408	491

In Run 2 and Run 3, biogas composition was not modified with respect to Run 1, ranging from 60 % CH_4 (v/v) to 64 % CH_4 (v/v) of biomethane in the three runs. Conversely, the AD kinetic of SBS was significantly affected by acclimatization of the inoculum carried out in this study (Table 3). Ultimate biogas yield calculated by adopting a kinetic constant obtained increases from 117 NL $kgVS^{-1}$ (Run 1) to 160 NL $kgVS^{-1}$ (Run 3), and a significant improvement of the apparent kinetic constant (k) was observed from Run 1 to Run 3 (+103 %). This caused a reduction of the time needed to obtain 80 % of the biogas yield of SBS (t_{80}) from 17 days (Run 1) to 11 days (Run 3).

Digestate characterization at the end of each AD run was carried out to understand the positive results obtained from the AD trials (Table 4). SBS digestates from the different runs did not differ significantly, and the SBS digestates composition did not differ significantly from the digestates from cellulose digestion. Consequently, the increased amount of biogas produced from SBS from Run 1 to Run 3 may not be attributed to a different chemical composition of the AD medium.Table 5..

Spectroscopic analysis of bioplastics residues (size > 2 mm) and the determination of mass loss after each AD run of SBS digestion were useful to highlight a modified degradation pattern during the three AD runs (see e-supplementary materials). Mass loss confirmed the results

Table 3
Kinetic analysis of biogas production during the three anaerobic digestion runs.

	Parameter	Run 1	Run 2	Run 3
Cellulose	B_0[c] (NL $kgVS^{-1}$)	733	691	717
	K[d] (d^{-1})	0.09	0.15	0.14
	R^2	0.9843	0.9943	0.9841
	t_{80}[e] (d)	15	12	13
SBS	B_0[c] (NL $kgVS^{-1}$)	117	140	160
	K[d] (d^{-1})	0.05	0.05	0.11
	R^2	0.9627	0.9746	0.9889
	t_{80}[e] (d)	17	18	11
PLA	B_0[c] (NL $kgVS^{-1}$)	n.a.[g]	n.a.	n.a.
	K[d,f] (d^{-1})	1.6	2.6	3.3
	R^2	0.9499	0.9746	0.9948
	t_{80}[e] (d)	21	20	20

[c] Ultimate biogas yield.
[d] Apparent kinetic constant.
[e] Time needed to obtain the 80% of the biogas yield of bioplastics.
[f] Kinetic constant calculated for the zero-order kinetic degradation (Cucina et al., 2021b).
[g] Not applicable.

Table 4
Digestate characterization (Av. ± St. Dev., n = 3). Means followed by the same letters are not statistically different according to Tukey test (P ≤ 0.05).

	Run 1			Run 2			Run 3		
Parameter	Cellulose	SBS[a]	PLA[b]	Cellulose	SBS	PLA	Cellulose	SBS	PLA
Total solids (%)	4.4[c] ± 0.2a	3.9 ± 0.1b	3.8 ± 0.2b	3.9 ± 0b	3.8 ± 0.1b	3.8 ± 0.3b	3.7 ± 0.2b	3.6 ± 0b	3.6 ± 0.1b
Volatile solids (% d.m.[d])	52 ± 1	54 ± 3	54 ± 2	56 ± 1	56 ± 1	53 ± 3	57 ± 2	55 ± 3	56 ± 0
Total organic C (% d.m.[d])	28 ± 1	26 ± 1	30 ± 1	31 ± 1	33 ± 1	33 ± 1	35 ± 0	37 ± 1	35 ± 1
pH (pH unit)	8.1 ± 0a	8.2 ± 0a	7.8 ± 0b	8.3 ± 0a	8.2 ± 0a	8.1 ± 0.1a	8.2 ± 0a	8.1 ± 0a	8.1 ± 0.1a
Titrable acidity ($gCH_3COOH\ L^{-1}$)	3.3 ± 0.3b	2.6 ± 0.2b	4.3 ± 0.3a	2.6 ± 0.4b	3.3 ± 0.4b	2.8 ± 0.2b	2.7 ± 0.7b	3.2 ± 0.2b	2.8 ± 0.4b
Total alkalinity ($gCaCO_3\ L^{-1}$)	32 ± 1	36 ± 2	36 ± 2	34 ± 3	37 ± 7	37 ± 3	32 ± 4	32 ± 2	38 ± 2
FOS/TAC	0.53 ± 0.06b	0.37 ± 0.05b	0.86 ± 0.03a	0.38 ± 0.05b	0.47 ± 0.06b	0.37 ± 0.04b	0.41 ± 0.08b	0.51 ± 0.07b	0.37 ± 0.04b
Ammonium-N ($gN\text{-}NH_3\ L^{-1}$)	1.6 ± 0.1b	1.5 ± 0.1b	2.6 ± 0.1a	2 ± 0.1b	1.9 ± 0b	1.8 ± 0.1b	1.9 ± 0.1b	2.1 ± 0.1b	2.2 ± 0.1b

