Science of the Total Environment 972 (2025) 179109

Contents lists available at ScienceDirect

Science of the Total Environment

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

# On the role of bacterial gut microbiota from supralittoral amphipod *Talitrus saltator* (Montagu, 1808) in bioplastic degradation

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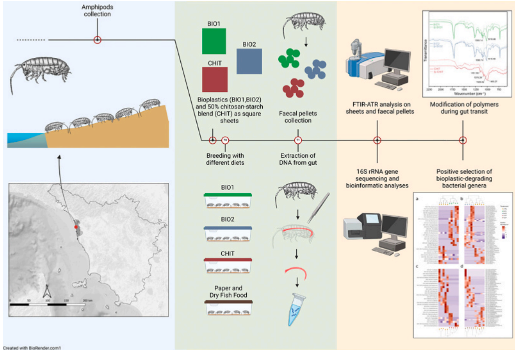
## HIGHLIGHTS

- Bioplastics ingested by *Talitrus saltator* undergo modification during gut transit.
- Gut microbiota of *T. saltator* is shaped by bioplastic feeding.
- The role of *T. saltator*'s gut microbiota in bioplastic modification is highlighted.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

Editor: Ouyang Wei

*Keywords:*
Starch-based bioplastic
Chitosan
Marine pollution
Gut microbiota
Bioplastic degradation
Microbiome

## ABSTRACT

Despite the promise of a reduced environmental impact, bioplastics are subjected to dispersion and accumulation similarly to traditional plastics, especially in marine and coastal environments. The environmental impact of bioplastics is attracting increasing attention due to the growing market demand. The ability of the supralittoral amphipod *Talitrus saltator* to ingest and survive on pristine starch-based bioplastic has already been assessed. However, the involvement of the gut microbiota of this key coastal species in making bioplastics a dietary supplement, remains unknown. In this study, we investigated the modification of *T. saltator* gut microbiota following bioplastic ingestion and the effect of this change on the modification of their chemical composition. Groups of adult amphipods were fed with: 1 — two different kinds of starch-based bioplastic; 2 — a 50 %/50 % chitosan-starch mixture; and 3 — paper and dry-fish-food. Freshly collected, unfed individuals were used as control group. Faecal pellets from the amphipods were collected and characterized using ATR-FTIR spectroscopy.

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https://doi.org/10.1016/j.scitotenv.2025.179109
Received 24 January 2025; Received in revised form 25 February 2025; Accepted 10 March 2025

DNA was extracted from gut samples for metagenomic analysis. Spectroscopic investigation suggested a partial digestion of polysaccharide components in the experimental polymeric materials. The analysis of the gut microbiota revealed that bioplastic feeding induced modification of sandhopper's gut microbial communities, shifting the abundance of specific microbial genera already present in the gut, towards bacterial genera associated with plastic/bioplastic degradation, especially in groups fed with starch-based bioplastics.

Overall, our results highlight the involvement of *T. saltator*'s gut microbiota in bioplastic modification, providing new insights into the potential role of microbial consortia associated to sandhoppers in bioplastic management.

## 1. Introduction

Sandhoppers, detritivore and scavenger amphipods, are key components of the food web, playing a crucial role in the modification and fragmentation of organic matter (McLachlan and Brown, 2006). *Talitrus saltator* is considered bioindicator of environmental pollutants like heavy metals, trace elements, PBDEs, and anthropogenic pressure (Rainbow et al., 1989; Ugolini et al., 2005; Ugolini et al., 2008; Ungherese et al., 2010; Ungherese et al., 2012). More recently, their possibility to ingest microplastic, bioplastic, and chitosan-starch mixtures has been investigated (Ugolini et al., 2013; Hodgson et al., 2018; Iannilli et al., 2018; Battistin et al., 2023; Martellini et al., 2023; Ugolini et al., 2024). Gut microbiota is known to be important for various functions in vertebrates, as well as in crustaceans (Holt et al., 2021), and ingestion of plastic items can have harmful effects on marine organism and on their gut microbiota (Wright et al., 2013; Yang et al., 2021; Iurk et al., 2024; Fackelmann et al., 2023). Gut microbial communities are implicated in host immune response, modulation of pathogens, nutrition and health (Li et al., 2018; Zoqratt et al., 2018; Holt et al., 2021; Hernandez-Perez et al., 2022; Chen et al., 2023). For instance, gut microbial production of *short-chain fatty acids* (SCFAs) (Nagpal et al., 2018; Akhtar et al., 2022), induce epigenetic modification, such as changes in histone acetylation and DNA methylation (D'Aquila et al., 2020; Woo and Alenghat, 2022; Akhtar et al., 2022). Exposure to pollutants including microplastic and bioplastic is related to adverse effects in epigenetic regulation and cytokine expression (Vandegehuchte and Janssen, 2014; Sun et al., 2021), and this pollutant mediated effects are regulated by SCFAs produced by microbial communities (Fellows et al., 2018; Fellows and Varga-Weisz, 2020; Huang et al., 2020). Few studies investigated the gut microbiota of amphipods (belonging to different families: Alicellidae, Ampeliscidae, Ampithoidae, Eurytheneidae, Hirondelleidae, Hyalidae, Ischyroceridae, Pardaliscidae, Scopelocheiridae, Talitridae, Uristidae) (Cheng et al., 2019; Chan et al., 2021; Nakamura et al., 2022). In talitrid amphipods, gut microbiota is mainly dominated by *Vibrio*, *Pseudomonas* and *Pseudoalteromonas* (Nakamura et al., 2022), and the microbial composition of different species is shaped by species-specific interactions, probably related to different dietary habits (Abdelrhman et al., 2017).

