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Influence of Dietary Microalgae on *Acartia tonsa* Copepod Microbiome

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Abstract

This study investigates the effect of different microalgae diets on the microbiomes associated with the marine copepod *Acartia tonsa*. Copepods were fed with two different mixed-diet compositions: (i) *Isochrysis galbana* (ISO) and *Rhinomonas reticulata* (RHI)—(ISO + RHI) and (ii) ISO and *Rhodomonas baltica* (RHO)—(ISO + RHO). 16S rDNA metabarcoding and comparative statistic have been adopted to study microbial diversity associated with algae and copepods. Diversity index, taxonomic profiling, and statistically significant taxa differential abundances were evaluated with reference to the different algal and copepod microbiomes. Results showed that the different feeding regimes shape different copepod microbial communities. The abundance of *Vermiphilaceae*, OM190, KI89A_clade, *Cyanobium*_PCC-6307, and Cyclobacteriaceae increased in copepod microbiomes independently by the feeding regimes. On the other hand, *Tistlia* sp., Bradymonadales, and Alteromonadaceae were differentially enriched in copepod microbiomes in relation to the different feeding regimes. Differences in the microbial community composition between ISO + RHI and ISO + RHO were observed, suggesting that the specific algal diet plays a pivotal role in shaping microbiome structure.

Keywords: *Acartia tonsa*; *Isochrysis galbana*; *Rhinomonas reticulata*; *Rhodomonas baltica*; plankton; *Cyanobium*_PCC-6307; *Vermiphilaceae*; *Tistlia* sp.

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1. Introduction

Copepods, small crustaceans belonging to the subclass Copepoda, are the most abundant animals in the ocean [1]. They serve as a critical link between primary producers and higher trophic levels in marine food webs [2,3] and represent a cornerstone of marine ecosystem productivity and function. Copepods may account for over 60% of the biomass of zooplankton in marine ecosystem, underscoring their importance as a primary food source for higher trophic levels [4,5]. By dominating zooplankton biomass, copepods significantly contribute to global biogeochemical cycles, including carbon sequestration via the biological pump and nutrient recycling [6]. Their role extends beyond energy transfer, as

they are crucial vectors for essential compounds like polyunsaturated fatty acids (PUFAs), including omega-3 fatty acids such as DHA and EPA, which are vital for vertebrate health, particularly brain function and cellular integrity [7,8].

Acartia tonsa [9] is a widely distributed calanoid copepod which thrives in subtropical and temperate coastal waters and exhibits remarkable tolerance to salinity fluctuations (5–35 PSU), making it an integral species in estuarine and marine food webs [10,11]. As a primary food source for many fish larvae, *A. tonsa* directly influence fish recruitment and population dynamics [12,13]. Furthermore, its adaptability to laboratory conditions has established *A. tonsa* as a preferred model organism in ecotoxicology, aquaculture, and ecological research [14–20].

Acartia tonsa is a herbivorous species and phytoplanktonic microalgae provide essential nutrients for their growth and development [21]. Recently, the role of microbial communities, particularly bacteria, in health, nutritional metabolism, and immune functions of copepods is increasingly recognized. Microbial symbionts contribute to copepod survival and productivity by aiding in the digestion of food, synthesis of essential nutrients, and protection against pathogens [22–24]. Furthermore, the interactions between microalgae and bacteria influence algal growth and improve nutrient cycling [17,25]. Copepod-microbial interactions, shaped by dietary inputs like microalgae, may influence copepod physiology and functionality [26]. Microalgae–bacteria consortia further modulate these dynamics, as certain bacterial species provide essential vitamins, such as B12, and metabolites, facilitating the growth and survival of both algae and copepods [27–29].

In this study, we conducted 16S rDNA metabarcoding to analyze the microbiome characterizing three monoalgal cultures, *Isochrysis galbana* (ISO), *Rhinomonas reticulata* (RHI), and *Rhodomonas baltica* (RHO), used in two different combinations as food for *A. tonsa* copepod. More precisely, the microbiomes of *A. tonsa* fed two different mixed-algal foods: (i) *I. galbana* and *R. reticulata* (ISO + RHI) and (ii) *I. galbana* and *R. baltica* (ISO + RHO). ISO was used as the baseline diet in both feeding groups due to its high di-gestibility, balanced fatty acid profile, and widespread use in copepod larval culture. [30]. The choice of the dietary regime was selected for the following reasons. ISO ensured that copepods received adequate essential nutrients, particularly EPA and DHA, facilitating meaningful comparisons between treatments [31,32]. RHI and RHO were selected as secondary algae components because they both belong to the Cryptophyceae class but differ in pigment composition, biochemical properties, and potential functional properties. RHI has a unique ultrastructure and hydrocarbon degradation capacity, potentially providing unique bioactive compounds [33]. On the other hand, RHO is rich in phycoerythrin pigments, which stimulate feeding responses in zooplankton while also providing high quality polyunsaturated fatty acids [34]. Diversity indexes (Chao1, Hill–Shannon, Hill–Simpson), beta diversity (PCoA), taxonomic profiling, and statistically significant taxa differential abundances (ANCOM-BC) were evaluated with reference to the different algal and copepod microbiomes. The aim was to compare bacterial community structure and diversity across monoalgal and copepod mixed-diet feeding regimes, in order to evaluate how dietary complexity influences the microbiome of *A. tonsa*.

