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Modulation of Gut Microbiota and Enhancement of Growth in Fish Fed with *Stypopodium schimperi*-Enriched Diet

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Abstract

Algae have attracted increasing interest as feed additives in fish farming. In this study, *Stypopodium schimperi*, which was determined to have 81.39±0.88% ROS effect (X±SD) by DPPH test among representatives of three macroalgae classes, was used as a fish feed supplement. The algal extract showed 9-11 mm inhibition zones against tested pathogenic strains in disk diffusion experiments. In broth dilution tests, the highest antimicrobial efficacy was observed at a 1/16 dilution against *E. coli* ATCC 25922. The effects of the algae, incorporated into fish feed at a 5% concentration, on gut microbiota were assessed using 16S rRNA amplicon sequencing. Alpha diversity among groups was determined by Shannon and Simpson indices, while beta diversity was obtained by Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance matrices. The PCoA1 (81.13%) and PCoA2 (18.85%) axis separates group A (experimental group) from CI (day 0 control group) and CF (day 21 control group). This indicates that the microbial community structure of sample A is fundamentally different from the other two, and the microbial profile between CI and CF is relatively close. Notably, in the Group A, *Pseudomonadaceae* (75.31%) and *Moraxellaceae* (21.57%) were the most abundant bacterial families, whereas in the CF group, *Rhodobacteraceae* (36.34%), *Enterobacteriaceae* (11.66%), and *Pseudomonadaceae* (6.78%) predominated. The CI group, was primarily characterized by *Enterobacteriaceae* (35.08%), *Rhodobacteraceae* (26.55%), and *Pseudomonadaceae* (4.8%). While algae supplementation inhibited various pathogens, a significant increase in the genus *Pseudomonas* was observed. Overall, the addition of *S. schimperi* to fish feed induced notable changes in gut microbiota composition and led to a statistically significant improvement in the fish's Specific Growth Rate.

Introduction

Aquatic ecosystems cover approximately 70% of the Earth's surface and provide habitats for a wide range of organisms (Ebada and Proksch, 2011). Among these organisms, algae play a crucial ecological and economic role in both freshwater and marine environments. Algae are generally categorized into two groups: microalgae and macroalgae. Macroalgae, comprising around 9,800 species, are classified into three main group—Rhodophyta (red algae), Phaeophyceae (brown algae), and Chlorophyta (green algae)—based on their pigment composition, cell wall polysaccharides, and storage products (Bocanegra et al., 2009; Barsanti and Gualtieri, 2022).

Algae synthesize a variety of secondary metabolites as part of their natural defense mechanisms, and the composition of these compounds varies according to their taxonomic classification. These bioactive metabolites have been shown to exhibit a range of biological activities, including antimicrobial, antifouling, antifeedant, and cytotoxic effects (Li et al.,

2009; Pereira et al., 2021;). Owing to these properties, many algal-derived compounds have been utilized in pharmaceuticals, nutraceuticals, and the food industry (Adarshan et al., 2023). Furthermore, algae are widely consumed as food in several cultures, especially in East Asia. As a foundational component of aquatic food webs, algae are also a valuable nutritional resource for aquaculture species, offering compounds with functional bioactivity that may enhance animal health and performance (Cezero et al., 2021; Siddik et al., 2023). In this context, the use of both macro- and microalgae as dietary supplements in aquaculture has garnered increasing attention in recent years (Souza et al., 2020).

One of the major challenges in fish aquaculture is the occurrence of bacterial infections, which can lead to significant fish mortality. Although antibiotics are commonly used to control bacterial diseases in fish farms concerns over antimicrobial resistance and environmental impact have prompted the search for natural alternatives (Okocha et al., 2018). Among these, macroalgae have emerged as promising candidates due to their content of sulfated polysaccharides such as alginate, fucoidan, carrageenan, laminarin, ulvan, and galactan, which are known to stimulate immune responses. For instance, *Gracilariopsis persica* has been reported to enhance both growth and immune function in aquaculture species (Abbaspour et al., 2015). Similarly, the green alga *Ulva intestinalis* has demonstrated antimicrobial, immunomodulatory, and growth-promoting properties (Safavi et al., 2019). Studies on the red alga *Gracilaria gracilis* in zebrafish have shown increased expression of key immune-related genes such as *interleukin-1β*, *lysozyme (LYZ)*, and *tumor necrosis factor-alpha* (Hoseinifar et al., 2018).

