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Influence of feedstock source on the development of polyhydroxyalkanoates-producing mixed microbial cultures in continuously stirred tank reactors

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are the new frontier of bioplastic production; however, research is needed to develop and characterise efficient mixed microbial communities (MMCs) for their application with a multi-feedstock approach. Here, the performance and composition of six MMCs developed from the same inoculum on different feedstocks were investigated through Illumina sequencing to understand community development and identify possible redundancies in terms of genera and PHA metabolism. High PHA production efficiencies (>80% mg COD_{PHA} mg^{-1} $COD_{OA\text{-}consumed}$) were seen across all samples, but differences in the organic acids (OAs) composition led to different ratios of the monomers poly(3-hydroxybutyrate) (3HB) to poly(3-hydroxyvalerate) (3HV). Communities differed across all feedstocks, with enrichments in specific PHA-producing genera, but analysis of potential enzymatic activity identified a certain degree of functional redundancy, possibly leading to the general high efficiency seen in PHA production from all feedstocks. Leading PHAs producers across all feedstocks were identified in genera such as *Thauera*, *Leadbetterella*, *Neomegalonema* and *Amaricoccus*.

1. Introduction

Polyhydroxyalkanoates (PHAs) are renewable, biodegradable and bio-based linear polyesters produced, under limiting conditions, by a wide array of prokaryotes as energy and carbon (C) reserves and as stress (i.e. low temperature and freezing, heat shock, osmotic shock, oxidative pressure, UV protection and heavy metals toxicity) resistance mechanism [1]. PHAs are considered green polymers and are expected to replace conventional petrochemical plastics in the near future [2]. However, PHA production has not yet been sufficiently optimized to achieve production efficiencies and cost-effectiveness comparable to those reached by the production of synthetic polymers [3].

More than 300 species (75 genera) of bacteria and archaea are known to have the ability to store PHAs. Currently, PHAs are industrially produced by selected bacterial strains in pure cultures through fermentation of high purity substrates. Production under these conditions (i.e. sterility) comes however at high costs. By contrast, the selection, through an engineered and selective process environment, of an efficient mixed microbial community (MMC) enriched in PHA-producing bacteria can be an alternative to the use of selected and pure bacterial strains [4,5]. The use of MMCs further provides a higher level of adaptability, resilience and metabolic diversity, which is essential in the context of resource recovery and upcycling [6]. When alternating the presence and absence of C-sources, adapted PHA-producers store C when C-sources are available, for later use when C-sources might be unavailable. Prokaryotes able to store C as PHAs

Abbreviations: ADB, *Arundo donax* biomass; ANOVA, One-way analysis of variance; AVS, Amplicon sequence variant; CCWP, Concentrated cheese whey permeate; COD, Chemical oxygen demand; CSTR, Continuously stirred tank reactor; DO, Dissolved oxygen; HRT, Hydraulic retention time; MMCs, Mixed microbial communities; NMDS, Nonmetric multidimensional scaling; OA, Organic acid; OFMSWD, Organic fraction of municipal solid waste and digestate; OLR, Organic loading rate; PERMANOVA, Permutational analysis of variance; PHA, Polyhydroxyalkanoate; PhaA/PhbA, Acetyl-CoA C-acetyltransferase; PhaB/PhbB, Acetoacetyl-CoA reductase; PhaC/PhbC, Polyhydroxyalkanoates polymerase; PhaP, Polyhydroxyalkanoic acid inclusion protein; PhaZ, Polyhydroxyalkanoates depolymerase; POFMSW, Pulped organic fraction of municipal solid waste; SBR, Sequencing Batch Reactor; SCW, second cheese whey; TSS, Total suspended solid; VS, Volatile solid; WWTP, Wastewater treatment plant; 3HB, Poly(3-hydroxybutyrate); 3HV, Poly(3-hydroxyvalerate).

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have therefore a metabolic advantage on other prokaryotes and enhanced stress resistance and robustness [7]. These advantages enable the use of non-pure cultures and the reduction of sterilization costs as PHA-producers thrive, leading to their enrichment, while other prokaryotes are hindered. MMCs have a benefit in terms of plasticity and versatility, while the use of pure cultures and of efficient selected PHA producers may lead to better performances. Specific microorganisms will have different substrate preferences, while at the same time different MMCs may also have preferences [8]. In terms of PHA production, preferences might affect performance. Organic acid (OA) mixtures will affect the PHA monomer composition (i.e. OAs characterized by an even number of C atoms tend to produce polyhydroxybutyrate (PHB) while those with an odd number tend to produce PHB and polyhydroxyvalerate (PHV) copolymers) [5]. The use of non-sterile conditions, a possibly variable feedstock and an MMC might therefore lead to a variable PHA composition over time when compared to production under sterile conditions, a constant and pure substrate, and a pure culture.