2. Materials and Methods

2.1. Microalgal Culture

Three species of microalgae, *Isochrysis galbana* [35]., (ISO), *Rhinomonas reticulata* [36] (RHI), and *Rhodomonas baltica* [37] (RHO), were cultured separately in 1 L sterile conical flasks [35–38]. The cultures were aerated to maintain suspension and provided with a 14:10 h light/dark photoperiod, as described in Zhang et al. [21]. The culture medium used was F/2 medium [39], prepared with 0.45 µm filtered seawater (FSW) with a salinity of

30 Practical Salinity Units (PSU), corrected with MilliQ water. The cultures were maintained at a stable temperature of 20 ± 1 °C. For DNA extraction, monoalgal cultures (100 mL) were collected at the concentrations of 8.48 × 10^5, 3.13 × 10^5, and 20.0 × 10^5 cells/mL, respectively, for ISO, RHI, and RHO. Samples were centrifuged at 14,000× *g* for 15 min, and the resulting pellets were transferred into 1.5 mL sterile centrifuge tubes and stored at −80 °C until DNA extraction.

2.2. Copepod Culture

One thousand eggs of *A. tonsa* were collected from the main copepod culture reared in ISPRA. Two groups of 500 eggs each were transferred to a 500 mL glass beaker filled with 0.22 µm FSW at the same salinity (30 PSU) and temperature (20 ± 1 °C) of the main culture. Hatched copepods in each group were then fed as follows: one group with a mixture of ISO + RHI and the other with ISO + RHO. Mixed algae were supplied at 3000 µg Carbon/L for the first three days. On the 4th day, nauplii and egg stages were observed, and the cultures were transferred to 1 L beakers. The feeding concentration was then reduced to 1500 µg Carbon/L supplied every two days. After 11 days, nearly all individuals became copepodites and after 14 days a new generation of nauplii (F1) was observed and the cultures were transferred to 2 L beakers. After almost 80 days, copepod culture (F3) from each feeding group was filtered and 500 individuals > 130 µm size were stored at −80 °C for DNA extraction. For each experimental group, three replicates were considered.

2.3. DNA Extraction and 16S rRNA Sequencing

Almost 500 *A. tonsa* copepods > 130 µm were collected, as described above, for DNA extraction to analyze their associated microbiome, following a modified protocol from Moisander et al. [40], which combines mechanical freezing–thawing cycles with enzymatic lysis. All collected copepods were processed to evaluate variability due to the diet regime on the animals, in cases where the diet itself might be a source of variability in dietary supply, while avoiding unnecessary analysis of variability between individual animals. After removing the samples from the freezer, 360 µL of Lysis Buffer ATL (QIAamp DNA Micro Kit, Qiagen, Germantown, MD, USA) was added to each sample. The samples were subjected to three cycles of freezing at −80 °C for 30 min, followed by thawing at 65 °C for 10 min to ensure effective cell lysis. To assess whether microbiomes were carried by the copepods or the food algae, we also extracted DNA from monoalgal cultures. Approximately 0.02 g of high-concentration centrifuged algae was used per sample for DNA extraction.

The quantity of DNA was measured using a Qubit 3.0 Fluorometer (ThermoFisher Scientific, Milan, Italy). The DNA purity and quality was determined spectrophotometrically (Biotek Powerwave Xs Microplate Spectrophotometer, Milan, Italy) by measuring absorbance at 260/280 and 260/230 nm. The primers used for amplifying the 16S rRNA gene were as follows: the forward primer 515F-GTGCCAGCMGCCGCGGTAA and the reverse primer 907R-CCGTCAATTCCTTTGAGTTT, amplifying 374 bp the fourth and fifth hypervariable (V4–V5) region of the 16S rRNA gene [41]. The sequencing library was prepared by Novogene company (Hong Kong). Illumina sequencing was performed using the Illumina NovaSeq 6000 platform, amplifying 250 bp forward and reverse of the V4-V5 374 bp amplified region. The raw sequencing data have been submitted to the NCBI database under the accession number PRJNA1288420.

2.4. Bioinformatics Analyses

The 250 bp paired-end reads generated from sequencing the 16S libraries of different metagenomes were first demultiplexed based on unique barcodes assigned to each sample by Novogene. These barcodes were then removed using Cutadapt v4.6 [42]. The resulting

reads were then clustered into Amplicon Sequence Variants (ASVs) using the DADA2 plugin v1.26 with QIIME2 v2023.2 [43–45]. The forward and reverse reads were merged, followed by quality filtering (requiring a minimum Phred-scaled quality score of 10), and the removal of potential chimeric sequences. Reference data from SILVA 138.2 (99% OTUs, 16S rRNA) (https://www.arb-silva.de/ accessed on 1 September 2025), targeting the relevant hypervariable region, were processed and curated using the QIIME 2 RESCRIPt plugin v2023.2.0 [46] to build a region-specific Naive Bayes classifier, which was then used to assign taxonomy to the DADA2-derived ASVs. Chloroplast-derived 16S rRNA gene sequences—here barely co-amplified by bacterial 16S primers—were removed during the quality control filtering step as they do not represent bacterial community members.