More recently, the role of probiotics and gut microbiota in promoting fish health has gained significant research interest. The gut microbiome plays a fundamental role in regulating host immunity, nutrient absorption, vitamin synthesis, and protection against opportunistic pathogens. Metagenomic analyses, particularly those based on 16S rRNA amplicon sequencing analysis, have been employed to identify microbial communities and natural probiotics involved in these processes. Notably, the interactions between dietary algae and gut microbiota have become a topic of active investigation (Tapia-Paniagua et al., 2019; Siddik et al., 2023). For example, a study involving *Schizochytrium sp.*—a microalga—demonstrated that its inclusion in Nile tilapia fish diets led to notable shifts in gut microbial composition and hematological parameters (Souza et al., 2020). However, the effects of macroalgae-derived dietary supplements on fish gut microbiota, immune function, and metabolism remain incompletely understood.

The present study aims to evaluate the antimicrobial potential of *Stypopodium schimperi* and to investigate its effects on gut microbiota composition and growth performance in zebrafish when used as a functional feed additive. To reach this aim, a macroalga specimen exhibiting high reactive oxygen species (ROS) activity were selected from three different taxonomic groups and incorporated into fish feed at a 5% concentration. The formulated diets were administered to the model organism *Danio rerio* (zebrafish), and the resulting changes in gut microbiota composition were analyzed using 16S rRNA gene-based metagenomic sequencing.

Material and Methods

Sample Collection and Preparation

Macroalgal samples were collected by SCUBA diving at a depth of 10 meters from two locations: Kaş (Antalya) and Gökova Bay (Muğla). Following collection, the algae material were thoroughly cleaned to remove epiphytes, rinsed with sterile seawater, and air-dried at room temperature for 2–3 days. Dried samples were then stored in airtight ziplock bags at room temperature until further analysis.

A total of 10 grams of the each dried algal sample was powdered using a mortar and pestle. The powdered material was mixed with 100 mL of distilled water and incubated in a water bath at 100°C for 1 hour. After cooling to room temperature, the resulting extract was filtered through Whatman No. 5 filter paper. The extracts were stored at +4°C until Disc diffusion and MIC analysis.

Determination of ROS Scavenging and Antimicrobial Activity of Algae Extract

ROS scavenging activity of the macroalgae was evaluated using a modified method of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, as described by Sharma and Bhat (2009). Macroalgae samples were extracted in methanol to obtain a final concentration of 1 mg/mL. For the assay, 525 µL of the algal extract was mixed with 175 µL of DPPH solution in a cuvette and incubated in the dark at room temperature for 30 minutes. Following incubation, absorbance was measured at 520 nm using a UV-Vis spectrophotometer. Trolox was used as the positive control. Trolox equivalent antioxidant capacity (TEAC) was taken as reference to evaluate the results of DPPH radical scavenging capacity of algae extracts. The percentage of DPPH radical inhibition was calculated using the following formula, where Ac is the absorbance value of the control and As is the absorbance value of the sample;

$$\text{Inhibition } (\%) = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \times 100$$

The antimicrobial activity of algae extracts were assessed using the disc diffusion method, originally described by Bauer et al. (1966), which is widely used for

evaluating the susceptibility of rapidly growing microorganisms. In this study, Gram-positive bacteria (*Bacillus subtilis* NRLL-B 209, *Bacillus cereus* ATCC 7064, *Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Proteus vulgaris* NRRL B-123), and the fungus *Aspergillus parasiticus* NRRL 1957 were tested. Test suspensions were prepared in Müller-Hinton broth from cultures grown in appropriate media, and their turbidity was adjusted to 0.5 McFarland standard. A volume of 200 µL of each suspension was spread onto agar plates using the spread plate technique. Sterile paper discs were placed onto the inoculated plates, and 25 µL of the algal extract was applied to each disc. The algal extract was prepared as described previously, and the dried extract was dissolved in 5 mL of DMSO (dimethyl sulfoxide). DMSO served as the negative control, while standard antibiotic discs (Nx: Neomycin, AMP: Ampicillin, C: Carbapenem) were used as positive controls. Plates were incubated at 37°C for 24 hours for bacteria and 48 hours for fungi. After incubation, the diameter of the inhibition zones around the discs was measured using a digital caliper. All experiments were performed in triplicate, and results were expressed as mean inhibition zone diameters.

