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More than meets the eye: microalgal-bacterial association in polyhydroxybutyrate (PHB) accumulating cultures of *Desmodesmus communis*

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ABSTRACT

The focus on microalgae within a circular economy framework has been growing due to their ability to utilize inexpensive substrates and produce high-value biomolecules. Among these, the biopolymer polyhydroxybutyrate (PHB) can be accumulated as a storage compound in several microalgal biomasses under nutrient- and light-limiting conditions. Herein, the Chlorophyta *Desmodesmus communis* was grown under mixotrophic conditions (low light, phosphorus-free medium, 1 g L^{-1} sodium acetate) in a 1 L semicontinuous system over 25 days. PHB accumulation progressively increased, reaching 57 % w/w on day 25. Since mixotrophic conditions also favor bacterial growth, the associated bacterial community was analyzed through 16S rRNA gene metabarcoding at different time points (day 0, 12, 21, 25) to investigate its potential contribution to PHB accumulation. A selection in bacterial genera was observed after prolonged cultivation. Moreover, antibiotic treatments markedly reduced bacterial diversity and PHB content, indicating a central bacterial role in PHB production and highlighting key genera likely involved. Environmental Scanning Electron Microscopy (ESEM) and Transmission Electron Microscopy (TEM) revealed close interactions between algal and bacterial cells, suggesting a potential endophytic presence of PHB-accumulating bacteria within *D. communis* cells. These findings propose a new perspective on microalgae-associated bacterial communities, viewing them not as contaminants but as essential contributors to microalgal growth and valuable metabolites production. The results emphasize the importance of considering the bacteria-microalgae consortium in PHB production research, rather than focusing solely on photoautotrophic organisms.

1. Introduction

Microalgae present a complex community of bacteria that live in association and symbiotic relationship with their host [1]. Although the importance of the symbiosis between algae and bacteria is well known, and its exploitation is appealing for biotechnological applications [2,3], the specific composition, role and function of the microbiome associated with microalgal cells are still underinvestigated. This interaction influences both physiology and metabolism of the involved organisms, since microalgae can produce organic compounds that bacteria assimilate, like dissolved organic carbon (DOC) [2,3], sugars including mono and disaccharides [4,5], and extracellular polymeric substances (EPS) [3]. On the other hand, bacteria can synthesize important compounds for algal growth stimulation, like cobalamin, thiamine, and biotin (vitamin B12, B1, and B7, respectively), spore germination, morphogenesis, and pathogen resistance [6], with a net positive effect that

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symbionts can provide to the algal growth and biomass productivity [7–9]. Bacterial organic carbon respiration (converted into CO_2), and phosphorous and nitrogen remineralisation encompass a major source of nutrients for algal cells and play a key role in biogeochemical nutrient cycling in aquatic environments [10,11].

Given this close interchange of nutrients and molecular signals, the phycosphere of microalgae can be considered a specialized habitat for some bacteria. Different microalgae might actively shape the phycosphere characteristics to create a more favourable environment for specific and /or beneficial bacteria [12]. In support of this, closely interacting bacteria strongly differ from loosely attached ones [13]. Moreover, attempts to completely remove bacteria or fungi from microalgae have frequently been inconclusive and, when achieved, resulted in impaired algal growth [14]. On the contrary, when artificially assembled consortia of growth-promoting bacteria are added to algal cultures, a significant increase in biomass yield can usually be observed [3,9,15], suggesting a strong and mutual co-dependency between the two.

In this context, advancing the knowledge on algae-bacteria associations, including the role of the alga and the microbiome, both individually and as a community, could be crucial to improving algal cultivation towards higher production of compounds of biotechnological interest.

Polyhydroxyalkanoates (PHAs) are biodegradable and bio-based polyesters produced as carbon and energy storage within cells of various microorganisms. Due to their biodegradability, biocompatibility, and thermoplasticity, PHAs could be potentially employed in numerous industrial applications [16]. The structure and composition of PHAs are affected by the carbon source used to feed bacteria, as well as by the microbial strain, medium composition, and fermentation process [16]. Among PHAs, polyhydroxybutyrate (PHB) is the first short-chain homopolymer isolated and characterized [17] and can be intracellularly accumulated by different species of bacteria, both heterotrophic and autotrophic [18]. Among the heterotrophic ones, *Pseudomonas*, *Bacillus*, *Cupriavidus*, and recombinant *Escherichia coli* are the most employed [4,19–23] for which high PHAs production yields are often observed under nutrient-limiting conditions (e.g., nitrogen and phosphorus depletion) [24], although species-specific even within the same genus [25].

Cyanobacteria are the most studied autotrophic species for PHB accumulation [26–28]. As an example, *Anabaena* sp. grown in mixotrophic conditions and phosphorous deficiency can accumulate PHB up to 40 % by weight [28]. Some microalgal biomasses, especially those belonging to the Chlorophyta phylum, like *Chlorella sorokiniana* SVMIICT8 [29], *Botryococcus braunii* [30], *Scenedesmus* sp. [31] and *D. communis* [32], have been reported for the accumulation of PHB under stress conditions. The growth conditions employed for PHB accumulation in microalgal biomasses are analogous to those that result in PHB enrichment in bacteria (i.e., nutrient limitation and organic carbon supply). Nonetheless, research conducted on the production of PHB from cyanobacteria or microalgae frequently focuses exclusively on the photoautotrophic component, while the heterotrophic bacterial role in this context is largely overlooked (Table S1), raising questions about the actual PHB producer under these conditions.