The ASV abundance data obtained through the QIIME2 pipeline were normalized using the coverage-based method (0.9950 ± 0.0025), implemented with iNEXT v3.0.2 (https://besjournals.onlinelibrary.wiley.com/doi/10.1111/2041-210X.12613 accessed on 1 September 2025). Alpha diversity was evaluated as the Hill–Simpson, Hill–Shannon diversity, and the Chao1 richness estimator, using the metagMisc_R package v0.5.0 [47–49]. Statistical significance of diversity indices was assessed using non-parametric measures ANOVA, followed by post hoc Dunn tests, implemented with the nparLD v2.2 and rstatix v0.7.2 packages [50]. Furthermore, rarefaction curves for observed species were constructed based on the coverage threshold. Beta diversity was estimated through Principal Coordinate Analysis (PCoA) using weighted UniFrac distances, conducted with the Phyloseq v1.41.1 and Vegan v2.6–4 packages [51,52].

In the taxonomic heatmap, the color scale represents the Z-score values of each taxon across the samples. The Z-score is calculated as the difference between the relative abundance of a taxon in a given sample and its mean relative abundance across all samples, divided by the standard deviation. Positive Z-scores indicate that a taxon is more abundant in that sample compared to its overall average, while negative Z-scores indicate that its abundance is lower than the mean. This standardization highlights relative differences in abundance patterns, allowing the identification of taxa that are enriched or depleted in specific samples or groups, regardless of their absolute abundance.

The core microbiome was defined as taxa with a relative abundance greater than 0.1% in each sample and present in at least 50% of all samples. More specifically, a taxon was considered part of the core microbiome if it was detected in at least two of the three sequencing replicates per sample, using the microbiome package v1.24.0 [45].

3. Results

3.1. Bacterial Community Diversity

In relation to the16S rDNA metabarcoding analysis, a total of 963,710 raw reads were processed. These latter corresponded to a medium value of 64,247 total reads per microbiomes (ISO 63,708; RHI 63,434; RHO 63,577; ISORHI 65,911; ISORHO 64,605). The filtering processes returned a total of 893,966 reads grouped in the following medium value per microbiome: ISO 60,004; RHI 59,204; RHO 59,751; ISORHI 61,667; ISORHO 57,361. The merging returned a total of 770,121 reads grouped in the following medium value per microbiome: ISO 57,503; RHI 54,910; RHO 56,310; ISORHI 53,468; ISORHO 34,514. The chimera removal step returned a total of 713,119 ASVs, grouped in the following medium value per microbiome: ISO 48,491; RHI 53811; RHO 54,950; ISORHI 48,462; ISORHO 31,991. These latter reads corresponded to 3,085 unique ASVs which will hereinafter be referred to as taxa (see Supplementary Information: ASV table, Taxonomy table, ASV sequences).

The core microbiome analysis (Figure 1) showed that only 64 ASVs were shared among the five microbiomes, indicating that most of the bacteria were specifically distributed in the different groups. The copepods fed with mixed algae ISO + RHI (1136 ASVs) and

ISO + RHO (1219 ASVs) had the largest number of ASVs, with 819 and 857 unique ASVs, respectively. In contrast, ISO microalgal culture exhibited the lowest bacterial richness, the total number of ASVs (347) and unique ASVs (222).

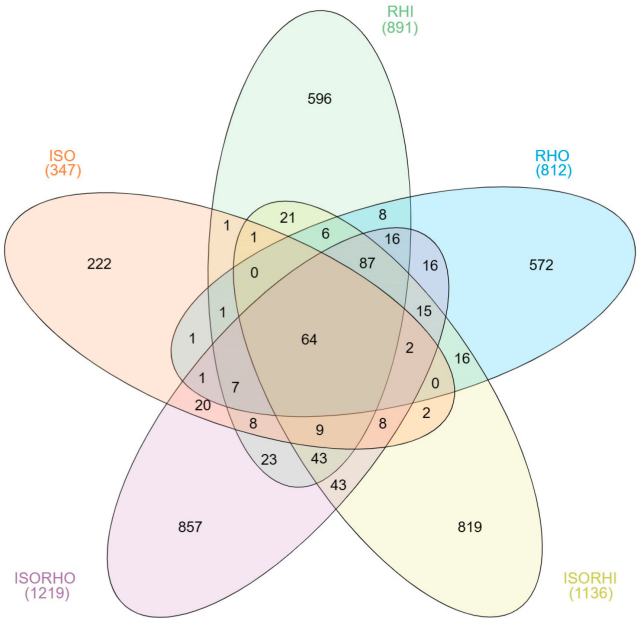

Figure 1. Venn diagrams show the unique and shared ASVs among the three microalgae and the two copepod core microbiomes.