The urgent need to replace traditional disposable plastic products linked to the increasing awareness of plastic and related pollutants led to a rapid increase in the development and production of bioplastic (European Bioplastics, 2020; Atiwesh et al., 2021; Melchor-Martínez et al., 2022; Döhler et al., 2022). Among these, chitosan is currently gaining interest. It can be produced from waste products from the fishing industry (Hamed et al., 2016), holding appealing properties such as water solubility at low pH, adhesion to mucosal surface, and antimicrobial actions (Ke et al., 2021), and finding applications in various fields, including food, agriculture, industrial, pharmaceutics and medicine (Kanmani et al., 2017; Sahariah and Másson, 2017; Zoe et al., 2023; Guo et al., 2024; Edo et al., 2024; Costa et al., 2024), and, not least, in product packaging (Van den Broek et al., 2015; Cazón and Vázquez, 2019; Oladzadabbasabadi et al., 2022). Bioplastics are considered by consumers as an important sustainable and low-impact alternative to traditional petroleum-based products, due to the inaccurate belief that bioplastic could easily and quickly be degraded in the environment. However, biodegradability properties of bioplastic vary greatly depending on the kind of bioplastic, temperature and humidity conditions. Therefore, not all bioplastics are biodegradable in open environment (European Bioplastic, 2016; Dilkes-Hoffman et al., 2019a; Dilkes-Hoffman et al., 2019b; Tong et al., 2022). Although the favourable aspects of bioplastic compared to traditional plastic materials — such as the lower reliance on petroleum, the reduced carbon emissions (Atiwesh et al., 2021), and the less energy required for bioplastic production (Thiruchelvi et al., 2020), bioplastics still arise concerns and controversy regarding their environmental impact (Islam et al., 2024). Indeed, similarly to petroleum-based plastic, bioplastic can fragment in smaller particles and enter in the food web (Ribba et al., 2022; Piyathilake et al., 2024), with detrimental effects on organisms (e.g., Green, 2016; Chagas et al., 2021; Charoeythornkhajhornchai et al., 2023). In this context, marine supralittoral environments are particularly endangered by the deposition and accumulation of plastic and bioplastic waste, carried by river flow, winds, and currents (Thompson et al., 2004). The supralittoral zone of sandy beaches represents an ecologically important ecotonal environment, threatened by waste, either biodegradable or not, of both marine and terrestrial origin (McLachlan and Brown, 2006). This, together with the growing bioplastic production, lead to an increased interest on the effect of bioplastic ingestion by aquatic, intertidal and estuarine organisms (Venâncio et al., 2022; Tao et al., 2024), and on the degradation mechanisms of these materials in the environment. Despite this, little is known about the fate of bioplastic debris in littoral environments and the effects of its ingestion by marine and coastline organisms.

We previously assessed the capacity of the supralittoral amphipod *T. saltator* to ingest and fragment both starch-based bioplastics and chitosan-starch mixtures, free by the fouling of microbial biofilm, assessing that these amphipods can partially modify starch-based bioplastic (Martellini et al., 2023; Ugolini et al., 2024).

In this study, we tested whether the ingestion of two different starch-based bioplastics and a 50 %–50 % chitosan-starch blend by *T. saltator* could induce a modification of the gut microbial composition. For this purpose, a laboratory experiment in controlled conditions of photoperiod and temperature was conducted, by feeding groups of *T. saltator* individuals with two starch-based different bioplastics commonly used for the production of disposable shopping bags and a chitosan-starch blend. A group of sandhoppers kept in captivity and fed with paper and dry-fish-food, and a group of sandhoppers collected in nature and not treated, were used as controls. The bioplastic materials before and after ingestion (as faecal pellets) were characterized by ATR-FTIR spectroscopy, and compared the gut microbiota composition changes among groups.

Results showed that ingested bioplastics were differently degraded during gut transit of *T. saltator*, and we observed a statistically significant increase of bacterial ASVs associated with plastic/bioplastic degradation, especially in sandhopper's group fed with starch-based materials. Thus, the involvement of *T. saltator*'s gut microbiota in bioplastic modification could be hypothesized.

## 2. Materials and methods

### 2.1. Amphipods collection and experimental setup

Adults *T. saltator* individuals were collected on the sandy beach of Regional Park of Migliarino-San Rossore-Massaciuccoli, Tuscany, Italy (43°44′53″ N, 10°16′33″ E), in summer 2024 using entomological aspirators and transferred alive in laboratory.

The map of Tuscan coast showed in the graphical abstract is generated using QGIS version 3.30 (https://qgis.org). *Talitrus saltator* picture in graphical abstract was adapted from Calman (1911).

The experiment was conducted under controlled conditions ($T = 25 \pm 2$ °C; artificial photoperiod L:D 12:12 in phase with the natural one). Individuals were divided into five groups for each treatment in Plexiglass boxes with artificial sand. Different experimental treatments represented the only food sources, and consisted of the following: 1 — two different starch-based bioplastics commonly used in supermarkets carrier bags, referred to as BIO1 and BIO2, and both classified as biodegradable and compostable according to UNI EN 13432 (Martellini et al., 2023) (n = 25 individuals); 2 — a 50 %–50 % chitosan and starch blend (hereafter referred to as CHIT) (Ugolini et al., 2024) (n = 25) and 3 — paper and dry fish food (hereafter referred to as PDFF) (n = 25), a standard food to maintain sandhoppers in captivity to simulate the detritivore diet of *T. saltator*, as previously described (Scopetani et al., 2018; Ciofini et al., 2020; Martellini et al., 2023; Ugolini et al., 2024). This control group is crucial to exclude possible confounding effects due to keeping animals in captivity. Main chemical composition of BIO1 and BIO2 was previously characterized by Martellini et al. (2023) and also reported in Table S1.

BIO1, BIO2, CHIT, and PDFF were presented as 4 × 3 cm sheets that were previously sterilized under UV light for 30 min, for each side. Groups were kept in experimental conditions for 7 days, an optimal time to investigate modification in *T. saltator* gut microbiota, as previously stated (Abdelrhman et al., 2017). However, CHIT group was exposed to the treatment for 4 days. This was the maximum time to ensure 100 % survival of *T. saltator* individuals exposed to this treatment, due to the previously observed high mortality associated with this material (Ugolini et al., 2024). After the treatment, individuals' guts from each group were extracted using sterile forceps and grouped into 5 pools, consisting of 5 guts each. Similarly, guts were extracted immediately after amphipod collection from the beach (untreated individuals) and merged into 5 pools consisting of 5 guts each. They were used as an untreated control group (CTRL). Guts pools were fully immersed in RNALater solution (ThermoFisher Scientific, Waltham, Ma, USA) and stored at −20 °C. All instruments were sterilized in advance with 70 % ethanol to avoid contamination (Cini et al., 2020).

### 2.2. Chitosan-starch blend sheets preparation

The 50 %–50 % chitosan-starch blend sheets (CHIT) were prepared using the solvent casting method (Matthew et al., 2006). Initially, the polymers were solubilized separately: 200 mg of chitosan powder (Sigma Aldrich, low molecular weight) was dissolved in 25 ml of 2 % acetic acid at 60 °C, while 200 mg of starch powder (Sigma Aldrich, from corn) was dissolved in 25 ml of distilled water at 80 °C.

The two polymers solution where the combined under continuous magnetic stirring, poured into 10 × 10 cm Petri dishes and left to dry. After complete solvent evaporation, thin films with an approximate thickness of 0.02 mm were obtained (Li et al., 2013).

### 2.3. Chemical analysis

Faecal pellets of amphipods resulting from BIO1, BIO2 and CHIT material ingestion were collected from boxes at the end of the experiment and pooled for characterization by Fourier Transform Infrared Spectroscopy (FTIR) in Attenuated Total Reflection mode (ATR). Measurements were performed on an IRAffinity-1S by SHIMADZU equipped with the ATR sampling accessory (MIRacle™ PIKE Technologies). The high polymeric content in faecal pellets derived from exclusive feeding of amphipods with BIO1, BIO2, and CHIT allowed the direct identification of characteristic polymeric absorptions in the ATR-FTIR spectra of collected samples.