In the context of the circular economy and waste valorisation, using MMCs enables the use of non-sterile wastes as C-sources, lowering costs by avoiding the use of raw materials and lowering the energy requirements for their preparation [9]. However, wastes need to be pre-treated to make them readily available to the MMCs [10]. MMCs have been reported to use available OAs to produce PHA [10–12] at high yield (i.e. 90% of the cell dry weight) [13]. Therefore, in most cases, the use of organic wastes starts with their fermentation, producing biodegradable and safer substrates, such as OAs, which are then used as feed for the enriched MMCs. Dark fermentation and anaerobic digestion are biological treatment processes used in biorefinery platforms, able to transform a variety of different substrate into OAs, thus representing a process able to “equalize” different substrates, making them all suitable for MMCs [6].

Research on multi-feedstock and multi-product biorefineries is of the utmost importance [6]. Within PHA production, it has been shown that not all OAs are consumed simultaneously, with a preference for butyrate and valerate over acetate and propionate [14]. Influence of substrate type on the selection and composition of MMCs, the interaction of multiple microorganisms and their resistance and resilience, has been little explored, and the effect of other operating parameters on the produced PHAs blends is still under-studied [15]. MMC compositions are generally affected by substrate alterations: gradual or pulsed feedstock shifts drive community changes, with MMC structure being affected while the PHA storing performance is maintained due to a reliable level of functional redundancy [16–19].

Even though MMC characterizations are commonly carried out, there is still a lack of understanding in terms of the impact of variations in MMC composition on the overall PHA storage capacity [20]. Further characterization is needed to consider the various steps of the PHA production processes (i.e. enrichment in PHA-producing species and PHA accumulation steps) [20]. Moreover, it is still unclear how different feedstock mixes affect different MMCs developed from the same inoculum, and vice versa, under the same environmental parameters.

This study aims at the characterization of different PHA-producing MMCs grown from the same inoculum on multiple OA feedstocks produced from different feeding mixtures, to understand whether communities, when grown on different substrates (different OAs), develop similarly once they go through the process of fermentation with the selection of specific microorganisms, or follow pathways independently from the initial substrate.

2. Materials and methods

2.1. Feeding mixtures and OAs production

Samples for this study were collected from a series of previous studies aimed at PHA production from: (i) second cheese whey (SCW, a liquid by-product of *Ricotta* production mainly composed of.

lactose and mineral salts) [12], (ii) concentrated cheese whey permeate (CCWP), (iii) *Arundo donax* biomass (ADB) [10], (iv) a mixture of organic fraction of municipal solid waste and digestate (10:1 w/w) (OFMSWD) [21] and (v) pulped organic fraction of municipal solid waste from two separate full-scale plants (POFMSW1 and POFMSW2) [22].

To convert the feeding mixtures derived from SCW, CCWP and ADB into a readily available sugars source suitable for the following fermentation step, samples were subjected to an enzymatic hydrolysis, preceded by an ionic liquid pre-treatment. The hydrolysis was then followed by filtration, to recover the liquid fraction for ADB [10] or by a centrifugation to remove suspended solids for SCW and CCWP [12]. POFMSWs, representing already fermented substrates, were only subjected to flocculation, by the addition of a cationic flocculant (powder by Kemira®, the Netherlands) dissolved in deionized water (1 g l^{-1}) to a final flocculant concentrations of 150 ml l^{-1} and solid to solvent ratio of 10% wt, followed by filtration (100 μm nylon mesh) to separate liquid and solid fractions [22].

For the production of OAs, dark fermentation processes were carried out for treated SCW, CCWP and ADB in continuously stirred tank reactors (CSTR (Tecnovetro, Monza, Italy), working volume: 1.5 L, agitation: 200 rpm) to reach a fast sugar fermentation under anaerobic and thermophilic conditions (55 °C) and at a pH of 5.5–5.8 (see the above mentioned references). An anaerobic digestate from a full-scale anaerobic digester fed with corn silage and operated under thermophilic conditions and with a hydraulic retention time (HRT) of 40 d [11], was used as the source of the inoculum after a thermic pre-treatment (1 h at 100 °C) to isolate the spore-forming bacteria and eliminate methane-producing archaea. Reactors were operated in batches under anaerobic conditions for 3 d, with glucose as carbon source, to acclimatize the hydrogen-producing bacteria, then the reactors were fed with pre-treated SCW, CCWP and ADB at a 2:1 inoculum/feed ratio. After this batch-mode period, the continuous feeding of the reactors started. In the continuous operation mode (HRT: 2 d), two reactors were fed with SCW, two with CCWP and two with ADB. Controls were further set up with only glucose as the C-source. Three organic loading rates (OLRs) were used for SCW and CCWP (8, 11 and 15.2 g sugars L^{-1} d^{-1}) while one was used for ADB (13 g sugars L^{-1} d^{-1}). Each load lasted 10 d until the achievement of process stability with constant H_2 production (8th day) and after each feeding interval a portion of the inoculum (50 ml d^{-1}) was added to maintain a stable microbial activity.

OAs from OFMSWD were produced by anaerobic digestion. Similarly to dark fermentation, anaerobic digestion was carried out in a CSTR (working volume: 1.5 L, agitation: 90 rpm) under anaerobic and thermophilic conditions (55 °C) and at a pH of 8.8. The same inoculum was used while HRT lasted 40 d, then 30 and then 20 with ORLs of 3, 4 and 6 kg VS m^{-3} reactor d^{-1}) respectively. The CSTR was fed daily with the OFMSWD-digestate mixture (3 kg VS m^{-3} reactor d^{-1}).