from biogas production, as degradation increased from 22.9 ± 1.2 % w/w (Run 1) to 26.7 ± 0.4 % w/w (Run 2) and to 29.5 ± 0.2 % w/w (Run 3). About 30 % w/w degradation of SBS after 30 days of AD was expected since Cucina et al. (2022), obtained similar results when testing these materials under thermophilic AD using sewage sludge digestate as inoculum. This degradation performance was compatible with SBS composition, which is expected to contain about 20–40 % w/w of starch which is an easily degradable biopolymer. This was in accordance with the results obtained from spectroscopic analysis of SBS residues in this study (see e-supplementary materials). In fact, looking at the spectrum of SBS before AD, four diagnostic band/peaks were recognized: the bands at around 3,400 cm^{-1} and 1,018 cm^{-1} were attributed to O-H and C-O functional groups of starch, and the two bands at 1,710 cm^{-1} and 730 cm^{-1}, were assigned to the polyester component (Elfehri Borchani et al., 2015) (see e-supplementary materials). After Run 1, diagnostic peaks attributed to starch almost disappeared as starch had been preferentially metabolized by microorganisms with respect to the polyester component (the ratio between the areas of diagnostic peaks of starch and polyester decreased from 1.07 ± 0.09 to 0.58 ± 0.07). During Run 2, the same pathway of degradation was observed (the ratio between the areas of diagnostic peaks 1018 cm^{-1}/1710 cm^{-1} after Run 2 was 0.58 ± 0.01). These results were in accordance with Cucina et al. (2022; 2021b) who observed the same behaviour during mesophilic and thermophilic AD of SBS co-digested with sewage sludge and organic wastes, respectively. Interestingly, the ratio between the areas of diagnostic peaks at 1018 cm^{-1}/1710 cm^{-1} after Run 3 increased significantly to 0.65 ± 0.04, probably because of a possible increase of degradation of the polyester component. This hypothesis needs further confirmation, using appropriate tools that may be able to highlight the activation of metabolic pathways suitable for the degradation of recalcitrant biopolymers such as the polyester components of SBS.

3.2. *Effects of microbial acclimatization on PLA-based bioplastics degradation and biogas production*

PLA digestion during Run 1 resulted in the production of 395 ± 3 NL $kgVS^{-1}$ of biogas (233 $NLCH_4\ kgVS^{-1}$) in about 30 days, which was a result comparable to certain literature findings (which have sometimes resulted in conflicting results, above all for thermophilic AD) (Table 2)

Table 5
Starch-based shoppers mass balance and qualitative modifications after anaerobic digestion as estimated by the ratio between diagnostic Fourier-transform infrared spectroscopy peaks areas (Av. ± St. Dev., n = 3). Means followed by the same letters are not statistically different according to Tukey test (P ≤ 0.05).

Parameter	Unprocessed SBS	Run 1	Run 2	Run 3
Degradation (% w/w[b])	–	23[c] ± 1c	27 ± 0b	30 ± 0a
1018 cm^{-1}/1710 cm^{-1}	1.1 ± 0.1a	0.58 ± 0.07c	0.58 ± 0.01c	0.65 ± 0.04b