The present study investigates the bacterial community associated with the green microalga *Desmodesmus communis* during the accumulation of PHB and explores the hypothesis that the microalgal-bacterial association might create a favourable biomass for a self-sustaining PHB-producing consortium, redefining the microbial role in biopolymer production. A “two-module system”, combining microalgae/cyanobacteria and autotrophic and heterotrophic bacteria, has already been hypothesised as promising for PHB yield improvement [19]. To this end, a 16S rRNA gene metabarcoding analysis was performed at different time points of the semicontinuous mixotrophic growth, namely day 0, day 12, day 21 and day 25, to investigate the most abundant bacterial taxa selected in *D. communis* cultures under conditions favouring PHB accumulation. Antibiotic treatment was also applied to understand the role of the bacterial component in the production of PHB. Furthermore, electron microscopy analysis was performed on samples to gain deeper insight on the interactions between the two microorganisms.

2. Materials and methods

2.1. D. communis growth conditions

A strain of the Chlorophyta *D. communis*, previously described as a PHB producer [32], was selected for the semi-continuous cultivation under mixotrophic conditions. The *Desmodesmus communis* strain used in this study was isolated from a freshwater pond in the province of Forlì-Cesena (Emilia Romagna, Italy) in February 2009 and identified at the species level as previously described [33]. Two sets of experiments were conducted using a long-established culture and a culture treated with antibiotics.

2.1.1. Semi-continuous mixotrophic cultivation

After a phototrophic growth phase (PGP), cultures were prepared in triplicate with an initial biomass concentration of about 0.2 g L^{-1}. The growth medium employed was modified CHU13 [34] without the addition of phosphates and supplemented with 1 g L^{-1} sodium acetate (NaOAc) [32]. Cultures were kept at 20 ± 1 °C with a 16:8 h light:dark photoperiod, low light at 20–30 μmol m^{-2} s^{-1} and continuous aeration with filtered (0.22 μm) air at a flow rate of about 1 L min^{-1}. Culture dry weight (DW, g L^{-1}) was measured every 4–5 days. The semi-continuous mixotrophic cultivation was performed as in the previous study [32], harvesting aliquots of the culture (a third of the total volume) every 3–4 days (except for day 18, after 6 days) and replacing with fresh medium without phosphate, supplemented with NaOAc (1 g L^{-1}).

Aliquots of the culture were centrifuged (7500 RPM/10395 RCF, J5 rotor, Beckman Coulter Avanti J-26S XP centrifuge, 15 min), and the pellet was stored at −20 °C for determining the PHB content.

2.1.2. Antibiotic treatment

For the antibiotic treatment, cultures (100 mL) were treated for 48 h with penicillin 30 μg mL^{-1}, streptomycin 7.5 μg mL^{-1}, gentamycin 7.5 μg mL^{-1}. The starting culture was then kept in sterile conditions, diluted 1:20 and let recover for 10 days in a complete CHU13 medium. At day 10, such culture was inoculated to a concentration of 0.2 g L^{-1} in PHB accumulation-inducing medium (CHU13 without phosphates and with 1 g L^{-1} NaOAc) in mixotrophic, semicontinuous growth. Two batches (2 biological replicates) of 1 L cultures were inoculated. At day 18, 300 mL aliquots were harvested by centrifugation (7500 RPM/10395 RCF, J5 rotor, Beckman Coulter Avanti J-26S XP centrifuge, 15 mins) and stored at −20 °C until processing for PHB content evaluation.

2.2. PHB content quantification

The PHB content in the algal biomass was determined as reported in the literature [35]. Briefly, freeze-dried algal samples (10 mg) or standard PHB (1–2 mg; Biomer, Germany) were charged in screw-cap vials (4 mL volume, 50 mm high) and then placed on a heating plate at 350 °C. At this temperature, PHB depolymerized into (*E*)-2- butenoic acid (i.e., crotonic acid) that was used as the molecular fingerprint of PHB for the quantitative analysis. After 20 min, the vials were removed from the heating plate and cooled down to RT before adding the internal standard (2-ethylbutanoic acid, 0.1 mL of a solution 5000 ppm in acetonitrile). The sample was then diluted with acetonitrile (4 mL) and analyzed by GC–MS to quantify the amount of crotonic acid in each sample, and then the amount of PHB. GC–MS analysis was performed using an Agilent 7820 A gas chromatograph connected to an Agilent 5977E quadrupole mass spectrometer. The injection port temperature was 280 °C. Analytes

were separated on a DBFFAP polar column (30 m length, 0.25 mm i.d., 0.25 μm film thickness), with helium flow of 1 mL min^{-1}. Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan/s within the 29–450 $m\,z^{-1}$ range. The temperature of the column was set to 50 °C (5 min) and increased to 250 °C (10 °C min^{-1}).

PHB content was expressed as a percentage of dry biomass weight (gPHB per gbiomass %). Specific PHB productivity (gPHB $gbiomass^{-1}$ day^{-1}) was calculated based on the cultivation period (days), following the equation [28]:

$$PHB\ \ productivity\left(\frac{g\ \ PHB}{g\ \ biomass\ \ \ day}\right)=\frac{PHB\ \ content\left(\frac{g\ \ PHB}{g\ \ biomass}\%\right)}{cultivation\ \ \ period\ \ (days)}$$

2.3. Metabarcoding samples collection

At days 0, 12, 21, and 25, 20 mL of culture were filtered using 0.22 μm sterile cellulose-nitrate filters (Ahlstrom, Helsinki, Finland) (3 biological replicas per each time) and stored at - 20 °C until DNA extraction. Filtration apparatuses were previously autoclave-sterilized, and the procedures were conducted in a biological safety cabinet.