### 2.4. 16S rRNA sequencing

Microbial DNA extraction was carried out using DNeasy Powersoil Kit (QIAGEN) following manufacturer instructions (Wei et al., 2023). Genomic DNA quantification was then assessed using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Ma, USA) 1× dsDNA High Sensitivity kit, for quality and quantity. Due to low yield of gDNA quantity, a nested-PCR amplification of 16S rRNA gene V3–V4 hypervariable regions was performed, using primers 341f (5′-CCTACGGGNGGCWGCAG-3′) and 805r (5′-GACTACNVGGGTWTCTAATCC-3′) for 30 cycles, preceded by amplification of the entire 16S rRNA sequence using primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525r (5′-AAGGAGGTG ATCCAGCC-3′) (Weisburg et al., 1991) for 10 cycles (adapted from Ogai et al., 2018). Sequence libraries were prepared according to Illumina Protocol 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B; URL: http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Paired-end 2 × 300 bp sequencing was performed on Illumina MiSeq Platform (Illumina Inc) using MiSeq Reagent Kit v3 (600 cycles).

### 2.5. Sequence processing and biodiversity analysis

The primers were removed by using CUTADAPT (Martin, 2011). Raw sequences were first quality-filtered and cleaned using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2016). The reads were then merged and cleaned from the presence of chimeras. Amplicon Sequence Variants (ASVs) were also determined with DADA2.

Taxonomic classification of sequences was performed using DECIPHER (Wright, 2016) with the Silva 138.1 database (Yilmaz et al., 2014). Microbial community analyses were conducted on R software using the packages phyloseq v.1.42.0 (McMurdie and Holmes, 2013), microbiome v.1.21.1 (Lahti et al., 2017), and tidyverse v.2.0.0 (Wickham et al., 2019).

Alpha diversity was evaluated using the number of observed ASVs and inverse Simpson indices.

Beta-diversity indices were calculated using Bray-Curtis distances with a PCoA approach using the metaMDS function from vegan v.2.6.4 (Oksanen et al., 2024). Differential abundance of microbial taxa was assessed with the Analysis of Compositions of Microbiomes with Bias Correction (ANCOMBC) package v.2.4.0 (Lin and Peddada, 2020) with an adjusted $p$-value threshold of 0.05.

To perform network analysis, the dataset was split into one dataset for each treatment, and then ASVs were filtered based on their prevalence (threshold = 0.6) and abundance (threshold = 0.001). Associations were calculated using the NetCoMi package (v.1.1.0) (Peschel et al., 2021) using "cclasso" as method, due to its ability to consider the compositionality of microbial data. Only associations with a significance $<0.05$ were considered for the subsequent analyses. Analysis of nodes and edges' statistics was done using the igraph package (v.2.1.1) (Csardi and Nepusz, 2006).

The ggplot2 package v.3.4.2 (Villanueva and Chen, 2019) was used for data visualization and Cytoscape (v.3.10.2) software (Shannon et al., 2003) for networks' graphic representation.

### 2.6. Reads number and quality

From an initial total number of reads of 3,952,056 (mean per sample

= 135,972.6), the first quality filtering returned 2,984,155 reads (mean per sample = 119,366.2, 87.8 % of the total). After denoising and merging, the total number of overlapping sequences obtained was 2,885,934 (mean per sample = 1,115,437.4, 84.9 % of the initial number). After the chimera removal, the resulting final number of reads obtained was 2,789,018 (mean per sample = 111,560.7, 82 % of the initial number of reads).

### 2.7. Statistical analysis

The significance of differences among alpha-diversity indices was assessed using the Wilcoxon test.

Significant correlations between community composition and treatment were evaluated with Permutational Analysis of Variance (PERMANOVA) using the adonis2 function built in the vegan package.

## 3. Results

### 3.1. Bioplastic and faecal pellets characterization

Faecal pellets of *T. saltator* treated with BIO1, BIO2 and CHIT were examined by ATR-FTIR analysis to characterize the polymeric matrices and confirm the presence of the polymeric items in the animal excretions. In Fig. 1a the FTIR spectra collected for native BIO1, BIO2 and CHIT are compared. Spectra of BIO1 and BIO2 polymers are very similar to each other, with a FTIR profile that can be traced back to the one of a typical polyester (PE). The absorptions centered at 1712, 1252–1103 and 723 cm$^{-1}$ can be indeed respectively attributed to the characteristic ester C═O stretching, C—O stretching and aromatic C—H out-of-plane bending of a PET (polyethylene terephthalate)-like scaffold (Chércoles Asensio et al., 2009; Jung et al., 2018). Along with terephthalic acid (TPA)-like molecules, spectra are also compatible with the presence in the formulation of polysaccharides (Kwon and Jeong, 2020; Arundati et al., 2024) and/or polyhydroxyalkanoates (PHA) in the analysed matrices (Porras et al., 2014). In particular, the absorption at 1016 cm$^{-1}$ may indicate the C—O stretching of a sugar component. Similarly, the spectrum of CHIT clearly displays the simultaneous presence of the characteristic absorptions of the two polysaccharide constituents, starch and chitosan (Ugolini et al., 2013). Of particular interest in the fingerprint region are the absorptions between 1151 and 995 cm$^{-1}$, associated with the asymmetric stretching of the C—O—C bridge and C—O stretching. In this region, the peak at 995 cm$^{-1}$ is likely associated to the C—O stretching of the starch component (Wang and Xie, 2010), being significantly shifted (of ca. 30 cm$^{-1}$) towards lower frequencies relative to the corresponding vibration in pure chitosan, located at 1028 cm$^{-1}$ (Queiroz et al., 2014; Ugolini et al., 2024).

Fig. 1b reports the FTIR spectra obtained after sandhoppers' intestinal transit (dotted lines), compared to the ones of the native polymeric materials (straight lines). As shown, all the characteristic absorptions of native polymers are still observable in the spectra of their corresponding faecal pellets, thus indicating the presence of these polymeric items in animal excretions. However, small but significative differences can be also appreciated before and after the gut transit. Among the most relevant, in the spectra of faecal pellets of BIO1 and BIO2, the decrease of absorptions at 1080 and 1016 cm$^{-1}$ can be observed, whereas a net decrease in intensity of the peak at 995 cm$^{-1}$ is evident in the spectrum obtained after CHIT-feeding, with the one centered at 1027 cm$^{-1}$ (reasonably ascribable to the chitosan component, vide supra) that becomes predominant.

### 3.2. Comparison of diversity and gut bacterial community composition among feeding groups

By Principal Component Analysis (PCOA) based on Bray-Curtis distances, we noted a separation of samples according to treatment groups (Fig. 2a). This result was corroborated by PERMANOVA test which showed a statistically significant effect of the treatment on microbial communities in each treatment group ($R^2 = 0.27$, $F = 1.84$, $p$-value = 0.003). Moreover, pairwise PERMANOVA carried out among groups (Fig. 2b) showed that every feeding group had a significant different gut bacterial community compared to the control. To note, BIO2 displayed significant differences when compared to BIO1 ($p$-value $< 0.033$) and PDFF ($p$-value $< 0.008$).