POFMSWs were not subjected to any additional OAs production steps as they represented naturally fermented substrates; they were however screened for the characterization of OAs through high-performance liquid chromatography (HPLC).

Effluents and extracts samples rich in OAs were retained and frozen at − 20 °C for subsequent use for the PHA production process. For more exhaustive information see the original publications [10,12,21,22].

2.2. Bacterial enrichment and PHA production

In order to produce PHA, the first step aimed at the selection of PHA-storing bacteria within an MMC, while the second step focused on PHA accumulation, using the selected community as inoculum.

For bacterial selection, dark fermentation and anaerobic digestions effluents and POFMSWs liquid fraction (feeding solutions) were diluted to a chemical oxygen demand (COD) concentration of 1500 mg COD L^{-1} (1900 mg COD L^{-1} for ADB) with deionized water and NH_4Cl, while for

PHA accumulation it was diluted to 7500 mg COD L^{-1} [10,22]. For SCW and CCWP, the OAs produced from OLR III was used, while for OFMSWD a mix of effluents from OLR I-II-III was used [12,21].

Briefly, the selection of PHA-storing bacteria was performed starting from an inoculum of activated sludge (8 g total suspended solids-TSS L^{-1}) from a wastewater treatment plant (WWTP). The enrichment in PHA producing bacteria was carried out in a Sequencing Batch Reactor (SBR) (working volume: 1 L (0.75 L for OFMSWD and 2 L for POFMSWs) with an aerobic dynamic feeding strategy. The community selection trend for PHA-producing bacteria was monitored by the determination of the feast phase duration by the dissolved oxygen (DO) levels in the media. During this phase, 500 ml of activated sludge were used as feed for each cycle with 500 ml of the effluents. The selection process lasted between 29 and 50 days depending on the media.

The second step of PHA accumulation was achieved via feed-batch assays in a glass reactor (working volume: 500 ml (200 ml for OFMSWD, 650 ml for POFMSWs) with continuous aeration (6 L min^{-1}) and stirring (110 rpm) at room temperature. The fermented streams were added to the enriched cultures with a pulse-wise feeding method when DO show a strong increase. As substrate, total C was calculated by C to microorganisms ratio that had to be identical to the selection phase. Accumulation cycles were considered completed when no DO variations were reached after substrate addition. For more exhaustive information see original publications [10,12,21,22].

2.3. Next generation sequencing (NGS)

Once the first step of selection of PHA-storing bacteria was completed and before the second step of PHA accumulation, samples for DNA extraction were collected directly from the CSTRs using sterile disposable pipettes. One biological replicate was collected from each one of the two CSTRs used for every treatment. Similarly, one biological replicate for the inoculum was collected before the selection step of each experiment. Over the years, inocula were taken from the same treatment plant [11] to ensure a certain level of similar characteristics, also in terms of microbial community, for the different trials. Each inoculum was however included in the NGS analyses to account for variability over time.

Samples were pelleted and stored at − 80 °C to allow for the possibility of a genomic study. Samples were then retrieved, and DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The yield and purity of the extracted DNA was quantified on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) while eventual fragmentation was determined through gel electrophoresis 1% (w/v) 1 ×TAE agarose gels. DNA was stored at − 80 °C until analyses.

Illumina sequencing was performed on all samples for prokaryotic communities at Stab Vida Lda (Lisbon, Portugal). For bacteria, the 16 S rRNA gene was selected and amplified using primers 341 F and 785 R [23]. The generated DNA libraries were sequenced with MiSeq Reagent Kit Nano in the lllumina MiSeq platform, using 300 bp paired-end sequencing reads. The nucleotide sequences generated and analysed are available at the NCBI SRA repository (Accession number: PRJNA932826). The sequences resulting from the NGS were quality checked through the FastQC software and analysed using DADA2 for R [24]. Reads were truncated at 280 (forward) and 245 (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 [25].

2.4. Statistical analyses

All statistical analyses were performed on R studio (version 4.1.2) with packages vegan [26] and FactoMineR [27]. Taxonomic summaries were performed using the phyloseq package [28]. Observed richness, Chao1 and Shannon and Simpson diversity indexes were calculated. Shapiro-Wilk test was used to test normality, then differences among samples of normally distributed data were tested by one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test ($P < 0.05$) while not normal data were analysed through a non-parametric Kruskal-Wallis test, followed by Dunn's Test for multiple comparisons. For pairwise comparison, T-test and Wilcoxon signed rank test were used for normal and not normal data respectively.

Multivariate analyses were performed on amplicon sequence variants' (AVSs) relative abundances. To test the effect of inocula on beta diversity, first, a nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances was applied and then results were confirmed through a permutational analysis of variance (PERMANOVA). Furthermore, pairwise comparisons were carried out with the package 'pairwiseAdonis' [29]. Co-occurrences were constructed through the package cooccur [30] to reveal intra-kingdom interaction. The prokaryotic pathway of the enzyme profile was investigated through iVikodak [31].