2.4. DNA extraction from filtered samples

DNA from material collected on sterile 0.22 μm filters was extracted using DNeasy PowerWater Kit (QIAGEN, Hilden, Germany) following the manufacturer's extraction protocol. DNA quantification was performed with a Qubit (Invitrogen, Carlsbad, CA, USA) fluorometer using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Nanopore library preparation, sequencing and data analysis

Sequencing library preparation was carried out starting from 10 ng of purified DNA from each sample using the 16S Barcoding Kit (SQK-16S024) from Oxford Nanopore Technologies (ONT, Oxford, UK), following the manufacturer's instruction. The protocol leads to the generation of 16S rRNA gene amplicons using primers 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′). The amplification was conducted using the following cycling conditions: initial denaturation 1 min at 95°C (1 cycle); denaturation 20 s at 95°C (25 cycles); annealing 30 s at 55°C (25 cycles); extension 2 min at 65°C (25 cycles); final extension 5 min at 65°C (1 cycle). The samples were processed following the manufacturer's instructions with no modifications. Pooled libraries were then sequenced on a MinION platform (ONT, Oxford, UK) using the Nanopore MinION Spot-on flow cell (FLO-MIN106D, version R9) and sequenced until reaching 5.68 Gb (3.03 M reads). Base-calling was automatically performed by MinKNOW 4.3.12 (ONT, Oxford, UK) software. Raw reads were obtained in fast5 and fastq formats from which "pass" quality reads were subjected to further analysis. The base-called data (fastq) were further processed using the 16S workflow available in the cloud-based data analysis platform EPI2ME (ONT, Oxford, UK) with 'Fastq 16S Analysis' (wf-metagenomics workflow). Data obtained were rarefied based on the least abundant sample (70819 reads for d0, d12, d21 and d25; 181437 reads for antibiotic-treated cultures) using Rstudio suite [36] through 'phyloseq' package. The reads were clustered at genus level. The relative abundance of each genus within each sample was calculated, and the genera were sorted in descending order by relative abundance, retaining only the taxa with a relative abundance higher than 0.5 % [37].

2.6. Statistical analysis

The 16S rRNA gene metabarcoding rarefied relative abundance data were transformed by square root.

Permutational multivariate analysis of variance, PERMANOVA, [38, 39] was performed to test for differences in microbial community structure at different time points of semicontinuous mixotrophic growth. PERMANOVA tests were based on Bray–Curtis similarity. All PERMANOVA analyses were performed using unrestricted permutation of the raw data and 999 permutations.

The community structure was analyzed by non-metric multidimensional scaling (nMDS).

Genera that mostly contributed to the dissimilarity/similarity of bacterial communities at different time points were identified using the SIMPER analysis (70 % cut-off) [39].

The significance level was set at 0.05 (5 %) for all tests. All analyses were conducted with PRIMER v7, provided with the PERMANOVA+ add-on [38].

2.7. Electron microscopy investigation

2.7.1. Environmental scanning electron microscopy (ESEM)

Liquid algal cultures were collected in 1.5 mL tubes through centrifugation at 4000 rcf for 10 mins until a pellet of appropriate dimension was visible. The supernatant was discarded. The algal samples were washed with 1 mL of PBS (0.01 M, pH 7.4) and re-pellet at 4000 rcf for 10 mins. Samples were then re-suspended in 1 mL Glutaraldehyde 2.5 % in PBS (0.01 M, pH 7.4) and incubated at 4°C for 24–48 h. After centrifugation (4000 rcf for 10 min), the algal samples were washed four times with 1 mL PBS (0.01 M, pH 7.4) and three times with distilled water. The samples were then treated with increasing concentrations of EtOH (50, 70, 80, 90, 95, and 100 % for three times). Centrifugations between each EtOH concentration increase were carried out at 4000 rcf for 20 min. Samples were then dried under a laminar flow hood and mounted on ESEM stubs covered in carbon foil tape and coated with gold using a BIO-RAD Sem Coating System SC502 working at 12 mA for 1 min. Samples were then imaged through ESEM (FEG FOUR S, Thermo Fisher Scientific).

2.7.2. Transmission electron microscopy (ESEM)

Appropriate volumes of the samples were centrifuged at 3500 RPM/3142 RCF (ALC 4235 A centrifuge) for 20 mins and processed for ultrastructural study using the following protocol: the pellets were fixed by immersion in 2.5 % glutaraldehyde (DDK, ItaliaJT) in cacodylate buffer 0.1 M and post-fixed in 1 % OsO4 (EMS, Hatfiel, PA) in 0.1 M cacodylate buffer. Specimens were dehydrated in ascending ethanol and embedded in Araldite (Serva, Heidelberg, Germany). Thin sections, stained with uranyl acetate and lead citrate, were studied using a Philips CM100 Transmission Electron Microscope (Philips/FEI Corporation, Eindhoven, Holland).