Alpha-diversity analysis was performed using the number of observed ASVs and the inverse Simpson indices (Fig. S1). We observed significant differences in inverse Simpson indexes between BIO2 group

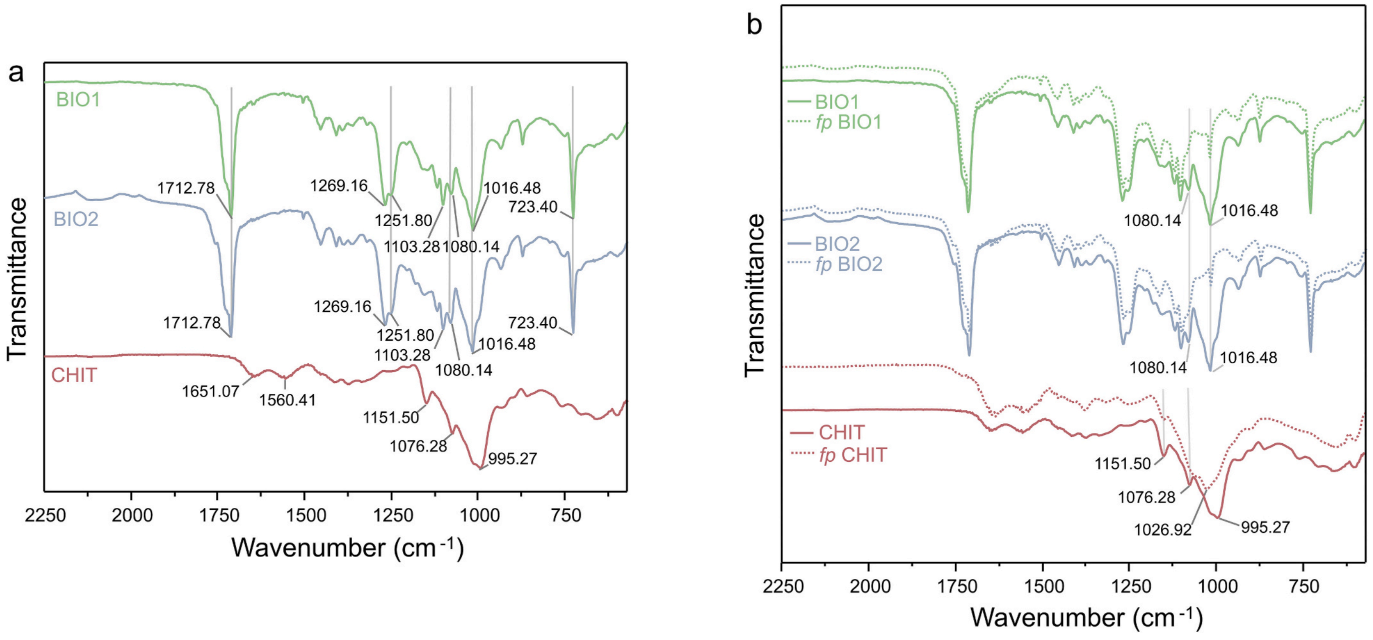

**Fig. 1.** ATR FTIR spectra of (a) BIO1 (green), BIO2 (blue) and CHIT (red) (vertical dotted lines highlight main absorption peaks common to BIO1 and BIO2) and (b) their comparison before (solid lines) and after (dotted lines) sandhopper's intestinal transit (most relevant differences highlighted by vertical dotted lines). Spectral range 2250–570 cm$^{-1}$ region.