3. Results and discussion

3.1. Feeding solutions and OAs characteristics

In terms of OAs profile, ADB showed the highest production of acetate and butyrate followed by lactate and formate in a 1:1:0.4:0.003 proportion, respectively (Table 1). ADB was the only sample to contain citrate (in a proportion acetate:citrate of 1:0.2) due to the buffer used during biomass pre-treatment [10]. SCW showed a similar composition to ADB (acetate:butyrate:lactate:formate, 1:0.7:0.1:0.05 proportion) while it differed from CCWP for the absence of lactate (acetate:butyrate:formate, 1:1:0.05). OFMSWD was characterized only by a lower concentration of acetate and the presence of propionate in a 0.5:1 ratio, respectively. The main OA in POFMSWs sample was lactate, while succinate was also produced (lactate:acetate:succinate:propionate:butyrate, 1:0.2:0.02:0.01:0.001 and 1:0.2:0.02:0.04:0.02 for POFMSW1 and POFMSW2, respectively).

SCW, CCWP and POFMSW1 produced and accumulated only 3HB while ADB and POFMSW2 contained also small amounts of 3HV (96:4 and 95:5 of 3HB:3HV, respectively). In contrast, OFMSWD showed an equal content of 3HB and 3HV, 47:53 (Table 1). Different PHA content depended on the OAs profile as the presence of propionic and valerate acids led to the presence of PHA containing 3HV as the "building block", as well described in the literature [32].

All cultures showed high levels of conversion of OAs to PHA (above 80%) indicating the development of well adapted and efficient communities (Table 1).

3.2. Dominant prokaryotic genera

An NGS analysis was carried out to evaluate the development of the prokaryotic community starting from the inocula and the differences among feedstocks. The sequencing of the 16 S rRNA gene produced between 78,590 and 139,591 prokaryotic reads, which accounted for a number of reads between 34,011 and 70,397 after DADA2 assignment (Suppl. Table S1). In terms of phyla, the inoculum showed a high abundance of Bacteroidota, Patescibacteria and Proteobacteria which were also maintained at high abundances in the PHA-production phase of almost all feedstocks (Suppl. Fig. S1). Up to now, most of the identified PHA-storing bacteria belong to the Proteobacteria (with classes such as Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria) together with Actinobacteria [20]. At genus level, inocula showed a high amount of low abundance genera (>2%), while main genera remained unclassified at genus level with the only presence of *Terrimonas* at 2.2% (Fig. 1).

When looking at ADB, the main genus was *Neomegalonema*, a genus that currently contains only one species, *N. perideroedes,* known from

Table 1
Organic acids profile of the feedstock used for both selection and accumulation steps, produced PHA profile and storage yield during the accumulation steps (average (± standard deviation), n = 2).

	ADB	SCW	CCWP	OFMSWD	POFMSW1	POFMSW2
OAs (% (mmol basis))						
Acetate	38.50 (± 2.37)	54.70 (± 8.33)	48.4 (± 0.57)	34.88 (± 0.68)	14.25 (± 0.93)	14.37 (± 5.59)
Citrate	6.68 (± 0.90)	–	–	–	–	–
Formate	0.11 (± 0.05)	2.50 (± 0.50)	2.40 (± 0.47)	–	–	–
Butyrate	37.68 (± 3.20)	36.30 (± 5.35)	49.15 (± 1.33)	–	0.06 (± 0.00)	1.90 (± 0.03)
Lactate	17.03 (± 1.03)	6.50 (± 0.60)	–	–	83.68 (± 5.64)	78.98 (± 0.25)
Proprionate	–	–	–	65.12 (± 1.94)	0.67 (± 0.00)	3.31 (± 2.46)
Succinate	–	–	–	–	1.35 (± 0.00)	1.44 (± 0.15)
PHA (%)						
3HB	96	100	100	47	100	95
3HV	4	0	0	53	0	5
PHA storage yield ($mg\ COD_{PHA}\ mg^{-1}\ COD_{OA\text{-}consumed}$)	1.15 (± 0.21)	0.84 (± 0.28)	0.82 (± 0.13)	1.29 (± 0.42)	Data not reported	Data not reported
References	[10]	[12]	[12]	[21]	[22]	[22]