3. Results and discussion

Results from this study highlight how the initially diverse bacterial community associated with the microalga *D. communis* undergoes significant changes over time under PHB accumulation-inducing growth conditions (low light, ØP, 1 g L^{-1} NaOAc). Isolation and maintenance of microalgal cultures under controlled laboratory conditions have been shown to select specific bacterial taxa, influenced by both growth conditions and algal exudates [12]. Thus, the bacteria identified in this work are likely to represent a subset of a wider range of bacteria capable of associating with this chlorophyte.

3.1. Bacterial community dynamics in semicontinuous mixotrophic growth

D. communis was cultivated in a semicontinuous setup under nutritional conditions suitable for inducing PHB accumulation (i.e., mixotrophy), following the growth for a total 25 days. Aliquots of total biomass were harvested every 4–5 days, and dry weight was also

measured at the same time points (Fig. 1). Progressively higher PHB accumulation was observed during growth (Fig. 2), reaching a maximum PHB content of 57 % w/w at day 25. The maximum productivity was achieved between day 18 and day 21, reaching values of 0.086 g PHB g biomass^{-1} day^{-1} (Table 1). These data are in line with the results previously observed by Pezzolesi et al., 2023 [32].

A 16S rRNA gene metabarcoding analysis was conducted on the bacterial communities present in the biomasses harvested at each time point and rarefied relative abundances (%) of the identified genera are reported in Fig. 3 (corresponding values in Table S2).

The nMDS analysis showed a clear separation of the communities at day 0 from the ones selected at later time points that, on the contrary, grouped from day 12 onwards (Fig. S1). PERMANOVA analysis supported this pattern (Table S3).

SIMPER analysis (Table S4) revealed that average dissimilarities between bacterial communities at each time point ranged from 19 % to 22 % (day 12 vs day 21–25 and day 21 vs day 25) and 52–54 % (day 0 vs day 12–21–25), confirming that the major differences are observed between samples collected on day 0 and those at subsequent time points.

The average dissimilarities between day 0 and PHB accumulating stages (day 12, day 21, day 25) were largely due to variations in the abundances of ten genera (1 % average dissimilarity contribution cut off): *Limnobacter, Limnospira, Nostoc, Gloeocapsopsis, Thermanaerothrix, Streptococcus, Hydrogenophaga, Annamia, Potamolinea,* and *Anaerolinea.*

The bacterial genera that underwent higher enrichment from day 12 onwards were *Hydrogenophaga* and *Limnobacter,* which include species known for PHB accumulation, like *Hydrogenophaga* sp. [40], *Hydrogenophaga palleronii* [41], *H. pseudoflava* [42] and *Limnobacter humi* [43].

Other bacterial genera that showed an increase in abundance, even if to a lower extent (but above 0.5 %, average dissimilarity contribution from SIMPER analysis) from day 0 to days 12, 21, and 25, include *Comamonas*, *Paraburkholderia*, *Achromobacter*, *Cupriavidus,* and *Pandorea*. Notably, most of these genera include species reported as PHB producers, such as *Comamonas acidovorans* [44] *C. testosteroni* [45], *Paraburkholderia sacchari* [46], *P. xenovorans* [47], *Achromobacter xylosoxidans* [48], and *Cupriavidus necator* [49].

Interestingly, the *Achromobacter* sp. CBA4603 has also been found to enhance the growth of the chlorophyte *Haematococcus pluvialis* due to its high auxin production [8], suggesting that this taxon may play an indirect role in promoting microalgal growth.

Between day 12 (15 % w/w PHB) and days 21–25 (54–57 % w/w PHB), dissimilarity between communities was mainly attributed to the following taxa *Limnobacter*, *Hydrogenophaga*, *Agrobacterium*, *Rhizobium*, *Sediminibacterium*, *Bosea Brevundimonas*, *Erythrobacter*, *Rhodobacter, Phenylobacterium, Pseudorhizobium,* and *Streptococcus.* Even though most of these genera had low relative abundances (around 1 % for

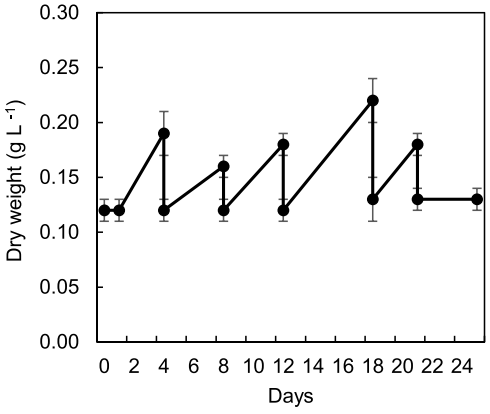

Fig. 1. *D. communis* mixotrophic semicontinuous growth (expressed as dry weight, g L^{-1}). Three biological replicates (n = 3) were evaluated for each time point.

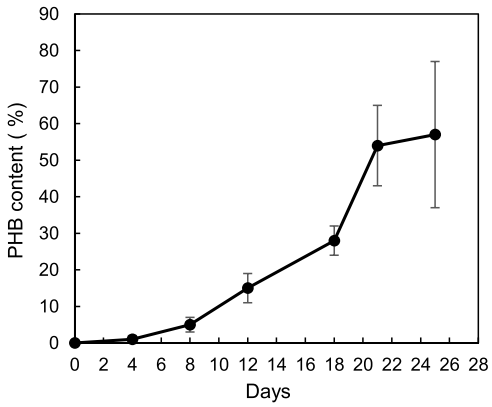

Fig. 2. PHB content (% w/w) measured in the biomass obtained in *D. communis* mixotrophic semicontinuous cultures. Three biological replicates (n = 3) were evaluated for each time point.