Alpha diversity analysis showed that there were no significant differences between the different analyzed microbiomes in terms of richness, biodiversity, and evenness, with the sole exception of the ISO microbiome that showed a lower richness with reference to the copepod group fed with the combination ISO + RHI (ISORHI) and ISO + RHO (ISORHO) (Figure 2A). Although any statically significant difference has been observed, a trend can be described. A tendency towards higher bacterial richness was observed in the two copepod culture groups. The Hill–Shannon index results showed that the diversity of the ISO microbiome was the lowest, followed by the RHO one (Figure 2B). The diversity indices of RHI, ISORHI, and ISORHO microbiomes were relatively high, with similar median values, even though the standard deviation of the ISORHO microbiome was relatively large. The trend of the Hill–Simpson index (Figure 2C) was similar to the Hill–Shannon one. The microbiome diversity of the microalgae ISO and RHO alone was relatively low compared with RHI.

Rarefaction curves assessed the adequacy of sequencing depth and confirmed the tendency towards higher bacterial richness of the microbiome associated with copepods fed two different algal regimes (Figure 2D).

Beta analysis revealed distinct bacterial community compositions across the five microbiomes (Figure 3). In particular, the microbiome associated with ISO was the most distinct and had the largest separation from all other groups. The microbiomes of RHI and RHO were closely clustered, indicating similar bacterial compositions. In addition, the copepod groups fed on mixed algae (ISORHI and ISORHO) showed similar microbiome compositions among each other but distinct from the one associated with the three monoalgal cultures.

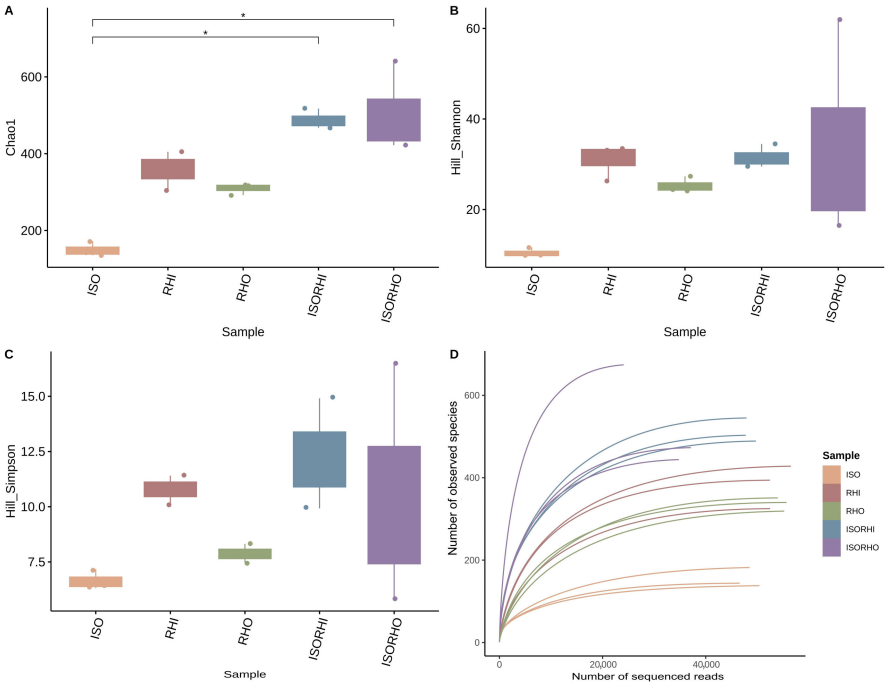

Figure 2. Chao1 index (**A**), Hill–Shannon (**B**), Hill–Simpson index (**C**), each calculated after rarefaction to a coverage of 99.5%, and rarefaction curves of observed species of bacterial community composition (**D**). Rarefaction curves display the relationship between sequencing depth and the number of observed species, allowing the assessment of sampling completeness and comparison of species richness among groups. Box and whiskers represent the minimum (Q0), 1st quartile (Q1), median (Q2), 3rd quartile (Q3), and maximum (Q4) of each group. Reported *p*-value is calculated by the Kruskal–Wallis test ($\alpha = 0.05$). Post hoc statistical test is based on the Dunn test with Benjamini–Hochberg correction for multiple comparisons. (*) Significant difference between the groups $p < 0.05$.