(Cazaudehore et al., 2022a). For instance, Hegde et al. (2018) and Bernat et al. (2021) reported a biodegradability and a biomethane production of 90 % and 400 $LCH_4\ kgVS^{-1}$, respectively, for PLA sheet and plastic cups digested by thermophilic AD, but Shrestha et al. (2020) reported null degradation and 20 L $kgVS^{-1}$ of biogas when degrading different sized PLA rigid pieces under similar AD-conditions. These contrasting results may be due, among other factors, to the different inocula used in the AD trials, the different composition of the bioplastics studied, and the different operative conditions used (i.e., OLR and HRT). Interestingly, biogas production from PLA significantly increased in this study at the end of Run 2 (600 ± 25 NL $kgVS^{-1}$, +52 % with respect to Run 1) and Run 3 (779 ± 13 NL $kgVS^{-1}$, +97 % with respect to Run 1). This means that after only three AD trials of acclimatization, the system was able to produce almost all the potential biomethane from PLA (Achinas and Euverink, 2016). These results, if further confirmed by testing other PLA-based materials, show the potential of the acclimatization of a microbial community to enhance anaerobic biodegradation of PLA and its conversion into biomethane. This is of the utmost importance if compared to the results of other strategies studied to enhance biogas production from PLA until now (i.e., pre-treatments, formulation of easy degradable composites). For instance, Vargas et al. (2009) obtained 225 $LCH_4\ kgVS^{-1}$ after pre-treating PLA-based commercial items through steam exposure (3 h, 120 °C) and Hegde et al. (2018) reported only 10–20 % of biogas production increase after including novel additives to PLA composites. Microbial acclimatization, being a no-cost strategy to increase biogas production from PLA-based bioplastics, thus appears to be a promising strategy to enhance circularity of bioplastics.

Differently from what was observed for SBS, during the three runs of PLA digestion an increase of biomethane concentration in biogas was observed in Run 2, but then in Run 3 the biomethane concentration was not significantly different from Run 1 (Table 3). To evaluate the kinetics of PLA digestion, a pseudo-zero order kinetic model was used as suggested in the literature (Cucina et al., 2021b). Although an enhancement of AD kinetic was evident from the kinetic constant that increased from 1.6 $mgC\ gC^{-1}\ d^{-1}$ (Run 1) to 3.3 $mgC\ gC^{-1}\ d^{-1}$ (Run 3), the time needed to obtain 80 % of the biogas production did not vary during the three runs due to the increased biogas production and ranged from 21 days (Run 1) to 20 days (Run 2 and Run 3).

Differently from what happened in SBS trials, in PLA-AD runs, a high solubilization of the materials tested (PLA-based glasses) occurred and thus, it was not possible to recover un-degraded PLA fragments to determine PLA degradation by mass loss calculation. Similarly, it was not possible to recover PLA fragments to be analysed by FT-IR spectroscopy (see e-supplementary materials), and only the PLA before AD was characterised. It was found that PLA-based glasses were mainly composed of polylactic acid since the spectrum was characterized by four diagnostic peaks that are commonly referred to polylactic acid (3,000, 1,750, 1,400 and 1,100 cm^{-1}) (see e-supplementary materials).

Nevertheless, some interesting results were obtained from digestate characterization (Table 4). Looking at the composition of PLA digestate

from Run 1, it was characterized by a significant reduction of the pH and a significant increase of titratable acidity and FOS/TAC ratio with respect to both cellulose and SBS digestates. This might be due to the solubilization of PLA in the medium and its hydrolyzation to lactic acid monomers, which because they were not readily degraded, accumulated in the media, leading to the slight acidification of the digestate. Nevertheless, due to the presence of alkaline buffers in the medium (i.e., bicarbonate and ammonia buffers), the pH was still in a sub-alkaline range (7.8). Contrarily, at the end of Run 2 and Run 3, the PLA digestates were not different from those coming from cellulose and SBS digestion, indicating that lactic acid monomers produced from PLA were efficiently and rapidly converted into biomethane, suggesting a successful acclimatization of the microbial population. This was consistent with the results of biogas production and digestion kinetic reported for PLA Run 2 and Run 3 (Table 2 and 3).

3.3. Prokaryotic communities' characterization

Composition and dynamics of the prokaryotic communities of all treatments (i.e. Inoculum, Control, Cellulose, SBS and PLA) were investigated for the three runs performed (i.e. Run 1, Run 2 and Run 3) through a 16 s rRNA sequencing analysis. Input reads across all samples ranged between 30,837 and 91,897 while between 21,006 and 64,190 after DADA2 assignment (see e-supplementary materials).

In general, all treatments seemed to show a trend of reduction in observed richness from Run 1 to Run 2 followed by an increase at Run 3. In term of diversity and evenness, treatments seemed to show the same trend, except for SBS and PLA where an overall decreasing trend was identified (see e-supplementary materials).