Table 1
PHB productivity achieved in *D. communis* semicontinuous cultivation.

Δ Time (day)	Productivity (g PHB g biomass^{-1} day^{-1})
0–4	0.002
4–8	0.011
8–12	0.025
12–18	0.022
18–21	0.086
21–25	0.008

Sediminibacterium, *Bosea*, *Brevundimonas,* and *Erythrobacter*, and between 0.2 % and 0.5 % for *Phenylobacterium, Pseudorhizobium,* and *Streptococcus*), some of them increased their abundance over time (i.e., *Hydrogenophaga*, *Sediminibacterium*, *Bosea*, *Brevundimonas, Erythrobacter, Phenylobacterium, Pseudorhizobium,* and *Streptococcus*).

Although its overall relative abundance was quite low (< 3 %), SIMPER analysis underlined the highest contribution of the genus *Sediminibacterium* in the dissimilarity between communities at day 12 (0.24 %) and day 25 (2.75 %), when a shift in PHB concentration from 15 % w/w to 57 % w/w was observed. This genus has not been reported as a PHB producer, but its presence has been observed in other studies on PHA production by bacteria; although its specific role remains unclear [50], it has been hypothesised as an important player in the balance of nutrient metabolism, environmental information exchange, as well as the resistance to contaminants in microalgal cultures, along with the abovementioned *Limnobacter* [51]. As for other taxa, studies have indicated *Bosea* spp. as a possible member of PHA-producing bacteria [52], and as a bacterial taxon often associated with cultured green microalgae like *Chlorella* [53].

Brevundimonas sp. isolated from *Chlorella ellipsoidea* UTEX 247 and re-inoculated in cultures of the same algal strain has had positive effects on microalgal growth [54], similar to what is observed for species of *Erythrobacter* [55] proven to be PHB-producers (e.g., *E. aquimaris* [56]), as well as *Streptococcus* spp. [57]. The *Phenylobacterium* genus has been previously found to be part of the microbiome associated with green microalgae (i.e., *Micrasterias crux-melitensis* [58]), while the role of *Pseudorhizobium* remains more elusive since no extensive literature is available on its possible interaction with microalgae, even though its close taxonomic relationship with *Rhizobium* and *Agrobacterium* (*Rhizobiaceae* family) might imply similar characteristics and activity.

Interestingly, the *Limnobacter* genus, even though its relative abundance switched from 0.36 % at day 0 to an average of 30 % at later time points, was observed to decrease in abundance between day 12 (35 %) and days 21 (32 %) and 25 (25 %), corroborating the idea that a complex trophic network is established among all bacterial genera present, and the selection would logically not exclusively be based on their PHB

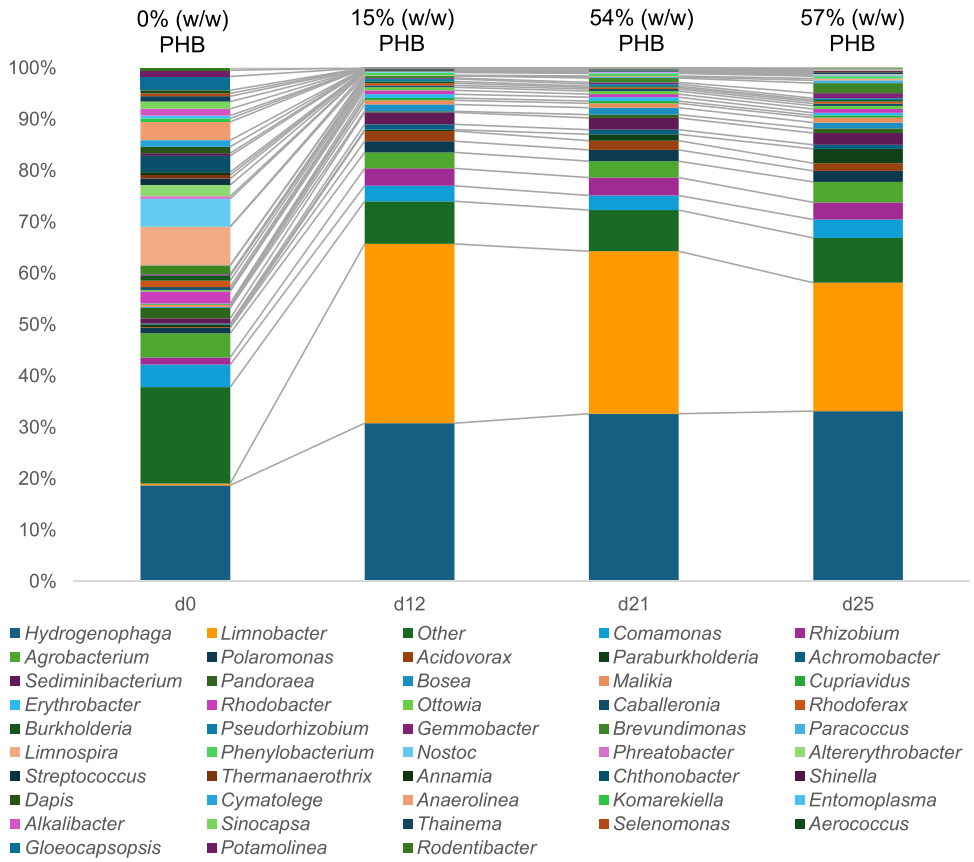

Fig. 3. Rarefied relative abundances of bacterial genera present in *D. communis* non-axenic, mixotrophic, semicontinuous culture at day 0 (d0), day 12 (d12), day 21 (d21) and day 25 (d25) of growth in PHB accumulation-inducing medium. Means from 3 biological reps are reported and a 0.5 % cut off was applied. The PHB content (% w/w) of the harvested biomass is reported at each time point.

accumulation capacity.