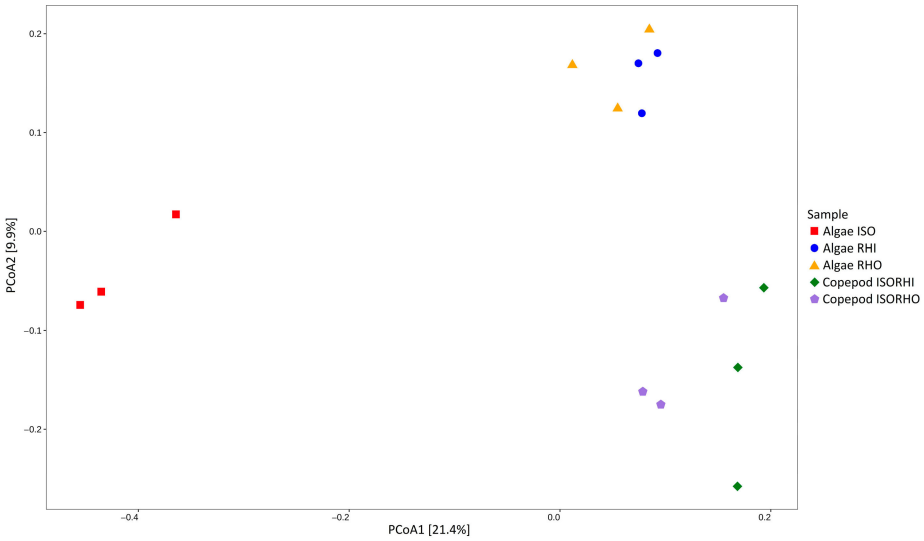

Figure 3. Principal component analysis (PCoA) represents microalgae and copepod microbiomes, based on weighted UniFrac distance for each sample replicate, without taxonomic filtering or aggregation. Colors and shape indicate time category for each datapoint. The percentage reported on axes represents the amount of total variance depicted by each.

3.2. Microbiome Taxonomic Composition

The heatmap of the 40 most abundant bacterial species showed differences in the microbial community structure of the five groups (Figure 4). Among the most represented taxa across all the microbiomes, the genera *Marivita* (14.26%), *Oceanicaulis* (7.83%), and

Muricauda (3.01%) showed higher abundance in the monoalgal ISO group with reference to the other groups. On the other hand, the dominant genus *Cyanobium*_PCC-6307 (13.45%) was significantly represented at high relative abundance in the ISO + RHI copepod microbiome, even though not highly represented in any of the monoalgal microbiome. In addition, genus *Phaeodactylibacter* (4.27%) and SMA102 (2.96%) showed higher abundance in the monoalgal ISORHI group with reference to the other. In the ISO + RHI copepod, the most represented bacterial genera were not dominant either in the three monoalgal microbiomes and in the ISO + RHO copepod microbiome. The genera *Jannaschia* (9.37%) and *Vibrio* (2.49%) showed higher abundance in the microbiome of copepod ISORHO. The genera *Winogradskyella*, *Tropicimonas*, and *Arenibacter* showed the highest relative abundance in the RHI microbiome. The genera *Gilvibacter* had the highest bacterial abundance in the RHO microbiome. However, these bacterial genera of monoalgal RHI and RHO were not dominant across all the microbiomes; the proportion ranges from 0.26% to 2.48%.

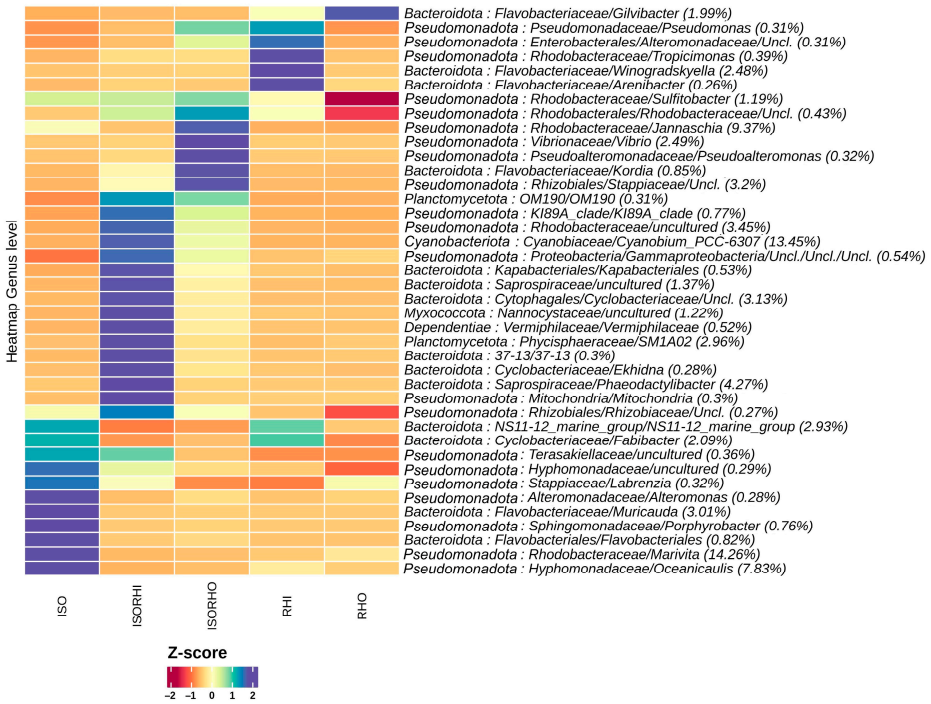

Figure 4. The heatmap of microalgae and copepod microbiomes showing the relative abundance of the 40 most abundant bacterial species in the different microbiomes at the genus level. Hierarchical clustering was performed on columns by Pearson correlation, based on Euclidean distance. The color scheme represents row-wise Z-scores of ASV counts. The percentage reported near ASV names represent the relative abundance of the sum of ASV counts per sample against total sum (i.e., Z = 0 matches reported percentage).

To better analyze possible differences in copepod microbiomes potentially related to dietary regime, an analysis of compositions of microbiomes with bias correction (ANCOM-BC) was performed to identify significant differences in abundance at the genus and family levels in the different microbiomes (Figure 5).