MIC of the algal extract was determined using the broth microdilution method in 96-well microplates, following established protocols (NCLL, 2007; Wiegand et al., 2008). Bacterial and fungal suspensions were prepared in Müller-Hinton broth and adjusted to 0.5 McFarland turbidity from freshly incubated cultures. The algal extract was serially diluted in the wells by adding 100 µL of the stock solution. Each well was inoculated with the test organism, and the plates were incubated at 37°C for 24–48 hours, depending on the microorganism (Sarker et al., 2007). Following incubation, microbial growth was visually evaluated, and the lowest concentration showing no visible growth was recorded as the MIC. To determine the MBC, 5 µL samples were taken from wells without visible growth and spot-inoculated onto Müller-Hinton agar plates. After incubation at 37°C for 24–48 hours, the lowest concentration that resulted in no visible colony formation (indicating 99.9% killing of the test organisms) was identified as the MBC.

Preparation of Fish Feed and Feeding Trial

After ROS, disc diffusion and MIC analysis one algae (*S. schimperi*) was selected out of three, with its promising antimicrobial and antioxidant activity as a study material. Dried *S. schimperi* thallus was ground into small pieces using a mixer and incorporated into standard fish feed at a concentration of 5% (w/w) with the addition of purified water to facilitate homogenization. The resulting paste was oven-dried at 40°C for 24 hours, re-pulverized, and stored at +4°C until use.

Zebrafish were acclimated for 10 days with a standard commercial diet upon arrival at the laboratory. Following acclimatization, fish were randomly divided into two experimental groups: a control group receiving standard feed and a treatment group receiving feed supplemented with 5% dried macroalga. Each group consisted of 25 fish housed in separate aquaria. Initial weight and total length measurements of the fish were recorded at the start of the experiment. Fish were fed 0.5–1 g of feed twice daily (morning and evening) over a period of 21 days. At the end of the feeding trial, fish were re-measured for weight and length, and growth performance indices such as specific growth rate (SGR) and length increment were calculated. While the average weight of the control group was 0.4 g, the average weight of the experimental group was 0.3 g.

Weight gain (g)= Final weight (g) – Initial weight (g)

$$SGR\ (\%) = [LnFinal\ weight - \text{İ}nitial\ weight]/days\ X\ 100$$

Viscera weights were also recorded to the nearest mg and VSI determined using the formula:

$$VSI\ (\%) = 100\ x\ [viscera\ weight\ (g)/whole\ fish\ weight\ (g)]$$

The environmental factor (CF), a metric that indicates the fish's physical condition, nutritional level, and adaptation to environmental conditions, was calculated using the following formula: W: Fish weight (g), L: Fish total length (cm).

$$CF= (W/L^3) \times 100$$

At the beginning of the experiment, normality was tested between the experimental and control groups using the Shapiro-Wilk-W test. Since the distribution did not differ significantly from the normal distribution, an independent samples t-test was applied between the two groups ($P>0.05$).The statistical analyzes were evaluated as mean±standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA). The critical value for statistical significance was $P<0.05$.

Determination of Fish Intestinal Microbiota by Microbial Community Analysis

Genomic DNA was extracted from fish intestinal samples obtained via surgical incision using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Cat. No: D6010, Zymo Research). The quantity and purity of the extracted DNA were assessed fluorometrically using a Qubit fluorometer. The 16S rRNA gene contains nine hypervariable regions (V1–V9), of which the V3–V4 regions are commonly used for profiling intestinal microbiota at the species level. In this study, the V3–V4 regions were amplified using the primer pair 341F–805R on a SimpliAmp™ Thermal Cycler (Almeida et al., 2019; Cerezo et al., 2022).