The *Rhizobium* and *Agrobacterium* genera, relevant in relative abundance, remained almost stable during bacterial growth. This might indicate that these genera may represent a stable, resident portion of the bacterial community associated with *D. communis*. Notably, it has been reported that *A. tumefaciens* transformation is effective in microalgae in an analogous way as in plants [59,60] and the *Rhizobium* genus includes known colonizers and growth promoters in both plants and microalgae [61]. Moreover, *Rhizobium* has previously been identified in association with *Scenedesmus* sp. [62] and cyanobacterial PHB-producing [63] cultures. This latest study advances the concept that a balanced coexistence between photoautotrophic and non-photoautotrophic bacteria is critical for achieving optimal PHB production in cyanobacteria-enriched microbiomes, consistent with the observations reported in the present work using a microalgal-bacterial system.

The main bacterial genera that exhibit an increase during the most productive growth phase (day 18 to day 21) might be considered the key players in PHB accumulation: *Hydrogenophaga, Sediminibacterium, Bosea* and *Erythrobacter*. It can be hypothesised that a well-established balance between PHB producers (possibly *Hydrogenophaga* and *Erythrobacter* as the most relevant) and other bacteria presenting growth-stimulation properties (e.g., *Achromobacter* and *Agrobacterium*) and nutrient balance-enhancement capacities (e.g., *Sediminibacterium*) is the key to the efficient production of carbon storage compounds.

3.2. Antibiotic treatment

After the antibiotic treatment, which did not generate completely axenic cultures, the most abundant genera identified through the 16S rRNA gene metabarcoding sequencing were (Fig. 4): *Bosea, Sphingomonas, Methylorubrum, Sphingopyxis, Erythrobacter, Novosphingobium,* and *Limnospira.*

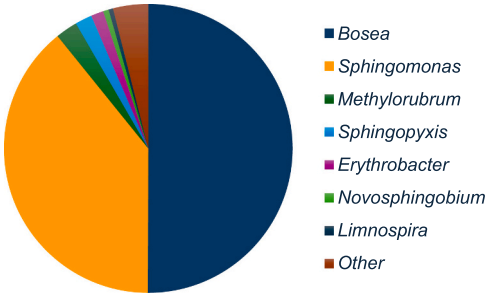

Fig. 4. Rarefied relative abundances (%) of bacterial genera present in a *D. communis* culture pretreated with antibiotics mix and grown in mixotrophic, semicontinuous conditions. Analysis was performed at day 18 after PHB accumulation induction. Means from 2 biological reps are reported and a 0.5 % cut off was applied.

Conversely, PHB content decreased in the antibiotic-treated cultures, reaching values of 10 % w/w after 18 days.

Among the observed bacterial genera, *Methylorubrum* spp. has been previously isolated from *Chlorella* cultures and has also shown a positive effect when co-cultured with the same microalgae [64], as well as PHB accumulation capacity [65]. *Sphingopyxis terrae* has been noted as a PHB producer too [66] and, when co-cultured with the Xantophyceae *Tribonema* sp., it effectively enhanced the chlorophyll *b*, carotenoid, and lipid contents of the microalgae [67]. In a study from Teeka et al., 2012 [68], *Novosphingobium* sp. was identified as a PHA producer and was able to enhance cyanobacterial growth [69]. Comparative genomics

analyses have revealed the presence of PHB-accumulating genes in *Limnospira* sp., to be used for carbon storage [70].

Overall, it appears that the more diverse the initial bacterial community, the stronger the selection of a more resilient bacterial-microalgal community capable of accumulating high PHB content. A marked decrease in bacterial abundance and diversity, including the nearly complete removal of *Hydrogenophaga*, a potential major PHB producer, may account for the lower PHB production observed after treatment. In untreated cultures, *Hydrogenophaga* made up 33 % of the bacterial population when PHB levels peaked at 54 %-57 % w/w (days 21 and 25). After antibiotic treatment, *Hydrogenophaga* was reduced to 0.06 % of the community, and PHB accumulation dropped to 10 % w/w (day 18), remarking its potential crucial role in achieving high PHB yields. This hypothesis is enforced by literature studies where *Hydrogenophaga* species have been reported for their ability to accumulate PHB if grown in chemotrophic conditions on different substrates [41, 42].

The common presence of *Bosea* and *Erythrobacter* in both antibiotic-treated and untreated cultures might suggest that these genera also play a significant role in PHB production.

3.3. Electron microscopy analyses

Environmental Scanning Electron Microscopy (ESEM) analysis of *D. communis* grown under either standard conditions or in semi-continuous mixotrophic growth confirmed the presence of bacterial cells strictly adhering to the surface of *D. communis* cells (Fig. S2).

A variety of morphologically distinct bacteria were found with Transmission Electron Microscopy (TEM) observations, differentiated by the abundance, morphology, and distribution of PHB intracellular granules (Fig. 5).