Results revealed that there were differences in bacterial abundance and adaptability between monoalgal culture and mixed-algal feeding copepod groups. In the comparison between ISO, RHI, and ISORHI (Figure 5A), the families *Vermiphilaceae*, *Cyclobacteriaceae*, genera *Tistlia*, *Cyanobium*_PCC-6307, OM190, KI89A_clade, the uncultured 51, uncultured 22, uncultured 20, and uncultured 2 were more abundant in the monoalgal RHI microbiome than in the ISO one. At the same time, the above bacteria abundance increased in the copepod ISORHI microbiome with respect to the monoalgal ISO and to RHI cultures.

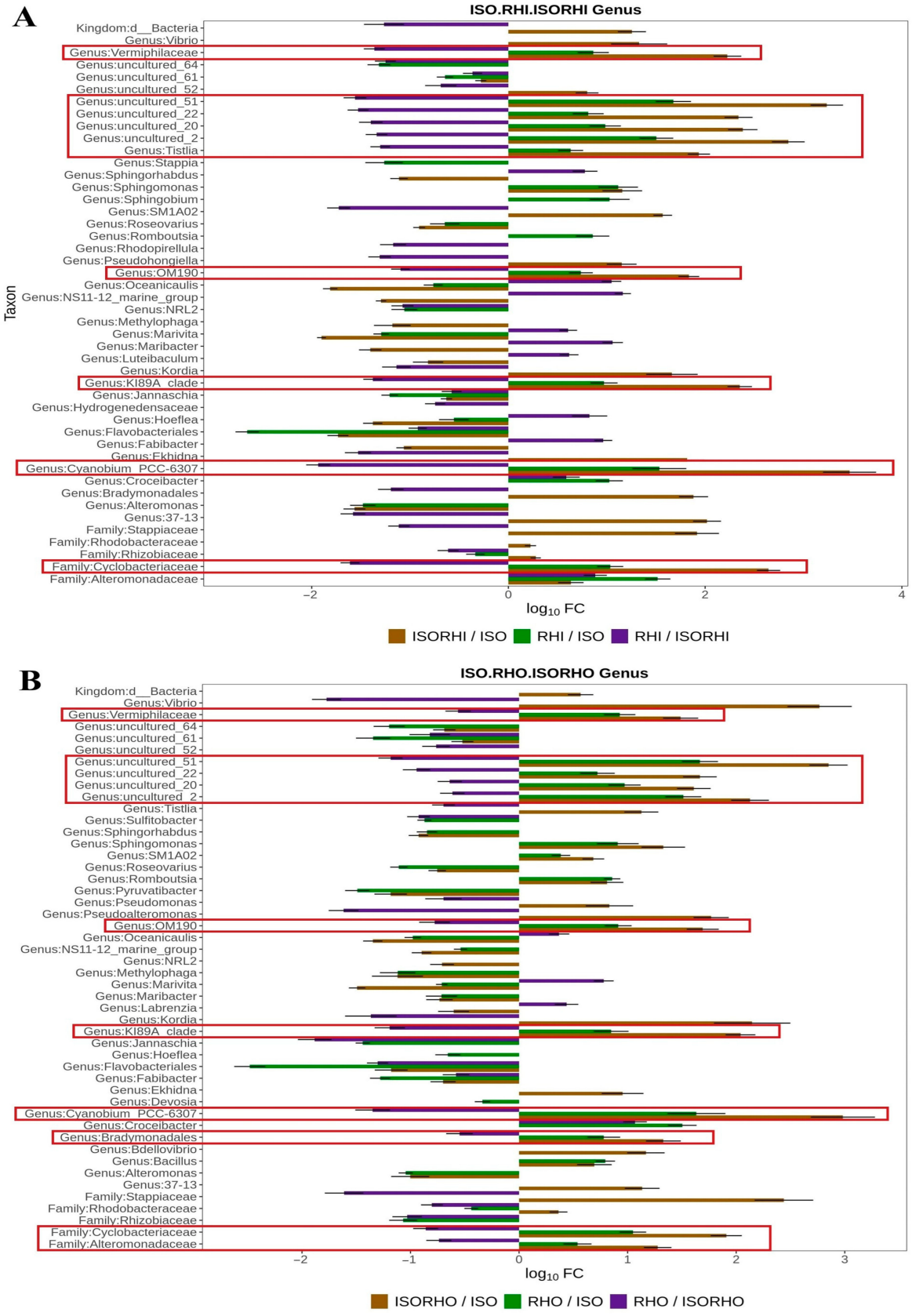

Figure 5. Log_{10} fold change (log_{10}FC) in the relative abundance of associated bacterial taxa comparing monoalgal cultures and copepods fed mixed-algal diets. (**A**) Comparisons among monoalgal ISO and RHI cultures and copepods fed the mixed-ISO + RHI diet. (**B**) Comparisons among monoalgal ISO and RHO cultures and copepods fed the mixed-ISO + RHO diet. Bars show taxa identified by ANCOM-BC2 as exhibiting a statistically significant increase or decrease in contribution in at least

one pairwise comparison; multiple-comparison correction is applied internally by the ANCOM-BC2 procedure. Positive $\log_{10}$FC values indicate higher relative abundance in copepods fed mixed algae versus monoalgal cultures, negative values indicate lower relative abundance. The red rectangles refer to the taxa that increase significantly in relative abundances in the copepod with reference to the algae providing dietary supply.