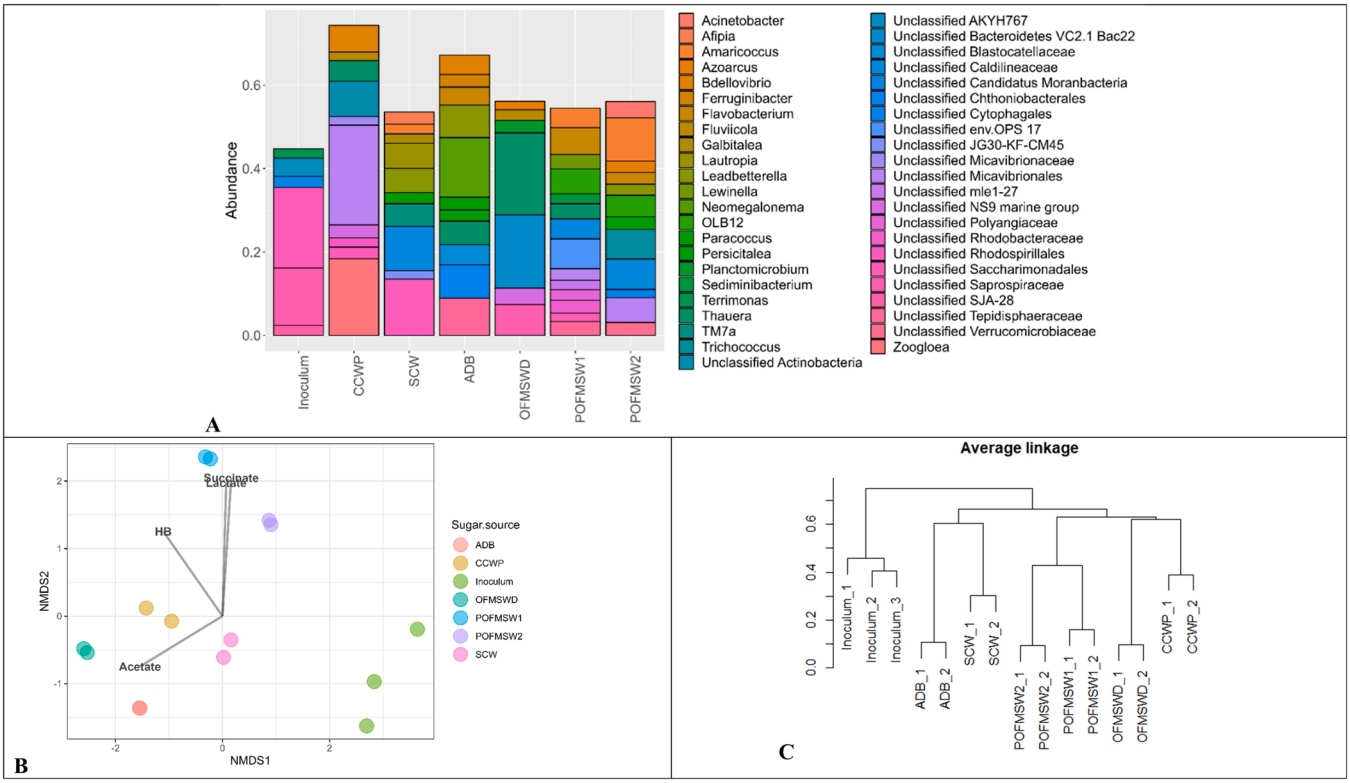

Fig. 1. A. Bacterial community composition at genus-level for the accumulations step. Relative abundance of the average values of two replicates are shown for each bar with a cut-off > 2%. B. Non-metric multidimensional scaling (NMDS) ordination plots of the sites with significant OAs and PHAs driving the distribution ($p < 0.05$). C. Bray-Curtis dissimilarity dendrogram.

other studies to accumulate high amounts of PHA and also to cause bulking problems in WWTP due to its filamentous morphology [33,34]. Other abundant genera were *Leadbetterella* (7.8%), *Thauera* (5.7%), *Bdellovibrio* (4.7%), *Flavobacterium* (4.3%), *Ferruginibacter* (3.0%), *Paracoccus* (3.0%) and *Persicitalea* (2.7%). *Leadbetterella* is again another genus with only one known species (*L. byssophila*) that has been linked to poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate production [35]. *Thauera* and *Paracoccus*, known bacteria involved in the N-cycle, have shown abilities to transform organic matter into PHAs and have therefore been found enriched in mixed cultures for PHA production [36]. *Paracoccus, Thauera* and *Azoarcus* showed a preference of butyrate, valerate and propionate over acetate, favouring the storage of OAs as PHAs with higher 3HB content. However, a higher abundance of *Thauera* has been linked to a higher 3HV content (with a possible similarity to OFMSWD) [37]. Additionally, although the other genera of *Ferruginibacter* and *Flavobacterium* have not been identified as PHA-producers, they have previously been described as "enriched" in PHA-accumulating reactors [38]. On the other hand, the genus *Bdellovibrio* is known to contain predators of PHA producers, which may lead to suboptimal PHA yields and to the collapse of the systems [39].

When analysing the communities of SCW, it could be seen that *Leadbetterella* and *Paracoccus* were again present as the most abundant genera (5.8% and 2.7%, respectively) accompanied by *Lautropia* (6.0%), *TM7a* (5.4%), *Afipia* (2.9%), *Amaricoccus* (2.3%) and *Galbitalea* (2.3%). *Lautropia* was previously found in mixed microbial culture for PHA production; however its role, as for *Persicitalea* and *Galbitalea*, remains obscure [40]. *Afipia* is a genus of mainly human pathogenic species [41] while *Amaricoccus* is often found in WWTPS and has shown PHA storing capacity [42].