The ultrastructure of microalgal cells grown under standard autotrophic conditions without nutrient starvation is reported in Fig. 6A, B, and C, where vacuole, pyrenoid, and starch granules are visible inside the cytoplasm. *D. communis* cells from semicontinuous mixotrophic growth in PHB accumulation-inducing medium are reported in Fig. 6D-I. Indications of bacterial cells (bc) containing PHB granules seem to be visible inside the microalgal cell boundaries, and a double-layered membrane was visible around the hypothetical bacterial cell inside *D. communis* (Fig. 6I), supporting the hypothesis of endophytic colonisation. Possible endophytic bacterial cells showed a diameter ranging between 400 and 750 nm, while single intracellular granules had a diameter of 100–180 nm. Although bacterial cells observed outside microalgal cells presented both smaller clustered PHB granules (Fig. 5A, B, and D) and larger PHB inclusions, often occupying the whole bacterial cell (Fig. 5A, C, and D), hypothetical endophytic bacteria appeared to contain the first type of smaller, clustered granules.

Notably, *Methylorubrum* was found to be among the most abundant bacterial genera identified by 16S rRNA gene metabarcoding after antibiotic treatment; *Methylorubrum extorquens* DSM13060 is an endophyte, and studies have shown that its ability to colonize the internal plant compartments is dependent on its PHB accumulation capacity, which supposedly provides it with a higher resistance to oxidative stress from the plant host [65].

These observations suggest that a very strict symbiosis is established between bacteria and microalgae. This underlines the strong interdependency of the two organisms in terms of reciprocal growth and sustainment, most of all under nutrient starvation and low light conditions.

Different types of PHB accumulators, based on differential morphological structures of PHB intracellular granules, seem to be present in association with microalgal cells. This enforces the idea of a complex community whose variability and diversity provide resilience and adaptability to the overall algal-bacterial consortium.

4. Conclusion

PHB production from microalgae and cyanobacteria cultivation is often treated as a direct product of the photoautotrophic cells (Table S1).

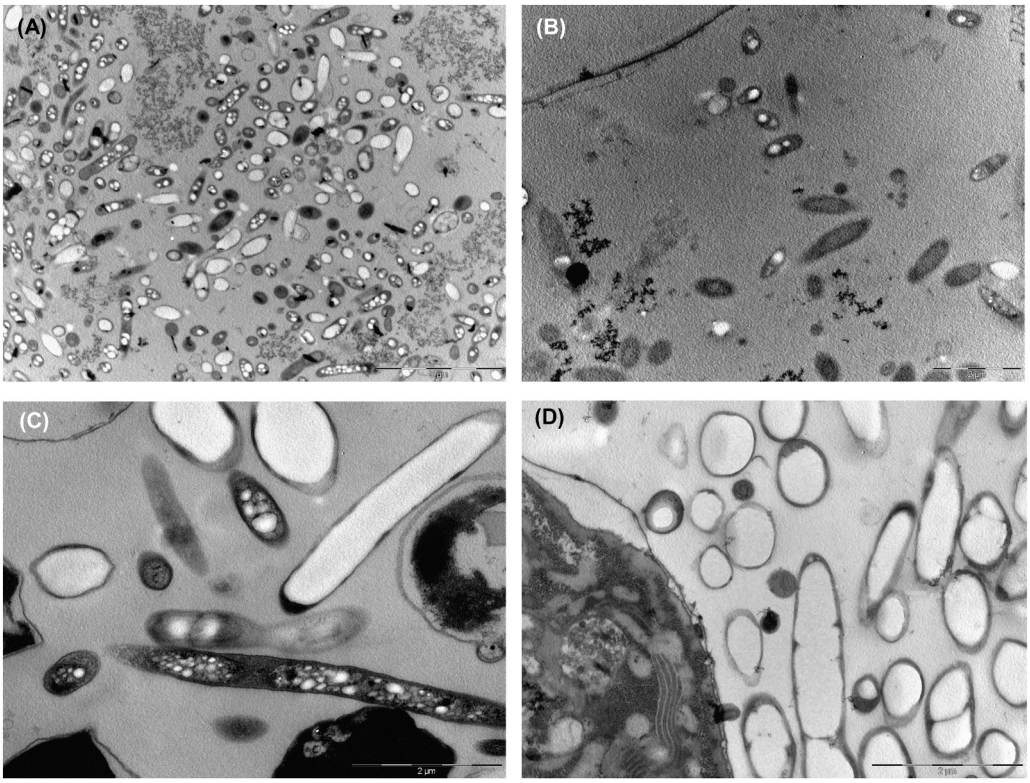

Fig. 5. TEM micrographs. (A), (B), (C) Bacterial cells in *D. communis* mixotrophic semicontinuous cultures. Most bacteria contain intracellular PHB granules of different shapes and dimensions. (D) Bacterial cells with PHB inclusions adjacent to a *D. communis* cell. Scale bar: 5 μm (A) 2 μm (B-C-D).