In comparison between ISO, RHO, and ISORHO microbiomes (Figure 5B), the bacterial order Bradymonadales, families *Vermiphilaceae, Cyclobacteriaceae*, and *Alteromonadaceae*, genera *Cyanobium*_PCC-6307, OM190, KI89A_clade, and the uncultured 51, uncultured 22, uncultured 20, uncultured 2 were more abundant in RHO microbiome when compared to the ISO one. At the same time, the above-mentioned bacteria were more abundant in the copepod ISORHO microbiomes with reference to the ISO and RHO microbiomes.

Results obtained showed that the bacterial taxa that increase in representativeness in the copepod microbiomes were more abundant in monoalgal RHI and RHO microbiomes with reference to the ISO one. Bacterial taxa increased significantly in the monoalgal RHI and RHO microbiomes and in the copepod microbiomes. Table 1 reports the similarities and the differences in the contribution of the different diets to the microbiomes of the copepods.

Table 1. Bacterial species that show a higher representativeness in both RHI and RHO monoalgal microbiomes with reference to ISO, increasing in representativeness also in the copepod microbiomes.

Bacterial Taxa	ISORHI	ISORHO
Vermiphilaceae	+	+
OM190	+	+
KI89A_clade	+	+
*Cyanobium*_PCC-6307	+	+
(NIatGL) Uncultured sp. 51	+	+
(NIatGL) Uncultured sp. 22	+	+
(NIatGL) Uncultured sp. 20	+	+
(NIatGL) Uncultured sp. 2	+	+
Cyclobacteriaceae	+	+
Bradymonadales		+
Alteromonadaceae		+
Tistlia sp.	+	

Notes: + indicates an increase in contribution to the copepods microbiomes with reference to bacterial taxa. NIatGL, not identified at genus level.

4. Discussion

Bacterial communities are crucial for maintaining copepod growth and well-being as they play key roles in pathogen resistance, immune regulation, and nutrient cycling [25,53]. The use of various microalgae diets to culture copepods and improve their nutritional intake enhances larval performance, e.g., monoalgal diets can negatively affect reproductive output and naupliar survival [21,30,54]. However, research on how differences in diets and changes in copepod-associated microbiota affect copepod development remains limited. Moreover, detailed studies of the changes in the copepod microbial ecology in response to different algal feeding regimes, in terms of taxonomy, are also still missing.

In this study, we compared the bacterial composition in monoalgal microbiomes and copepod cultures fed with different mixed algae. This comparison aimed to investigate the effect of dietary diversity in shaping the bacterial communities associated with the copepod *A. tonsa*.

We observed a unique bacterial community in the ISO microalgal culture, with the lowest richness and diversity among the different microbiomes observed. In contrast, the RHI and RHO monoalgal cultures were associated with a higher richness that increased significantly if the copepods were fed with the two different algal mixtures. Both the core microbiome analysis and the taxonomic profile of the most represented bacterial species in the five analyzed microbiomes evidenced significant differences in the microbial composition of the monoalgal culture and the two copepod microbiomes. These differences were associated with a particular clusterization of beta diversity, where the ecology of the monocultural ISO algae resulted to be distant from the one of the RHI and RHO. On the other hand, these latter were similar but distinct from the one of the copepods fed under the two diet regimes. In fact, the copepod microbiomes were clustering separately but showed more reciprocal similarity with respect to the other analyzed microbiomes. Copepods fed with mixed-algal diets exhibited higher bacterial richness and diversity of all the groups observed, suggesting the importance of dissecting the effect of the complexity of the diet in shaping the copepod microbiome. In this direction, our results showed that in copepod microbiomes of the ISORHI group, *Cyanobium*_PCC-6307, *Phaeodactylibacter*, and SMA102 were predominant. On the other hand, in ISORHO group, *Jannaschia* and *Vibrio* were more prevalent. Some of these highly enriched bacteria are also considered to be beneficial bacteria for aquatic organisms. *Cyanobium*_PCC-6307 acts as a probiotic by enhancing the photosynthetic efficiency of Symbiodiniaceae under high-temperature conditions, thereby supporting coral health and resilience to environmental stress [55]. *Phaeodactylibacter* contributes to improving water quality in crab larviculture by participating in nitrogen removal through heterotrophic nitrification and denitrification, highlighting its beneficial role in recirculating aquaculture systems [56]. SMA102 is a potentially beneficial symbiotic bacterium that promotes the recovery and stability of the core functional bacterium *Candidatus brocadia* in the reactor, thereby supporting the restoration of nitrogen removal performance [57]. *Vibrio* sp. 01, isolated from copepod carcasses, possesses ester bond hydrolysis capabilities and has the potential to participate in the degradation process of bioplastic PBSA [58]. However, *Jannaschia* showed to induce a low feeding and assimilation rate in copepods [59].