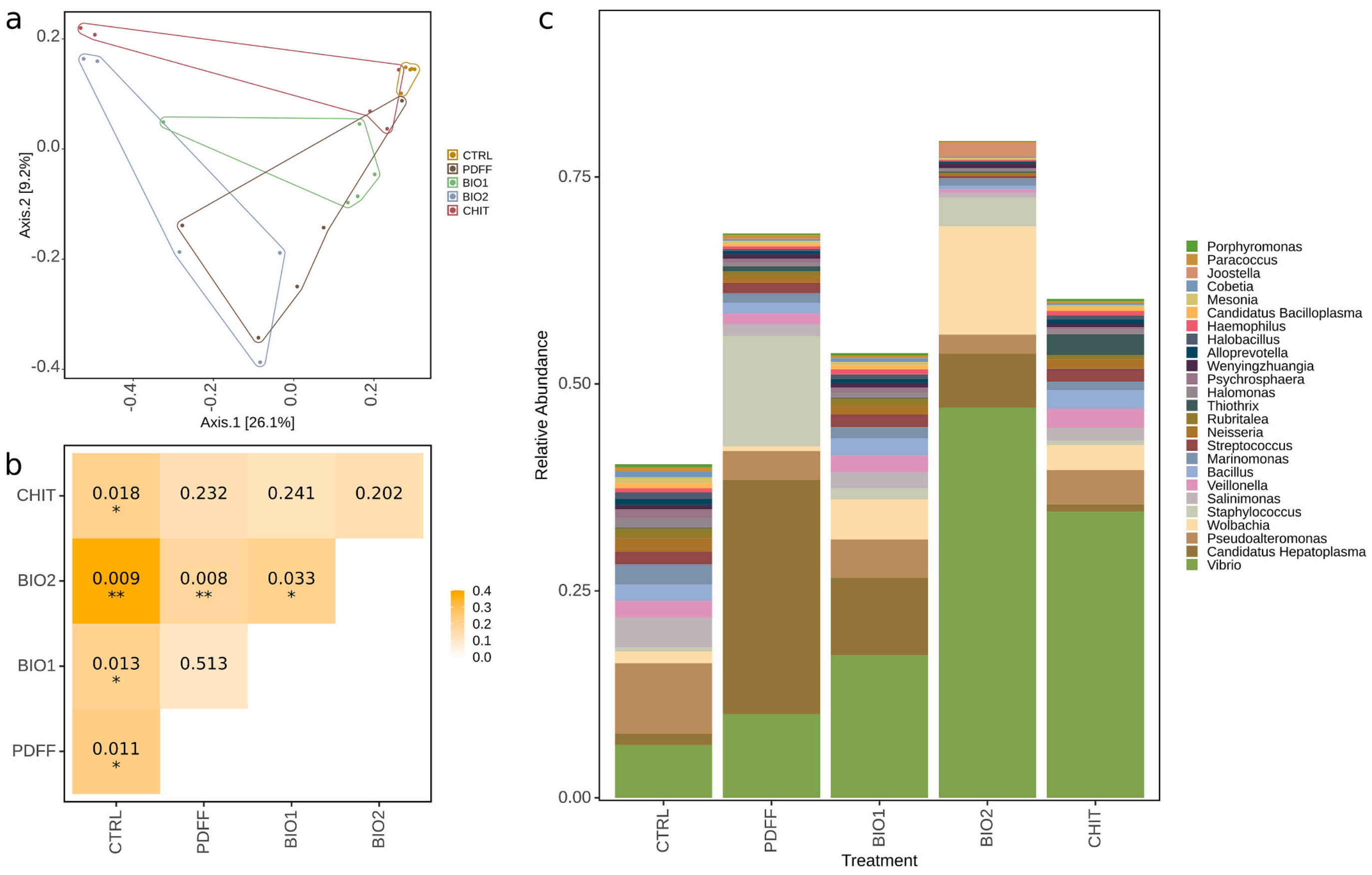

**Fig. 2.** Diversity analysis. a Multidimensional scaling analysis (PCoA) based on Bray-Curtis distances, according to treatment; b Heatmap displaying statistical significance and $R^2$ values from pairwise PERMANOVA for each comparison between groups. Colour scale indicates $R^2$ values of comparisons; statistical significances are reported using asterisks (*, $p < 0.05$; **, $p < 0.01$); c Barplots of the 25 most abundant bacterial genera across different groups. CTRL: Control; BIO1: starch-based bioplastic 1; BIO2: starch-based bioplastic 2; CHIT: 50/50 chitosan-starch blend; PDFF: paper and dry-fish-food.

and CTRL ($p$-value $< 0.05$).

Analysis of bacterial genera abundances showed that the microbiota composition of *T. saltator* is influenced by feeding treatments (Fig. 2c). Unlike CTRL group, in the 4 treated groups we observed that the 25 most abundant genera represent over 40 % of the overall microbial community composition. In untreated conditions (CTRL), the most abundant bacterial genera were *Vibrio*, *Pseudoalteromonas*, and *Salinimonas*. In the treated groups these genera were differently abundant. Especially, *Vibrio* was more abundant in BIO1, BIO2 and CHIT, while *Pseudoalteromonas*, and *Salinimonas* were reduced when compared to CTRL. Overall, we observed an abundance of Candidatus *Hepatoplasma* in BIO1, BIO2 and PDFF, while abundance of *Wolbachia* and *Staphylococcus* in PDFF.

### 3.3. Influence of treatments in the distribution of bacterial taxa

Differential abundance analysis was performed between each treatment group compared to CTRL (Fig. 4). Based on statistically significant ASVs abundance distribution ($q$-value $\leq 0.05$), all comparisons carried out with the CTRL group showed well-distinct clusters of samples according to the treatment variable. In each treated group, we observed bacterial ASVs abundant in at least 60 % (3/5) of samples (for the complete list, see Table S2). Among these, we found several ASVs that are known to be associated with plastic/bioplastic degradation. Compared to CTRL, BIO1 (Fig. 3a) showed an increased abundance in *Fusobacterium*, *Enteroccocus*, *Methylocystis*, *Planococcus*, *Zunongwangia*, *Gramella*, and *Cutibacterium* (Bauer et al., 2006; Park et al., 2021; Bandini et al., 2022; Hchaichi et al., 2020; Ascencio-Galván et al., 2023; Sharma, 2023; Chaimusik et al., 2024), and *Peredibacter* associated with plastisphere (Chen et al., 2022; Nguyen et al., 2023). Similarly, BIO2 (Fig. 3b) showed an increase in plastic/bioplastic degrading associated bacterial genera, such as *Marinomonas*, *Alcanivorax*, *Thalassospira*, *Rhodococcus*, *Oceanimonas*, as well as bacteria genera associated with plastisphere and plastic biofilm formation, including *Luteibaculum* and *Peredibacter* (Denaro et al., 2020; Delacuvellerie et al., 2021; Odobel et al., 2021; Joshi et al., 2022; Nguyen et al., 2023; Di Gregorio et al., 2024; Zampolli et al., 2024; Zhang et al., 2024), and *Joostella*, known for lignocellulosic material degradation (Dutta and Bandopadhyay, 2022). In CHIT (Fig. 3c), although a separate cluster of samples from CTRL group is observable, we did not find an increase in ASV abundance in at least 60 % of the samples. In PDFF cluster (Fig. 3d), we found ASVs associated with other genera known to be associated with plastisphere and/or bioplastic degradation such as *Mesoflavibacter*, *Shewanella*, *Roseovarius*, *Thalassospira*, *Alcanivorax*, and *Cutibacterium* (Ariole and George-West, 2020; Di Pippo et al., 2020; Fawcett et al., 2021; Nguyen et al., 2023; Chattopadhyay, 2022).

We observed that bacterial genera known for involvement in bioplastic degradation were present with different abundance in each group, both in treated and the untreated samples (CTRL). However, bacterial genera such as *Joostella*, *Alcanivorax*, *Thalassospira*, *Peredibacter*, and *Rhodococcus* were significantly increased especially in BIO2 group (Fig. 4).

### 3.4. Effects of treatment on gut microbiota interaction networks

Network analysis was performed to assess the microbial interactions within the community of each group. This analysis showed that the connections within the microbial communities of sandhoppers fed with the two starch-based bioplastic (BIO1 and BIO2) and starch-chitosan