As expected, the bacterial community showed a higher number of enteric bacteria often associated with engineered (e.g. bioreactors) and wastewater systems. Firmicutes was the most abundant phylum across all samples (max. and min. values across all samples: 71–78 %) followed by Bacteroidota (9–17 %) and Cloacimonadota (2–6 %) (see e-supplementary materials).

Communities were generally similar in terms of most abundant genera. Venn diagrams highlight a wide set of common genera among treatments (between 165 and 191 across the three runs) with a smaller number of genera specific for each treatment (between 4 and 18) (Fig. 1). The main common genera across all samples were *Fastidiosipila* (15–27 %), a proteolytic bacterium previously identified as a major genus in mesophilic AD treating different types of waste and involved in the production of acetic and butyric acids (Rasi et al., 2022), *Lentimicrobium* (4–7 %), a strictly anaerobic and mesophilic slow-growing bacteria, *Acholeplasma* (3–8 %), commonly found in copiotrophic environments such as anaerobic digesters rapidly feeding on dead bacterial biomass (Hanajima et al., 2015), *Streptococcus* (3–14 %), an unknown DTU014 and an unknown MBA03 (Fig. 1).

Members of the of the phylum Actinobacteria, such as *Pseudonocardiaceae, Micromonosporaceae, Streptomycetaceae, Streptosporangiaceae,* and *Thermomonosporaceae*, have been found to play an important role in bioplastic (especially PLA) degradation (Butbunchu and Pathom-Aree, 2019) under a wide range of temperatures and oxygen conditions. Within this study, they were retrieved at very low abundances (<0.003 %) across all samples. Considering specifically anaerobic digestion under thermophilic conditions, *Clostridium* (*sensu stricto*), *Streptococcus*, *Caldicoprobacter* and *Tepidimicrobium* were found to be driving PLA degradation (Peng et al. 2022; Cazaudehore et al., 2023). Across all these genera, *Streptococcus* and *Caldicoprobacter* were present across most samples at high abundance (>2%) while *Tepidimicrobium* was retrieved at high abundance only within the PLA treatments in Run 2 and Run 3. Cellulose and SBS were both characterised by the presence of *Hydrogenispora* and by *Halocella* and *Haloplasma,* respectively. *Hydrogenispora* are anaerobic fermentative bacteria using carbohydrates as substrates (Jin et al., 2023) therefore, it is more likely that their growth was enhanced in the presence of cellulose and maize starch (SBS). Similarly, *Halocella* are hemicellulose and starch degraders often found in thermophilic anaerobic digestors (Wahid and Horn, 2021), in which growth seems to be impaired by the presence of PLA (Zheng et al., 2023). Likewise, *Haloplasma*, an as yet under-studied genus, has been linked to amylase production (Duan et al., 2022). On the other hand, the PLA treatment was characterised by *Syntrophaceticus* and *Tepidimicrobium* at high abundance. The genus *Syntrophaceticus* contains syntrophic acetate-oxidizers which have shown an association with microplastics surfaces (Porter et al., 2020) while *Tepidimicrobium* has been identified as a main driver of lactate-utilisation from PLA decomposition during thermophilic anaerobic digestion (Tseng et al., 2020).

A linear discriminant effect size (LEfSe) analysis was further carried out to determine the genera most likely to explain differences between treatments. Similarly to the most abundant genera, the LEfSe analysis highlighted an enrichment in an unknown *Hydrogenispora* and *Haloplasma* for the SBS treatment, while of *Halocella* and of an *D8A* for the cellulose treatment, i.e. genera connected to starch and cellulose degradation (Fig. 1). The PLA treatments again showed *Tepidimicrobium* as the main enriched genus with the addition of three other prokaryotes: an unknown *Peptococcaceae*, the methanogen archaea *Methanothermobacter* and *Tepidanaerobacter*, a thermophilic and anaerobic bacterium that has been correlated to lactate conversion and increasing methane production from PLA within thermophilic reactors (Cazaudehore et al., 2022b).

The selected primers (341F and 785R), have being designed to maximize bacterial environmental coverage however they have been found to further amplify the methanogenic archaeal community (Watanabe et al., 2022). Within this study, main archaeal genera retrieved were Methanothermobacter, Methanoculleus, Methanosphaera and a Candidatus Methanoplasma, all however at abundances below 2 %.