Communities grown on CCWP were characterized by *Zoogloea*

(18.4%) as the dominant genus followed by *Bdellovibrio* (6.4%) and *Thauera* (5.0%) as in ADB, and by *Fluviicola* (2.0%). Both *Fluviicola* and *Zooglea* genus are often observed in PHA producing environments and are thought to possess the ability to degrade and produce PHA, respectively [15,43].

The community grown on OFMSWD was again characterized by *Thauera* (19.7%) followed by *Planctomicrobium* (3.0%), *Fluviicola* (2.5%) and *Azoarcus* (2.0%). *Planctomicrobium* is a relatively unknown genus that is possibly associated with PLA degradation [44]. while *Azoarcus* is a genus of well-known and important PHA accumulating microorganisms [45].

The POFMSW1 media was characterized by *Flavobacterium* (6.5%), *OLB12* (6.0%), *Amaricoccus* (4.7%), *Thauera* (3.7%), *Lewinella* (3.4%) and *Sediminibacterium* (2.4%). *OLB12* is a genus often found in anammox granules [46] while *Amaricoccus* has been shown to produce PHA [47]. On the other hand, *Lewinella* has often been found in WWTP connected to denitrification and recently was linked to possible PLA degradation [44]. *Sediminibacterium* presence was also detected in other PHA production studies, but its role is still unknown [48]. Similarly, the POFMSW2 media showed as the main genera *Amaricoccus* (10.3%), *Trichococcus* (7.1%), *OLB12* (5.1%), *Acinetobacter* (3.9%), *Paracoccus* (3.0%), *Flavobacterium* (2.8%), *Azoarcus* (2.7%) and *Leadbetterella* (2.7%). *Trichococcus* was found in PHA accumulating communities and in municipal sludge subjected to fermentative processes [49], while *Acinetobacter* has been proposed as a PHA accumulator [38].

In general, inocula showed high observed richness (species number in a community) ($p < 0.02$) while similar to POFMSW2 (Suppl. Table S2). Shannon (species diversity in a community) and Simpson diversity (accounting also for species relative abundance) further indicated a trend of highest diversity for the inocula and POFMSW2 followed by POFMSW1 and SCW. Pielou's evenness (closeness in numbers of each species in a community showed again the same trend. These trends are possibly an indication of selection and specialization of the communities.

3.3. Structure and beta-diversity

Permanova analyses indicated an influence of the feeding mixtures in shaping the microbial communities ($p < 0.001$). NMDS and dendrogram analysis showed the development of different communities starting from the inoculum with a higher difference between POFMSWs and all other samples (Fig. 1). The Bray-Curtis dissimilarity, calculated on genera and not on single ASVs, (0: the two sites have the same composition, 1: the two sites do not share any species) showed diversity above 60% among different feedstocks (Fig. 1).

An LEfSe analysis (all against all) was used to reveal statistical significance and biological relevance highlighting differences in (over-represented) genera between the communities developed from the different feeding solutions (biomarker discovery) (Suppl. Fig. S2). The LEfSe analysis highlighted multiple genera specific to each feedstock, with all samples characterized by an enrichment in a specific set of PHA-producers or bacteria linked to PHA production. ADB showed highest number of enrichments in multiple genera of PHA-producers or linked to PHA production (i.e. *Brevundimonas, Leadbetterella*, *Leucobacter, Luteimonas, Ferruginibacter*, *Persicitalea, Pseudoxanthomonas*). On the other hand, SCW was characterized by enrichments in the PHA producers *Hyphomicrobium* and *Bosea* and by *Lautropia* and *Devosia* (two genera found in MMC but not directly connected to PHA production [40]) while CCWP, by *Bdellovibrio* and *Edaphobaculum*. The presence of *Bdellovibrio* as a biomarker might point to possible problems within this system. OFMSWD was characterized by the PHA producer *Thauera*, together with *Luteolibacter*, *Sumerlaea* (possibly involved in degradation of organic substrates linked to dissimilatory nitrate reduction [46]) and *Bryobacter*. POFMSW1 was characterized by *Flavobacterium*, *OLB12* and *Terrimicrobium,* while POFMSW2 by *Amaricoccus*, *Niabella* (found abundant in PHA producing MMCs [15]) and *Arenimonas*, a denitrifier dominating environments characterized by high PHA utilization [50].

OAs and relative production of PHA driving the differentiation were analysed through an Envfit analysis, significant parameters ($p < 0.05$) were acetate, lactate, succinate and 3HB (Fig. 1). As expected, lactate and succinate characterized specifically the two POFMSWs samples. The other samples showed a higher concentration of acetate when compared to the two POFMSWs. Unsurprisingly, the production of 3HB was the driving factor in the diversity from the inoculum.

3.4. Contribution to PHA accumulation

Of the retrieved genera, 250 contributed to the butanoate metabolisms (Kegg reference pathway: map00650). Commonly known PHA accumulators were found at both high and low (<2%) abundances, they accounted for 37–14% of the total communities across all samples (2% in the inoculum) (Fig. 2). Most abundant genera were *Thauera*, *Leadbetterella*, *Neomegalonema* and *Amaricoccus*.