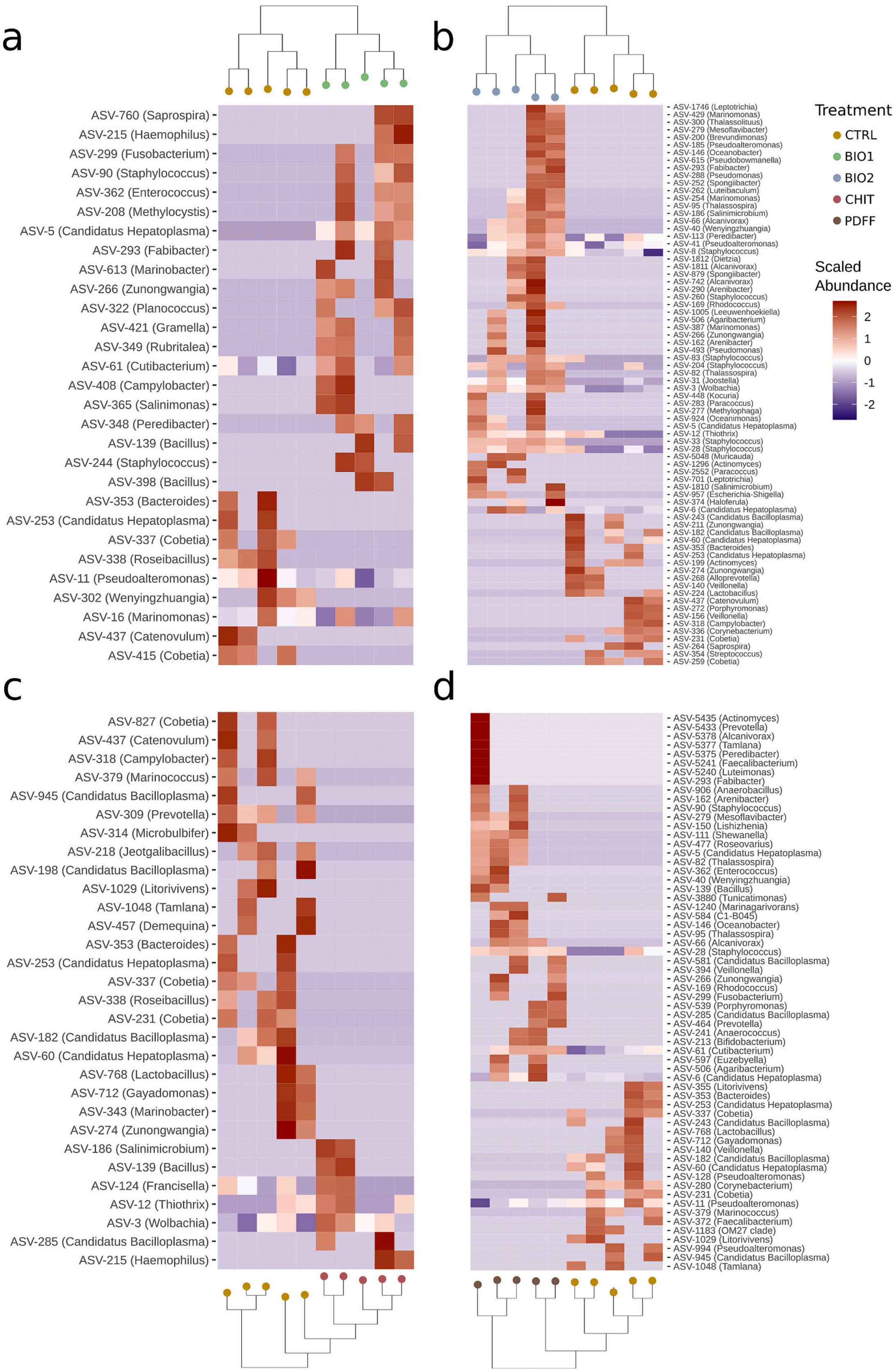

**Fig. 3.** Heatmap and cluster analysis of statistically significant ASVs ($q$-value $\leq 0.05$) from the pairwise differential abundance analysis of treatments versus control. a, BIO1 vs CTRL; b, BIO2 vs CTRL; c, CHIT vs CTRL; d, PDFF vs CTRL.

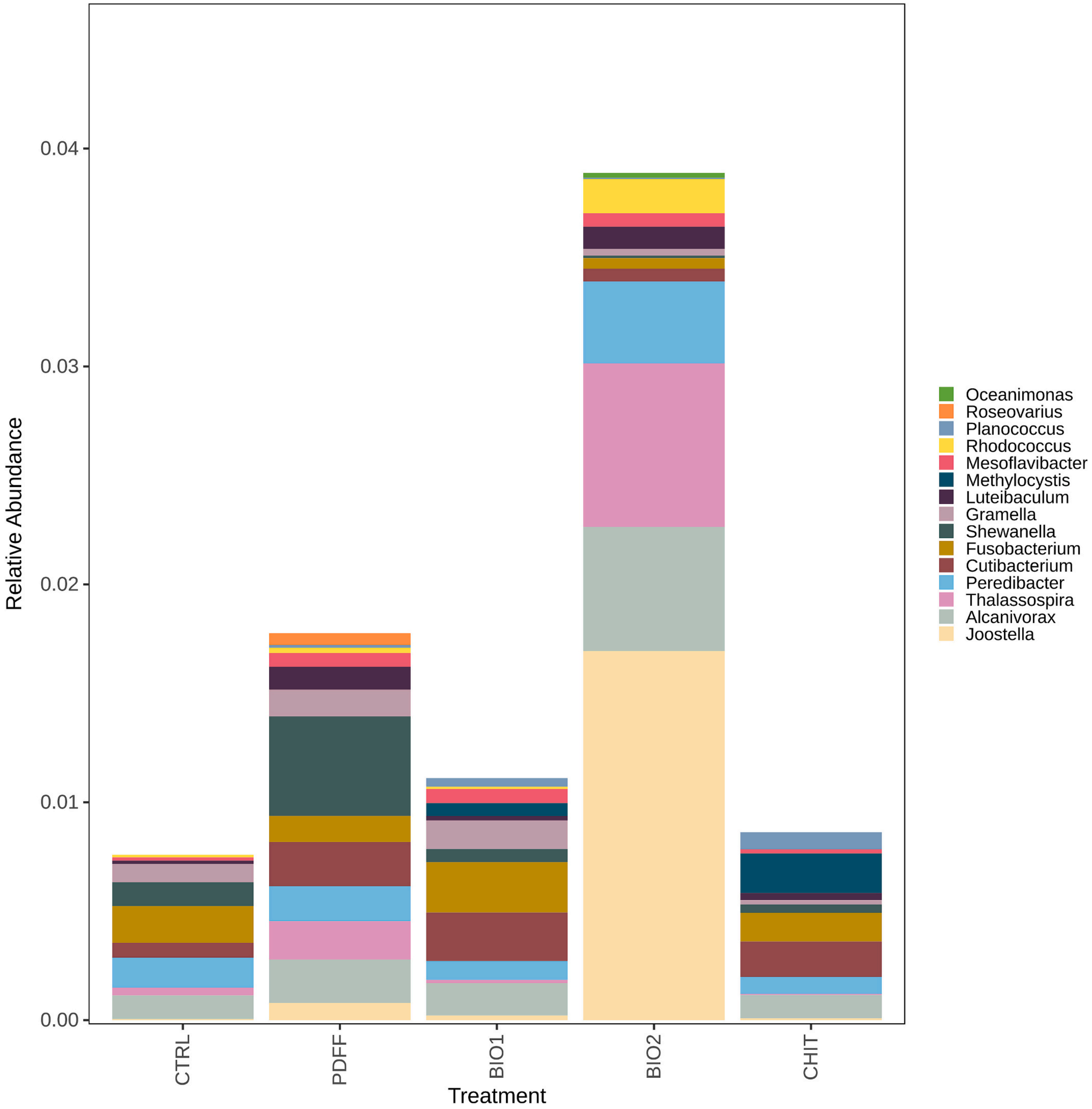

**Fig. 4.** Barplots of the relative abundance of treatments-linked genera of interest for their possible involvement in bioplastic degradation. Groups are reported according to the following legend, CTRL: Control; PDFF: paper and dry-fish-food BIO1: starch-based bioplastic 1; BIO2: starch-based bioplastic 2; CHIT: 50 %/50 % chitosan-starch blend.