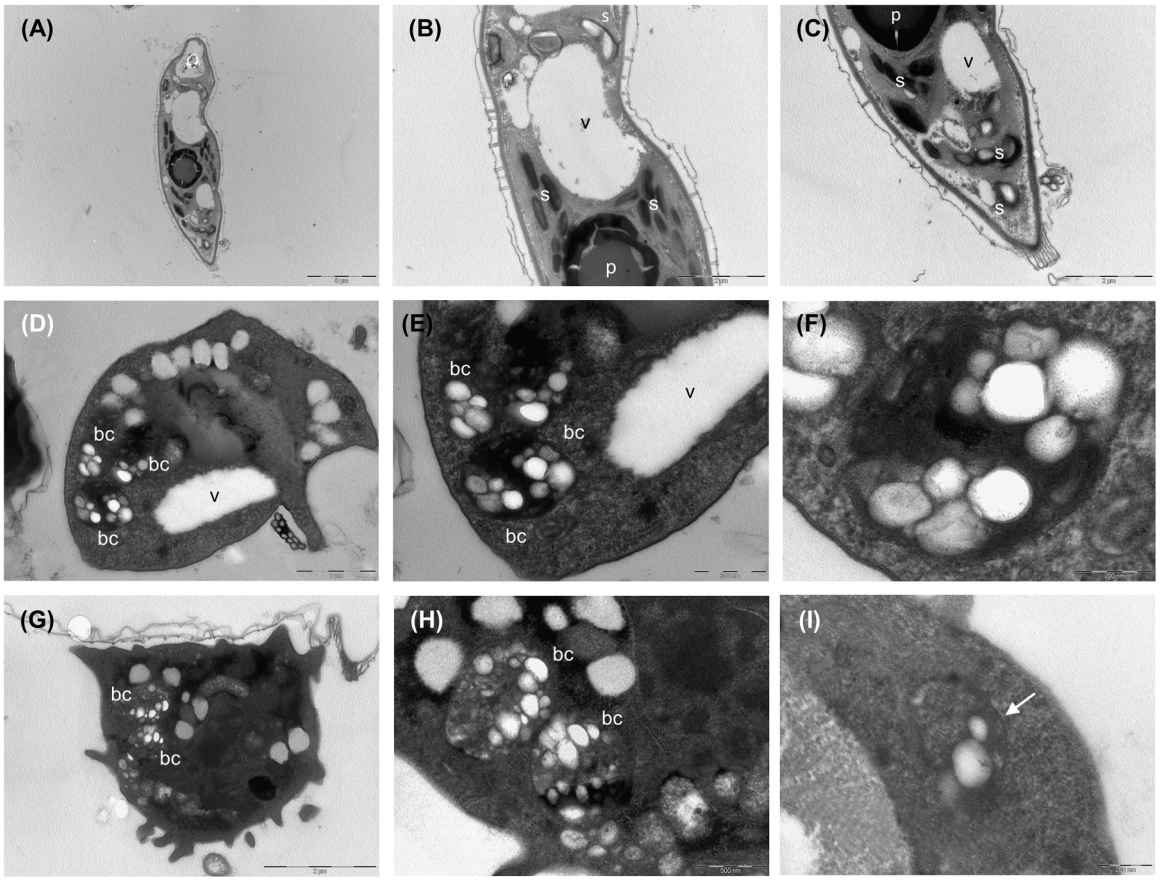

Fig. 6. TEM micrographs. (A) *D. communis* cell from control (CTRL) culture (grown in complete CHU13 medium). (B), (C) detail of D. communis cell from CTRL culture showing vacuole (v), pyrenoid (p), starch (s) granules. (D) to (I) bacterial cells (bc), with intracellular PHB granules, inside *D. communis* cells. (I) detail of a bacterial cell double-layered membrane (arrow) inside microalgal cells grown in PHB accumulation-inducing medium. Scale bar: 5 μm (A), 2 μm (B-C-G), 1 μm (D), 500 μm (E-H), 200 μm (F-I).

This work challenges the conventional view of PHB accumulation in autotrophic biomasses as a purely microalgal and/or cyanobacterial trait. We demonstrate that high PHB yields (up to 57 % w/w) in semi-continuous cultivation are linked to the enrichment of specific bacterial genera, and their removal drastically reduces PHB accumulation. By reframing microalgae-associated bacteria as co-producers rather than contaminants, this study opens new paths for designing bacterial-algal consortia that could improve biopolymer yields and stability. To our knowledge, this is the first report suggesting the potential endophytic colonisation of *Desmodesmus communis* by PHB-accumulating bacteria. Moreover, the observation of intracellular bacterial-like structures containing PHB granules further challenges the conventional view that microalgae-associated bacteria act only extracellularly and instead points to a far more intimate and possibly obligatory symbiosis. This finding redefines the role of the bacterial component in microalgal cultures, positioning them not merely as surface-associated partners, but as integrated contributors within the algal host.

In summary, employing an algal-bacterial consortium instead of solely bacteria for PHB production could be considered a valuable option due to the high yields of PHB, together with the possibility to improve wastewater treatment and co-produce additional metabolites, resulting in a more efficient, sustainable, and versatile process compared to using bacteria alone. The symbiotic relationship between algae and bacteria can enhance process stability, reduce energy consumption, and improve resource management, promoting a more sustainable production cycle.

CRediT authorship contribution statement

Martina Franchini: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Mara Simonazzi**: Writing – review & editing, Conceptualization. **Federica Costantini**: Writing – review & editing. **Valentina Papa**: Investigation, Data curation. **Rossella Pistocchi**: Writing – review & editing. **Stefano Ratti**: Writing – review & editing. **Chiara Samorì**: Writing – review & editing, Supervision. **Laura Pezzolesi**: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2026.01.005.

Data availability

Data will be made available on request.

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