Essential enzymes for the production of PHAs are (i) acetyl-CoA C-acetyltransferase (PhaA/PhbA – Kegg entry: E.C. 2.3.1.9), (ii) acetoacetyl-CoA reductase (PhaB/PhbB – E.C. 1.1.1.36) and (iii) PHA polymerase (PhaC/PhbC – E.C. 2.3.1.304/2.3.1.-) while the polyhydroxyalkanoic acid inclusion protein "phasins" (PhaP – E.C. not assigned) is responsible for PHA accumulation [51]. POFMSW2 and SCW showed a higher abundance of potential enzymes for butanoate metabolisms when compared to CCWP, OFMSWD and POFMSW1 ($p < 0.05$), while POFMSW1 showed a lower potential than all other samples, except for CCWP ($P < 0.05$) (Fig. 2). In regard to the three main enzymes, PhaA and PhaC were present at high abundances in all samples, while PhaB was relatively lower. PhaB showed highest potential in OFMSWD ($p < 0.05$) while the lowest potential was seen for POFMSWs which were similar to the inoculum. PhaA again showed the lowest potential in POFMSW1, while the highest in ADB, SCW and OFMSWD ($p < 0.05$). PhaC showed highest potential in ADB, SCW and POFMSW2 while lowest in OFMSWD ($p < 0.05$). When looking at the main depolymerase (PhaZ) potential, highest values were retrieved in CCWP ($p < 0.05$) possibly linked to *Bdellovibrio* ability to degrade the prey's PHA, which in CCWP was found more abundantly than in the other samples.

Interactions between PHA accumulating genera and the prokaryotic communities were investigated in terms of co-occurrence (Fig. 3). Across all PHA accumulating genera communities, the highest number of positive interactions were detected for *Xanthobacter* (16) and *Hyphomicrobium* (13) while of negatives for *Leucobacter* (5). For the most abundant genera, the highest number of positive interactions were retrieved for *Devosia* (8), *Nakamurella* (7), *SWB02* (7) and *Thrichococcus* (7) while highest number of negative interaction were found for *Neochlamydia* (5) and *Arenimonas* (4). Although there is currently no scientific evidence of PHA accumulation by *Devosia* and *Nakamurella*, these genera have been found dominant in multiple PHA-accumulating systems, in which they may possibly enhance PHA production [38,52].

4. Conclusions

MMCs grown from the same inoculum on multiple OA feedstocks produced from different feeding mixtures developed different communities on different substrates. The main differences between the POFMSWs inocula and the other samples were linked to a high lactate content with the presence of succinate from one side and of a high content of acetate from the other. A specific set of different PHA-producers or bacteria linked to PHA production characterized each MMC. The presence of *Bdellovibrio* at high concentrations might indicate an instability of some MMCs. However, analysis of potential enzymatic activity identified a certain degree of functional redundancy, possibly leading to the general high efficiency in PHA production.