mixture (CHIT) were changed when compared with the CTRL and with the PDFF group. The core (hubs) of the control group (CTRL, Fig. 5a) was the most diverse among the five networks, with *Veillonella*, *Streptococcus*, *Capnocytophaga*, *Salinimonas*, *Pseudoalteromonas*, *Alloprevotella*, *Neisseria*, and *Tamlana* as main nodes. In BIO1 (Fig. 5b), a switch in the network's core was evidenced, with an increase of ASVs belonging to plastic/bioplastic-degrading associated genera (e.g., *Wenyingzhuangia* and *Marinomonas*). Similarly, the network calculated on BIO2 resulted in orbiting around a number of hub ASVs with a role in bioplastic degradation, such as *Peredibacter*, *Wenyingzhuangia*, *Alcanivorax*, *Rhodococcus*, and *Thalassospira* (Fig. 5c). Moreover, the number of nodes and edges in this group were reduced compared to both BIO1 and CTRL. In CHIT group, the most central ASVs in the network were *Veillonella*, *Vibrio*, *Psychrosphaera*, *Paracoccus*, and *Alloprevotella* (Fig. 5d). Interestingly, ASVs belonging to the genus *Gayadomonas*, possibly having a role in starch degradation (Chi et al., 2013), and *Thiothrix*, which is a chitin-colonizing genus (Protasov et al., 2020) with potential starch-degrading enzymes (Boden and Scott, 2018) were also found to be part of the central nodes of the network. In sandhoppers fed with paper and dry fish food (PDFF), the main hubs were represented by *Staphylococcus*, *Vibrio*, *Cadidatus Hepatoplasma*, *Pseudoalteromonas*, *Shewanella*, *Cobetia*, and *Rubritalea* (Fig. 5e).

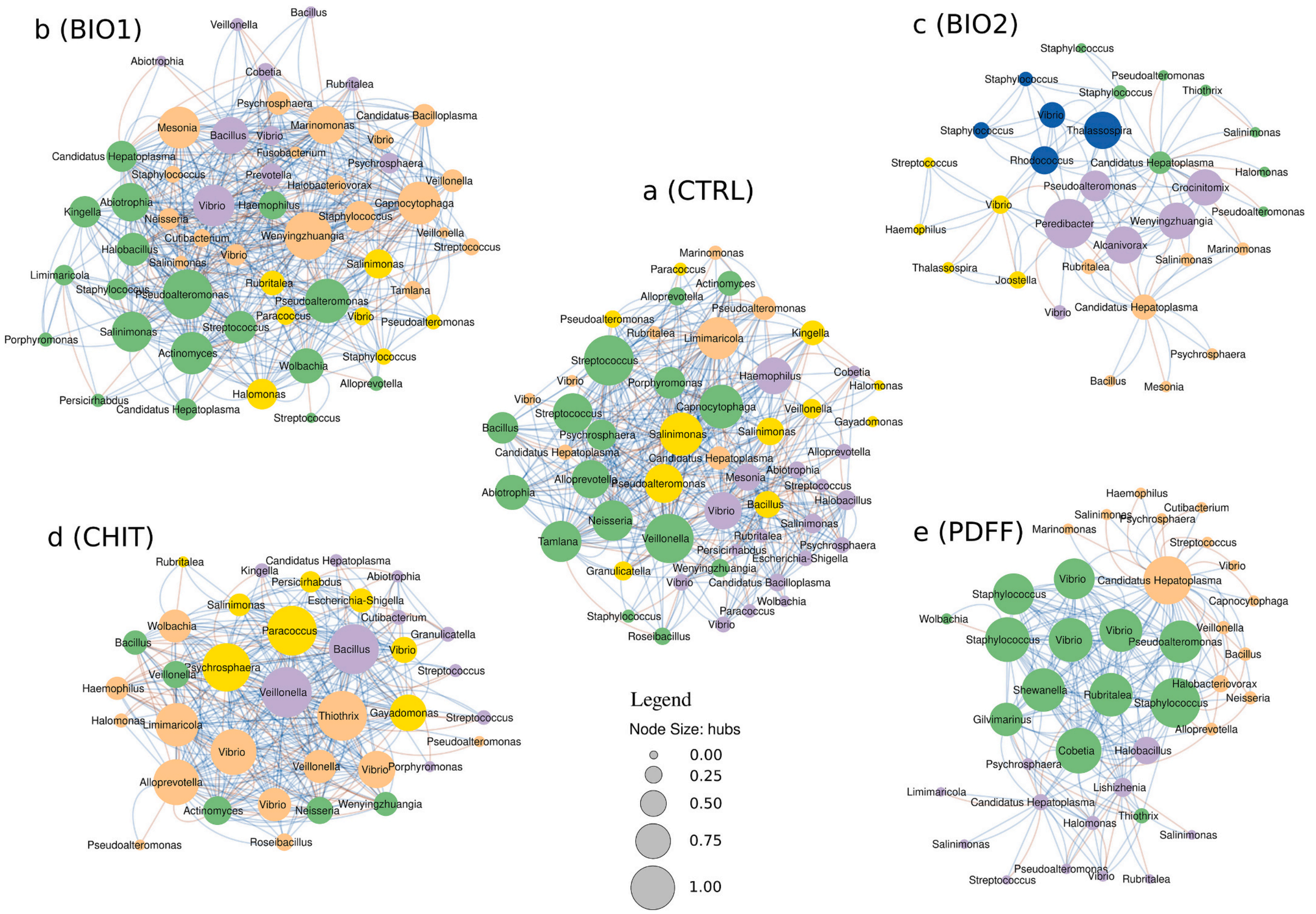

**Fig. 5.** Microbial interaction networks in the different groups. The connection network of dominant ASVs in: a, CTRL: Control; b, BIO1: starch-based bioplastic 1; c, BIO2: starch-based bioplastic 2; d, CHIT: 50 %/50 % chitosan-starch blend; e, PDFF: paper and dry-fish-food. Colors indicate different ASVs clusters, size of nodes (in legend) represents the hub score of ASVs.

## 4. Discussion

To date, few studies investigated bioplastic consumption by talitrid amphipods (Straub et al., 2017; Hodgson et al., 2018; Shruti and Kutralam-Muniasamy, 2019; Ugolini et al., 2024; Ugolini et al., 2024). Previous studies showed that microplastic, trace metals and other contaminants are mainly assumed by *T. saltator* through food (Ugolini et al., 2013; Ungherese et al., 2012; Scopetani et al., 2018). In prior studies, we evidenced that different bioplastics and chitosan-starch mixture have different effects on survival rate (Martellini et al., 2023; Ugolini et al., 2024). The observed ability to modify the structure of the ingested materials confirmed a potential role of amphipods in the degradation of bioplastics in supralittoral zone of marine environment (Martellini et al., 2023).

The bioplastic matrices characterized in our study showed spectra of a typical polyester, also compatible with the presence of polysaccharides in the formulation, confirming what previously reported (Martellini et al., 2023). Regarding CHIT, the obtained spectra clearly distinguish between characteristics peak of chitosan and the starch component. After gut transit, in both BIO1 and BIO2 faecal pellets, the absorptions of polymeric items were still observable. A slight but significative modification of the polymer's spectra were detectable, especially regarding a diminished absorption in the spectral range ascribable to the sugar component of the matrix. Interestingly, this was also found in the CHIT faecal pellets, that showed a predominant consumption of the starch component of this matrix compared to the one of chitosan.