The resulting amplicons were purified using the Column-Pure PCR Clean-Up Kit (Cat. No: D509, ABMGood). Library preparation was carried out using Illumina's Nextera XT DNA Library Prep Kit (Cat. No: FC-131-1096), and indexing was performed with the TG Nextera XT Index Kit v2 Set A (Cat. No: TG-131-2001). Sequencing was conducted on an Illumina Iseq100 platform using paired-end (2×150 bp) chemistry. FastQ files obtained from Illumina Iseq100 were obtained as demultiplexed raw reads for each sample. Illumina Iseq100 reads were analyzed with FastaQC software (Andrews et al., 2010) to assess sequence quality. They were then subjected to primer sequence trimming using Trimmomatic v0.32 (Bolger et al, 2014). Demultiplexed and raw reads were filtered from low-quality data using CLC Genomics Workbench (Qiagen, USA). They were then classified into "Operational Taxonomic Units, OTU" classes using the Kraken2 Metagenomics system, which assigns taxonomic labels to DNA sequences with high accuracy and speed (Wood and Salzberg, 2014). Alterations in microbiota composition associated with different dietary interventions were assessed by analyzing the relative abundance and alpha diversity of bacterial taxa, as measured by the Shannon and Simpson indices, at both the family and genus levels. The species richness curve was performed using OmicsBox software (BioBam Bioinformatics, ES), while the Principal Coordinate Analysis (PCoA) based on Bray–Curtis distance was performed using PAST software version 4.03 (Hammer et al., 2001). The analysis was conducted based on a correlation matrix to eliminate the influence of scale differences between variables. Heat maps and hierarchical cluster analysis (HCA) were applied to visually assess similarities between samples and variables. Nearest Neighbor was used as the clustering method, and Pearson correlation was used as the distance metric. Analyses were performed using OriginPro 2024.

Results

Sample Collection and Identification

Collected samples were morphologically identified upon arrival at the laboratory. Three species belonged to the phylum Heterokontophyta, two species were from Rhodophyta, and one species was from Chlorophyta. *S. schimperi*, *C. circinalum*, *S. vulgare*, and *P. pavonica* were collected from Kaş (Antalya), while *L. viscida* and *C. bursa* were collected from Gökova Bay (Muğla).

Determination of ROS Scavenging and Antimicrobial Activity of Algae Extract

ROS scavenging capacities of the collected macroalgae were evaluated using the DPPH assay. Based on the DPPH activity results (Table 1), brown alga *S. schimperi* exhibited the highest antioxidant activity. Therefore, *S. schimperi* was selected for incorporation into fish feed formulations.

The antimicrobial activity of *S. schimperi* extract was determined using different pathogenic microorganisms. The extract demonstrated significant antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as fungi. Inhibition zones of 10.5 mm were observed against *B. subtilis* and *B. cereus*, 9 mm against *E. coli*, 11 mm against *P. vulgaris*, and 11 mm against *A. parasiticus*. In comparison, neomycin exhibited inhibition zones of 12 mm against *B. subtilis*, 13 mm against *S. aureus*, and 9 mm against *P. vulgaris*. Notably, ampicillin and carbapenem showed no activity against the tested pathogens (Figure 1).

MIC and MBC of *S. schimperi* extract were determined using the broth microdilution method against different pathogenic microorganisms. The MIC and MBC results are summarized in Table 2.

The MIC values of *S. schimperi* extract, determined by the broth dilution method, demonstrated effectiveness at 1, 1/2, and 1/4 concentrations against all bacterial species, and at 1 and 1/2 concentrations against the fungus. Notably, the extract exhibited a bacteriostatic effect against *E. coli* up to a 1/16 dilution. The MBC values of *S. schimperi* extract at a 1% concentration inhibited all microorganisms, except *S. aureus*. At 1/2 and 1/4 concentrations, inhibition was observed only against *E. coli*.

Preparation of Fish Feed and Feeding of Fish

Commercial fish feed, with the addition of 5% algae, was administered to the experimental group of fish for a period of twenty-one days. At the beginning of the experiment, the weight and length of each fish were

Table 1. DPHH analysis results of algal samples

Algae species	Systematic group of species	%ROS effect (X±SD)	Impact area
Stypopodium schimperi	Heterokontophyta	81.39±0.88	+++++
Liagora viscida	Rhodophyta	24.69±1.85	++
Codium bursa	Chlorophyta	7.96 ± 5.02	+
Ceramium circinalum	Rhodophyta	ND	-
Sargassum vulgare	Heterokontophyta	ND	-
Padina pavonica	Heterokontophyta	63.3±0.02	+++
Trolox (positive control)		80.23±0.56	

+++++; very strong ++++; strong, +++; medium ++; weak, +; very weak, -; not determined (Trolox Equivalent Antioxidant Capacity (TEAC) was used in the classification).

measured. A normality test was performed between the experimental and control groups using the Shapiro-Wilk-W test, and P= 0.177 was obtained for both groups. These results indicate that the distribution does not differ significantly from a normal distribution, as P>0.05 is greater. An independent samples t-test was performed between the two groups, yielding a t-statistic of 0.0 and a p-value of 1.0. This result indicates that the means of the two groups are similar, and there is no statistically significant difference between them (P>0.05). Upon completion of the experiment, the final length and weight were recorded, and morphological characteristics such as specific growth rates and changes in length were assessed (Table 3).