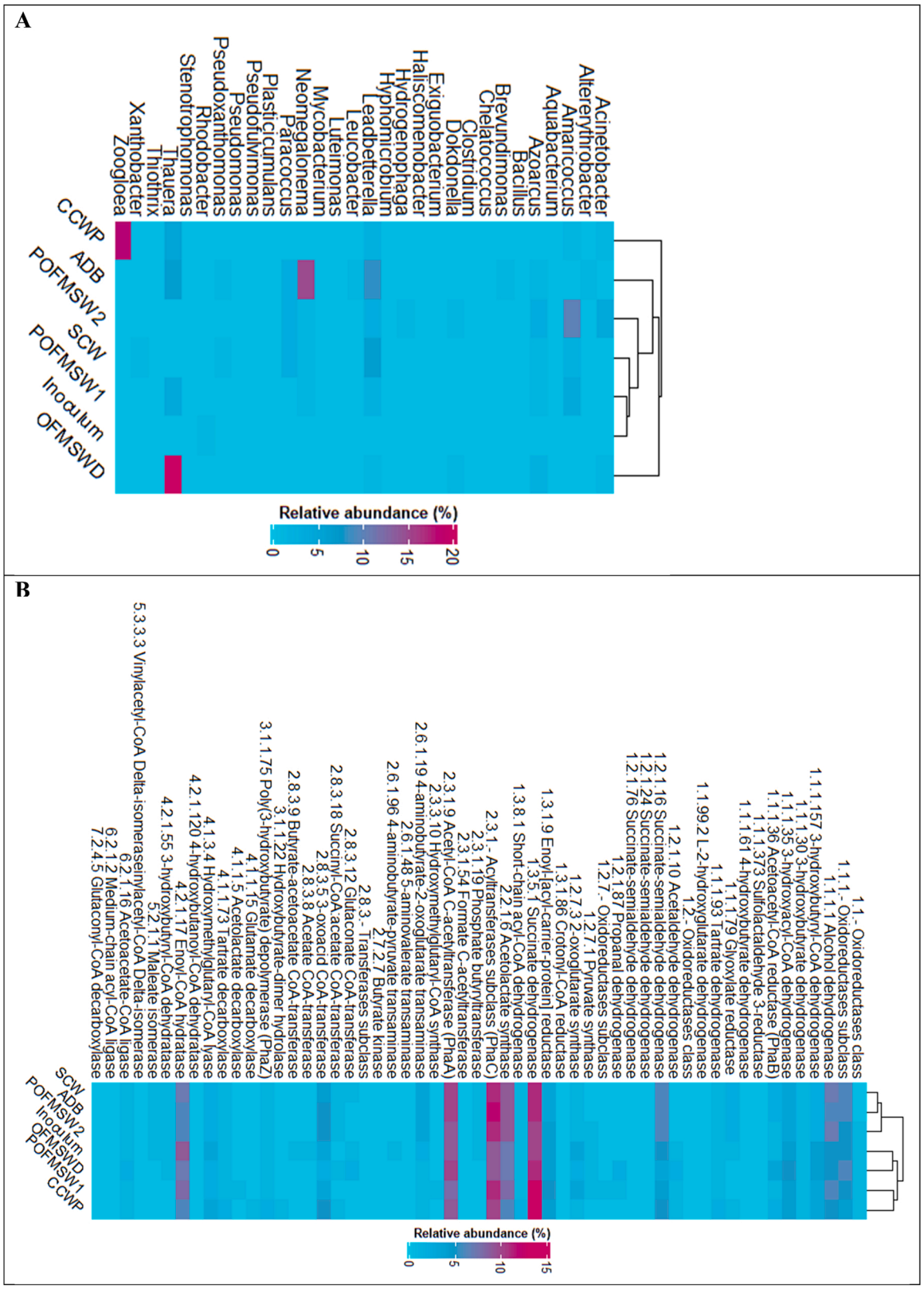

Fig. 2. A. Heatmap for the most widely known PHA accumulating genera present at the accumulations step. B. Enzyme abundance profile inferred for the butanoate metabolism.

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Declaration of Competing Interest

The authors declare that there are no competing interests associated with the contents of this article.

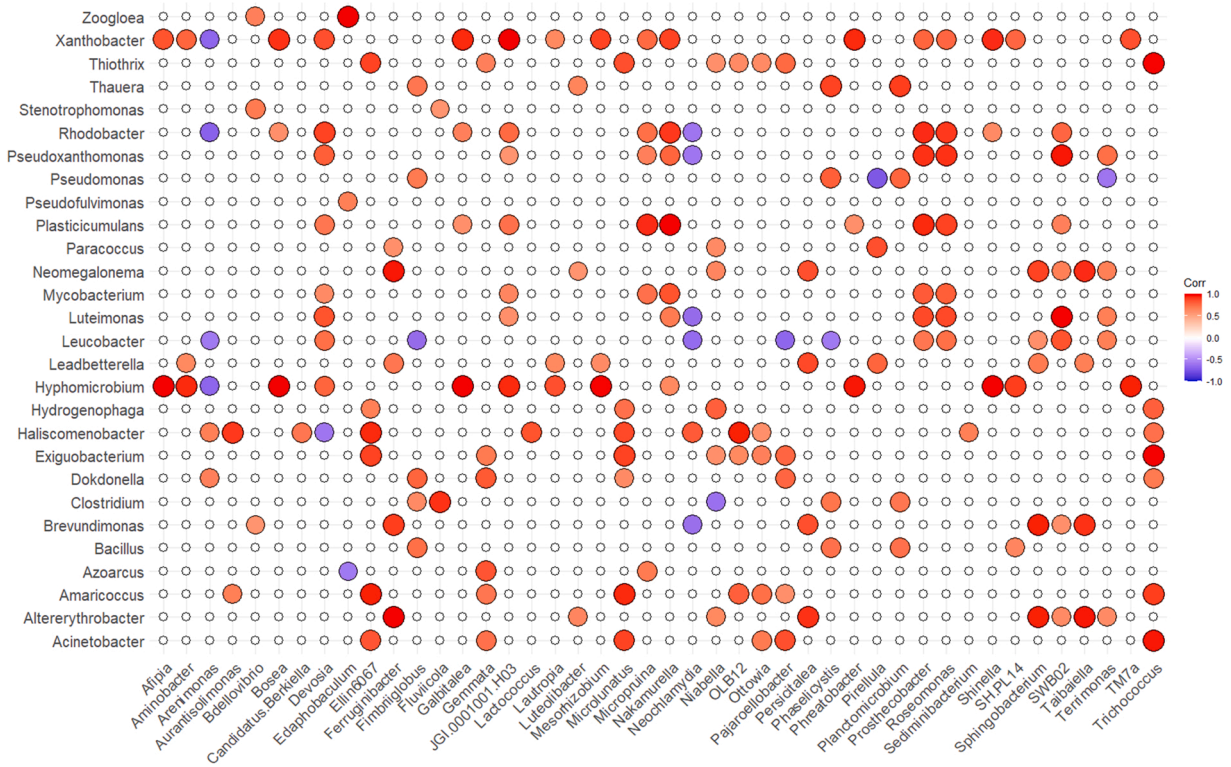

Fig. 3. Co-occurrence based on Spearman rank correlation index of PHA accumulating genera against the most abundant (>2% in at least one sample) prokaryotic genera for the statistically significant interactions (p value< 0.05).

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.05.005.

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