In accordance with what previously assessed (Ugolini et al., 2024), this result suggests a preferential consumption of the starch component of the CHIT material. Overall, notwithstanding the complexity of the faecal matrices makes it difficult to straightforwardly correlate the observed FTIR variations with possible different digestion capabilities of *T. saltator*, these data hint a partial digestion of polysaccharide components of the feeding polymeric materials.

Moreover, our previous study (Ugolini et al., 2024) demonstrated that chitosan is not a nutritional source for *T. saltator*. On the contrary, chitosan has a possible detrimental effect in the digestive process, threatening sandhopper's survival, possibly due to reduction of dietary fat absorption in the intestine (Cheung et al., 2015), or oxidative stress effect (Mosleh et al., 2007), or antimicrobial properties, being effective against the growth of some bacteria and fungi (Kendra and Hadwiger, 1984; Muzzarelli et al., 1990; Roller and Covill, 1999; Tsai and Su, 1999; Qi et al., 2004; Carlson et al., 2008; Xing et al., 2015; Kidibule et al., 2021; Dou et al., 2024).

According to the metabarcoding analysis, feeding with the three different matrices impact the gut microbiota composition of *T. saltator* compared to the untreated individuals (CTRL) and those fed with paper and dried fish (PDFF). However, alpha diversity analysis showed significant differences in bacterial richness only between BIO2 and CTRL group. The most abundant genera retrieved in all groups had already been characterized as part of sandhopper's gut microbiota by previous research, especially *Vibrio*, Candidatus *Hepatoplasma*, *Pseudoalteromonas* and *Wolbachia* (Abdelrhman et al., 2017; Nakamura et al., 2022). However, *Vibrio* spp. dominated the amphipod and crustacean gut microbiota, and we found a significant abundance of this bacterial genus after feeding with BIO1, BIO2 and CHIT, possibly related to bioplastic degradation as previously evidenced (Hchaichi et al., 2020; Marín et al., 2023; Hu et al., 2024). We also observed that *Pseudoalteromonas*, and *Salinimonas* were reduced in the treated groups, when compared to untreated individuals. In addition, when compared to CTRL group, in the treated groups we found a differential abundance of bacterial genera known for plastic and bioplastic degradation, bacteria associated with plastisphere and plastic biofilm formation. However, these bacterial genera were also present in the untreated samples, suggesting that these genera are naturally present in amphipods' gut (Russini et al., 2021; Nakamura et al., 2022; Lozada et al., 2023; Aires et al., 2023). This result highlights that bioplastic feeding (BIO1 and BIO2) enriches ASVs and microbial genera, often with specific plastic degrading properties, that are already present in the animal gut, but were not found to increase in CHIT. In addition, results of microbial network analysis show that in BIO1 and BIO2, ASVs involved in plastic degradation replaced the central core of the CTRL group. In particular, the microbial network of BIO2 included a fewer number of nodes and interactions, probably due to a peculiar increase of some specific taxa driven by the ingestion of bioplastic. This is consistent with the modification of the ingested material, as stated by the FTIR analysis on faecal pellets, suggesting a possible role of gut microbiota in the degradation of bioplastics. The observed differences in gut microbiota between the two bioplastic-fed groups might be due to the different chemical composition of BIO1 and BIO2. By our previous chemical characterization (Martellini et al., 2023), BIO1 and BIO2 differed by their filmogenic agent: 1,6-dioxacyclodecane-7,12-dione in BIO1 and p-dioxane-2,5 dimethanol in BIO2. These compounds are characterized by the presence of hydroxyl groups, which makes them more susceptible to degradation by environmental factors, as well as microorganism, due to the action of enzymes such as laccase, peroxidase and hydroxylase (Nguyen et al., 2021; Heris, 2024). In BIO2 the filmogen compound was present in higher amount than in BIO1, possibly increasing its degradability. This aspect might explain the differences observed in microbial composition between BIO1 and BIO2. Regarding CHIT material, the centrality of potential starch-degrading bacteria in the network might explain the partial degradation of chitosan of the starch component alone, as observed by FTIR analysis.

Given the role of *T. saltator* in the food chain of the supralittoral zone of sandy beaches (Griffiths et al., 1983; McLachlan and Brown, 2006), this species could provide a valuable contribution in the recycling/modification of starch-based bioplastics.

## 5. Conclusions

Sandhoppers are known to be a key component of the supralittoral belt of sandy beaches (McLachlan and Brown, 2006). These areas are notorious accumulation zones for various types of waste from both the sea and inland, including plastic waste (Hodgson et al., 2018). *T. saltator* is capable to ingest bioplastic and chitosan-starch blends (Martellini et al., 2023; Ugolini et al., 2024). In this study, we show that ingestion of these materials modifies the gut microbiota composition, with a significant enrichment in bacterial genera associated with plastic and bioplastic degradation. These bacterial taxa seem to assume a specific role in the community, functioning as hubs in the overall community interactions. The changes in the microbiome correspond to the alteration of bioplastics ATR-FTIR spectra in sandhopper's faecal pellets, confirming the modification of bioplastic materials, indicating a role of *T. saltator* gut microbiota in the degradation process. Our findings highlight the role of *T. saltator* microbiota in modifying and potentially recycling starch-based bioplastics in the supralittoral environment. These results open new perspectives for the use of *T. saltator* or its gut microbial consortia in bioplastic recycling, waste processing and management within the supralittoral environment.

## CRediT authorship contribution statement

**Alessandro Russo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Aldo D'Alessandro:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Monica Di Paola:** Writing – review & editing, Writing – original draft, Visualization, Methodology. **Benedetta Cerasuolo:** Writing – review & editing, Methodology. **Sonia Renzi:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Niccolò Meriggi:** Writing – review & editing, Methodology. **Luca Conti:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Jessica Costa:** Writing – review & editing, Writing – original draft, Methodology. **Rebecca Pogni:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology. **Tania Martellini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis. **Alessandra Cincinelli:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis. **Alberto Ugolini:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Duccio Cavalieri:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

## Funding

This work was supported by FishEUTrust project Horizon-CL6-2021-FARM2FORK01 (grant n. 101060712), Regione Toscana (Italy) with the project MICROPLASMED 2022, and Ministero dell'Istruzione, dell'Università e della Ricerca (MUR) to Consorzio Interuniversitario Biotecnologie (D.M. 09 agosto 2021; CIB N. 08-23 to C.B.)

## Declaration of competing interest

The authors declare that they have no conflicting interests that influenced this research.

## Acknowledgements

Thanks are due to the Ente Parco Regionale Migliarino, San Rossore, Massaciuccoli (Pisa, Italy) for authorization to amphipods collection.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179109.

## Data availability

The 16S rRNA raw sequences have been deposited to the European Nucleotide Archive (ENA) under the accession code PRJEB83643 and will be made public simultaneously with the publication of this article. All results from statistical analyses were reported as figures or tables in the main text and supplementary materials. Any other data will be made available on request.

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