When comparing weight gain, the fish fed with the algae mixture exhibited a higher increase in weight. A significant increase in length was also observed in the fish fed with *S. schimperi*. While a 1.2-fold increase in length was noted in fish fed with normal feed, a 1.32-fold increase was observed in those fed with the algae-enriched diet.

At the start of the experiment and at the end of the 21st day, the intestines of the fish were sampled for metagenomic analysis, and the bacterial flora was characterized using molecular methods.

Microbial Community Analysis of Fish Gut Microbiota

The rarefaction curves generated from the sequencing data of fish gut samples indicate sufficient sequencing depth, as evidenced by the curves reaching a plateau. This plateau suggests that the species diversity in the samples, including rare species, has been adequately captured. The species richness curve represents the total number of bacterial species identified in the microbiota (Figure 2).

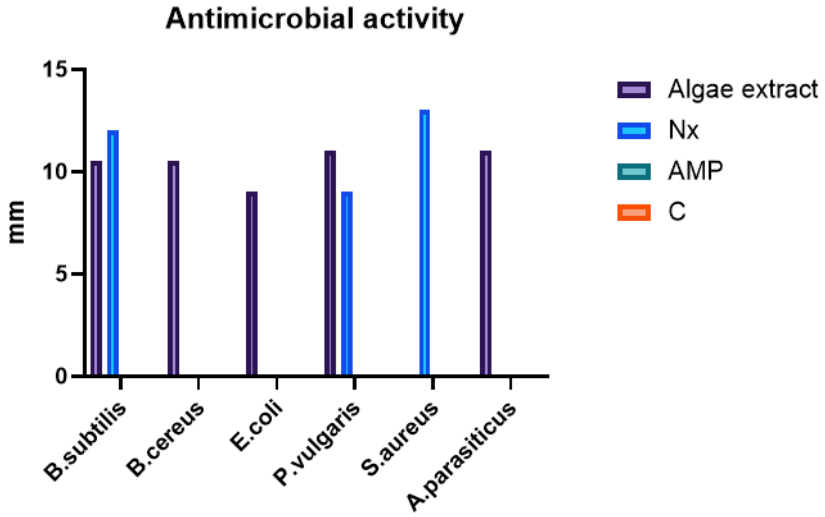

Figure 1. Antimicrobial effects of *Stypopodium schimperi* extract against various pathogens using the disc diffusion method (Nx: Neomycin, AMP: Ampicillin, C: Carbapenem).

Table 2. MIC and MBC values of *Stypopodium shimperi* extract against different pathogenic microorganisms

Dilution rate	*B. subtilis*		*B. cereus*		*E. coli*		*P. vulgaris*		*S. aureus*		*A. parasitus*	
µg/mL	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	-	-	-	-	-	-	-	-	-	+	-	-
1/2	-	+	-	+	-	-	-	+	-	+	-	+
1/4	-	+	-	+	-	-	-	+	-	+	+	+
1/8	+	+	-	+	-	+	-	+	-	+	+	+
1/16	+	+	+	+	-	+	+	+	+	+	+	+

+: There is growth; -: No growth.

Table 3. Growth parameters of Zebrafish fed and not fed with 5% *Stypopodium shimperi*

Groups	Weight gain (WG)/(X±SD)	Environment factor (CF)/(X±SD)	Specific growth rate (SGR)/(X±SD)	Viscerosomatic index (VSI)/(X±SD)
Control	0.124±0.01	12.76±0.11	1.463±0.12	1342±56.75
%5 algae	0.121±0.09	11.81±0.11	1.669±0.11*	2227±117.36*

*Represents a statistically significant difference of P<0.05 when compared with the control; SD, standard deviation.

The PCoA score plot clearly reveals the differences in microbial communities among the samples (Figure 3). The PCoA1 axis explains 81.13% of the total variance and primarily distinguishes experimental group on day 21 (A) from control initial (CI) and control final groups (CF). This indicates that the microbial community structure of sample A is fundamentally different from the other two. The PCoA2 axis accounts for 18.85% of the variance and reflects the distinction between CI and CF samples. Both samples occupy a similar position along PCoA1 (in the negative direction), suggesting that their microbial profiles are relatively close to each other compared to sample A. However, their dispersion in opposite directions along PCoA2 indicates that CI and CF emphasize different microbial groups and exhibit notable diversity in their community compositions. In conclusion, all three samples are distinct from one another and exhibit unique microbial profiles. The lack of overlap among the samples on the PCoA plot suggests that there is no shared dominant microbial structure; instead, each sample highlights different taxonomic distributions. In addition, the distinct density and different location exhibited by Group A indicate that the applied algae affect the diversity and abundance of the microbiota.