A suitable set of enzymes is required for microbial bioplastic biodegradation (García-Depraect et al., 2021). The identification of the enzymatic profile of prokaryotic organisms can improve the understanding of the metabolic capabilities and possible ecological roles of the whole community in terms of bioplastic degradation and efficiency since the functional potential of any community is a result of the sum of the total genetic pool of each single microorganism (Nagpal et al., 2019).

iVikodak was used to predict the prokaryotic enzymatic profile; however, in terms of core functions (i.e. carbohydrate metabolism, amino acid metabolism, replication and repair, nucleotide metabolism and energy metabolism) no particular differences were retrieved (see e-supplementary materials). Similarly, in the context of starch and cellulose degradation, no differences were retrieved within the enzymatic profile of the starch and sucrose metabolism (Kegg pathway: map00500) (see e-supplementary materials).

Knowledge on PLA-degrading microorganisms and their pathways is still limited. Most microorganisms involved in PLA degradation are Actinomycetes while a minority (28.5 %) are other types of bacteria. PLA biodegradation to carbon dioxide is mainly carried out through hydrolysis and then enters the pyruvate metabolism. Main enzymes that have been isolated from PLA-degrading microorganisms are proteases (such as carboxylesterase – e.g. mhqD gene, nap gene), lipases, deoxygenase (e.g. mhqP, mhqO, and mhqN), esterase (such as cutinase) and serine protease (e.g. besA gene) (Xu et al., 2022; Yu et al., 2023). In this study, PLA degradation (more specifically lactate) was investigated within the pyruvate metabolism, however again no significant differences were retrieved (Kegg pathway: map00620) (see e-supplementary materials).

In general, all samples showed a strong common set of prokaryotic genera. Starting from the same inoculum, each treatment developed and was characterized, by a small number of specific genera linked to the presence of the bioplastic substrate, with SBS moving towards starch degraders while PLA moved towards known PLA-degraders. In the context of improving the biodegradability of biodegradable plastics,