An accurate quantitative assessment of differential abundance in microbiome data by correcting for compositional bias is a crucial approach and tool for identifying truly significant microbial changes across the different microbiomes. Results obtained showed that the most significant effect of the diet on the copepod microbiome derived from the monoalgal RHI and RHO. In fact, several bacterial taxa shared between the three algae resulted to be more abundant in the RHI and RHO microbiomes when compared to the ISO microbiome. Interestingly the same taxa showed a significant increment in representativeness also in the copepod's microbiome with reference to the algal microbiomes as well. The bacterial taxa *Vermiphilaceae*, OM190, KI89A_clade, *Cyanobium*_PCC-6307, and *Cyclobacteriaceae* are consistently favored under both feeding regimes, as evidenced by their increased relative abundance in both ISORHI and ISORHO copepod microbiomes. These bacterial taxa may represent microbial groups particularly responsive to the presence of copepods or to trophic interactions associated with mixed-algal environments. Despite this overlap, distinct differences in the microbial community composition between ISORHI and ISORHO microbiomes were observed, suggesting that the specific algal diet influences the copepod microbiome structure.

Notably, the relative abundance of *Tistlia* sp. increased in the ISORHI microbiome, and Bradymonadales and Alteromonadaceae in the ISORHO one. As a beneficial microbe contributing to nitrogen fixation, the genus *Tistlia* holds ecological potential in saline environments [60]. Díaz-Cárdenas et al. [61] described *Tistlia consotensis* as a novel aero-

bic, nitrogen-fixing alphaproteobacterium with broad metabolic versatility and moderate halotolerance, suggesting its adaptability to saline and marine-like environments. These physiological traits indicate that *Tistlia* may play a beneficial role in nitrogen cycling and organic matter turnover within coastal or mild saline ecosystems. Gong et al. [62] found that Bradymonabacteria strains, under the order Bradymonadales, showed efficient predation ability under low-proportion mixed-culture conditions and were able to invade and eliminate adjacent prey colonies, indicating that they have strong ecological competitiveness and environmental adaptability. In combination with their broad-spectrum predation on a variety of prey, Bradymonabacteria shows potential in controlling drug-resistant pathogens and can be used as a new, environmentally friendly probiotic candidate in aquaculture. Moreover, Mu et al. [63] highlighted the ecological importance of Bradymonadales in saline environments, noting their widespread distribution and significant abundance in marine and salt-lake sediments. The distinct metabolic characteristics of Bradymonabacteria, including biosynthetic deficiencies and a strong starvation response, suggest a transitional strategy between obligate and facultative predation, with potential roles in both microbial community regulation and nutrient cycling. Smahajcsik et al. [64] demonstrated that members of the family Alteromonadaceae play a central role in the inhibition of aquaculture pathogens within complex algal-associated microbiomes. They found that Alteromonadaceae were consistently enriched in the most effective anti-*Vibrio anguillarum* communities derived from *I. galbana*, suggesting a direct or synergistic contribution to pathogen suppression. Previous studies have also reported that Alteromonadaceae are common members of the core microbiome of microalgae and copepods [65–68], highlighting their ecological relevance and potential as probiotic candidates in aquaculture.

5. Conclusions

In conclusion, this study demonstrates that bacterial taxa abundant in monoalgal cultures, particularly RHI and RHO, were also consistently abundant in the corresponding copepod microbiomes, suggesting possible microbial direct transfer from diet to host. The bacterial taxa *Vermiphilaceae*, OM190, KI89A_clade, and *Cyanobium*_PCC-6307 showed high relative abundance both in microalgae and copepods, suggesting their adaptability and potential ecological roles in nutrient cycling or algal degradation within the copepods. Differentially enriched bacteria in copepods fed with mixed-algal diets, such as Bradymonadales and Alteromonadaceae, were enriched specifically in the ISORHO microbiome, while *Tistlia* sp. exhibited an increased relative abundance exclusively in the ISORHI microbiome. These bacterial taxa have been described to enhance nitrogen fixation, organic matter cycling, and pathogen suppression, and might be useful in designing mixed diets according to specific ecological functional requirements. The results highlight the influence of dietary composition on copepod microbiome structure. Since copepods are hot spot of microbiome diversity in the sea, the putative functions of bacteria retained or enriched in copepods under the two different diet regimes interestingly play key roles in functions such as digestion, reproduction, and in antibiotic and marine biogeochemical cycles. Thus, diet-dependent microbial selection can be used to optimize microbiome composition in aquaculture applications. Since the copepod microbiome—composed of both the gut and external colonizing microbial communities as a whole—plays a role in providing beneficial microorganisms in aquaculture, our results are consistent with this assessment, and future dedicated experiments might provide new scenarios for cultivating beneficial microbiomes in organisms at higher trophic levels.

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Abbreviations

The following abbreviations are used in this manuscript:

ISO	*Isochrysis galbana*
RHI	*Rhinomonas reticulata*
RHO	*Rhodomonas baltica*
PCoA	Principal Component Analysis
ANCOM-BC	An analysis of compositions of microbiomes with bias correction
FSW	Filtered seawater
DNA	Deoxyribonucleic Acid
ASVs	Amplicon Sequence Variants
log_{10}FC	Log_{10} fold change

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