To further assess the species diversity at the species level, Shannon and Simpson indices were calculated for all bacterial species (Table 4). These indices reflect the species diversity within a population (Feng et al., 2017). The Shannon index for the experimental group on day 21 was 4.503, indicating high species diversity. Similarly, the samples from the control group on day 21 also showed high diversity. However, a slight decrease in species diversity was observed in the experimental group compared to the control group at the end of the experimental period. This decrease is likely due to the inhibitory effects of the added macroalgae on some microbial species, resulting in reduced diversity. Typically, the Shannon index ranges from 1.5 to 3.5, with higher values indicating greater

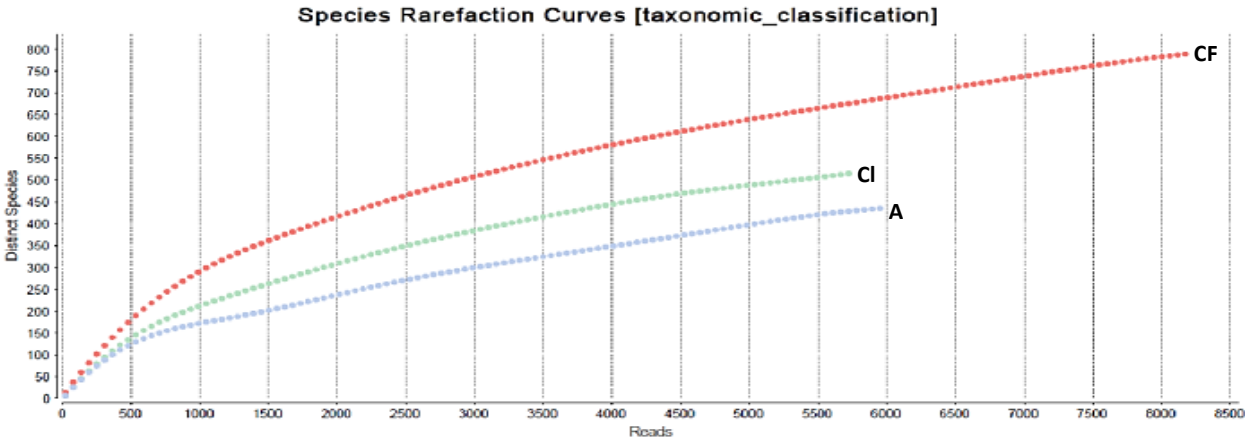

Figure 2. Species richness curve showing the number of different species (without considering population sizes) according to the number of OTU reads in fish gut samples (... :A, ... :CF, ... :CI).

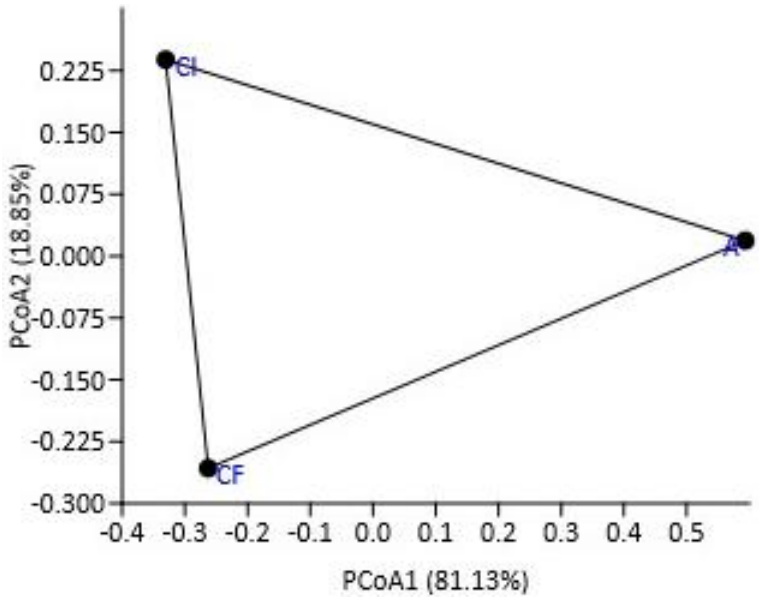

Figure 3. PCoA based on Bray-Curtis distance matrices a graph showing similarities and differences between fish gut samples.

diversity. Despite the observed inhibition in the experimental groups, the species diversity remained high compared to the initial microbiota of the control group.