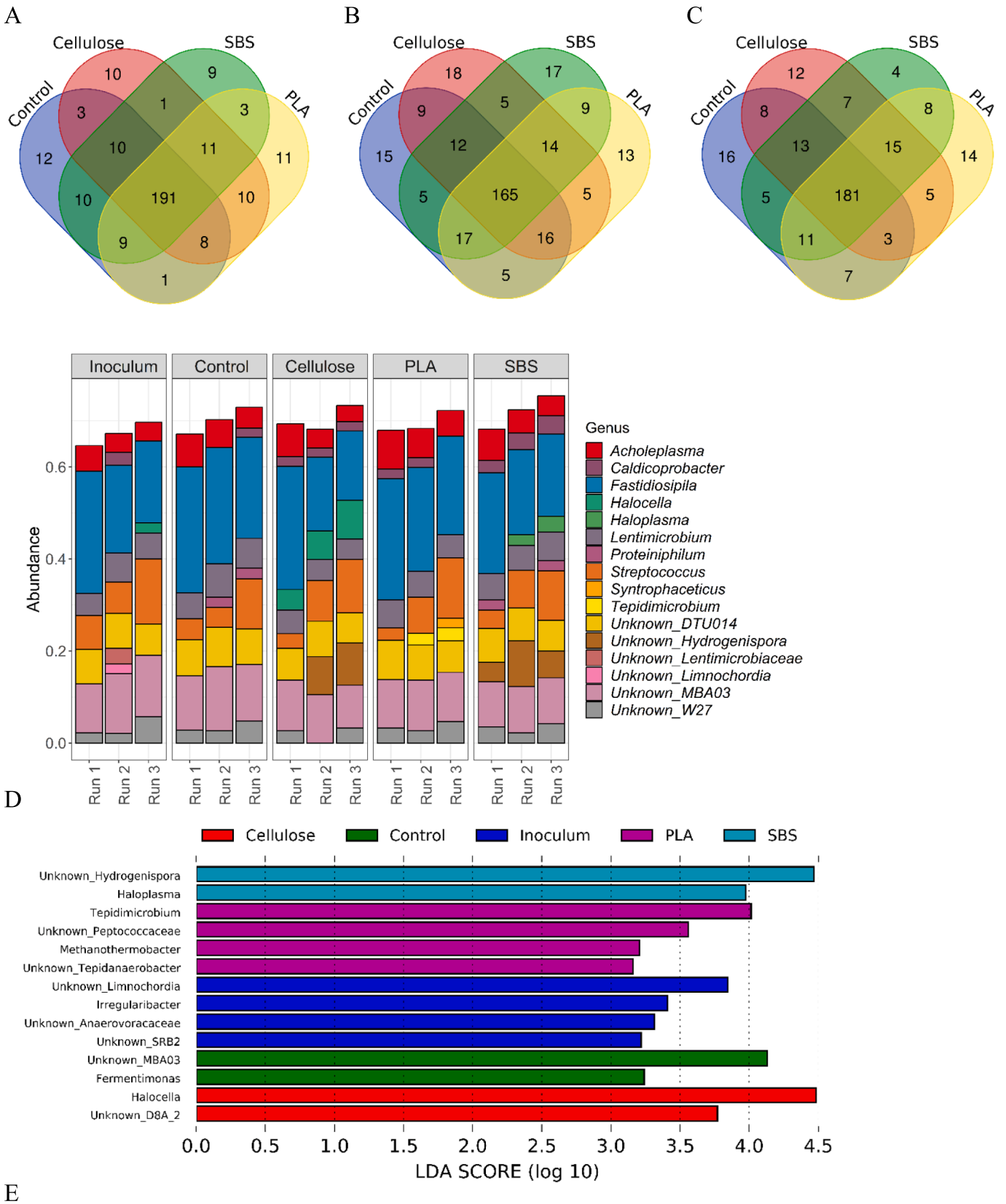

Fig. 1. Venn diagrams at genus level between treatments for run 1 (A), run 2 (B) and run 3 (C). Prokaryotic communities' composition at genus level with a > 2 % cut-off (D). Linear discriminant analysis (LDA) effect size analysis (LEfSe) at genus level for the prokaryotic communities (E).

there is still a lack of study considering inoculum origin and its acclimatization (Cazaudehore et al., 2022a) and this study is a first step in this direction. The growth of these specific genera probably contributed to the enhanced bioplastic degradation and biogas production through thermophilic anaerobic digestion that was registered at each cycle, highlighting the importance of inoculum acclimatization towards the development of a higher efficiency process.

Summarising, microbial acclimatization promotes anaerobic biodegradation and conversion into biogas of the two bioplastics studied. A more in-depth look at the Archaeal community needs to be carried out to confirm the findings of Venkiteshwaran et al. (2019), who reported that bacterial communities seem to be quicker in acclimatization in comparison to Archaea communities. Considering the results obtained from the SBS trials, it may be interesting to carry out more prolonged experiments to study the potential development of a microbial community specialized in polyester biodegradation.

Overall, a transcriptomic analysis might shed further light on the mechanisms leading the acclimatization achieved. Predicted function alone may not sufficient as it is based on known sequences and therefore on the presence/absence of specific genes withing an already sequenced bacterial species and therefore it does not consider the variables of gene expression, unknown species, and plasmid expression.

4. Conclusions

A first study of microbial acclimatization for bioplastics anaerobic degradation and conversion into biogas was carried out. The results showed that AD of starch-based and polylactic acid-based bioplastics was favoured by the acclimatization of the inoculum to the substrate. Starch-based bioplastic enhanced the growth of starch degrader bacteria, whereas polylactic acid-based bioplastic degradation seemed to be correlated to the growth of known PLA-degraders.

Considering the results obtained, microbial acclimatization appears a suitable and low-cost strategy to enhance bioplastics anaerobic biodegradability, and thus to promote bioplastics circularity. Therefore, this approach should be further investigated to be applied efficiently in practice.

Funding

This work was supported by Gruppo Ricicla – Università degli Studi di Milano - Grant number RV_RIMB16FADAN_M.

CRediT authorship contribution statement

Elisa Clagnan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mirko Cucina:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Raveena Vilas Sajgule:** Investigation. **Patrizia De Nisi:** Conceptualization, Investigation, Writing – review & editing. **Fabrizio Adani:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adani Fabrizio reports financial support was provided by University of Milan. Clagnan Elisa reports financial support was provided by University of Milan. Mirko Cucina reports financial support was provided by University of Milan. De Nisi Patrizia reports financial support was provided by University of Milan. Raveena Sajgule reports financial support was provided by University of Milan.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129889.

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