The Simpson index, another measure of species diversity, ranges from 0 to 1, with values closer to 1 indicating high diversity and values closer to 0 indicating low diversity. Analysis revealed that the Simpson index was 0.9762 on day 21 in the control group and 0.9769 in the experimental group. The control initial value was 0.9309, suggesting that species diversity was higher in both groups at the end of the experiment compared to the initial microbiota.

Three experimental groups were utilized to assess the microbiota composition. The initial microbiota of the control group was determined by analyzing intestinal content prior to any feeding regimen, while additional samples were obtained from the experimental group after the application of either macroalgae-added or unadded feed. Average values from two replicate studies were used for analysis. Graphs were created to visualize the results obtained from the metagenome analyses. The bacterial distribution of the ten species with the highest read sequences at the species level across the groups was depicted using heatmap graphs.

In the experimental group (A), *Proteobacteria* was identified as the most dominant phylum. In contrast, the relative abundances of *Actinobacteria* and *Firmicutes* were reduced compared to the control group. The results suggest that the algae suppressed the *Actinobacteria* and *Firmicutes* phyla, while promoting the increased abundance of the *Proteobacteria* phylum.

Family-Level Bacterial Composition

In Group A, the most abundant families were *Pseudomonadaceae* (75.31%) and *Moraxellaceae* (21.57%), while the relative abundance of all other families was below 1%. In the control group on day 21 (CF), *Rhodobacteraceae* was the most prevalent family at 36.34%, followed by *Enterobacteriaceae* at 11.66%, and *Pseudomonadaceae* at 6.78%. On day 0 of the control group (CI), *Enterobacteriaceae* dominated at 35.08%, followed by *Rhodobacteraceae* at 26.55%, and *Pseudomonadaceae* at 4.8% (Figure 4a).

Genus-Level Bacterial Composition

At the genus level, *Pseudomonas* (74.34%) and *Acinetobacter* (22.54%) were dominant in Group A, while *Rhodobacter* (15.05%) and *Pseudomonas* (8.63%) were the most abundant in the CF group. In all three groups (A, CI, and CF), the genera *Pseudomonas* (A: 74.34%, CI: 5.56%, CF: 8.63%), *Salmonella* (A: 0.18%, CI: 13.72%, CF: 4.43%), and *Escherichia* (A: 0.18%, CI: 18.16%, CF: 6.51%) were detected at varying levels. *Pseudomonas* showed a significant increase in Group A compared to the CI group, whereas *Salmonella* and *Escherichia* were notably reduced in Group A compared to the CI group. The absence of genera such as *Shigella*, *Gordonia*, *Paracoccus*, and *Exiguobacterium*—which include pathogenic species—in Group A suggests that the algae inhibited these groups. Additionally, the relative abundance of *Rhodobacter* increased from 8.34% in the CI group to 15.05% in the CF group, while *Faecalibacterium* increased from 2.52% in the CI group to 3.43% in the CF group % (Figure 4b).

A total of 651 genera were identified through metagenomic analyses, of which 297 (45.6%) were classified within the phylum Proteobacteria. In group A, 139 genera were detected, while 118 genera were identified in the CI group. After excluding genera represented by only one or two samples, 82 genera remained in group A and 52 genera in group CI. The CF group exhibited the highest diversity, with a total of 143 genera identified (Supplementary Table 1). Although a 63% increase in diversity was observed in group A compared to group CI, the increase in diversity in group A was lower than that observed in group CF. This is thought to be due to the alg used in the diet, which inhibits certain pathogenic groups while promoting the growth of *Pseudomonas* and *Acinetobacter* genera (Figure 5).

Species-Level Bacterial Composition

In the CI group, *E. coli* (20.1%) and *S. enterica* (14.62%) were the most abundant species, whereas in the CF group, *E. coli* (8.49%) and *Rhodobacter capsulatus* (6.81%) were the predominant species. In Group A, *E. coli* was found at a significantly lower level of 1.67%. Similarly, *S. enterica* was also detected at much lower levels in Group A, with a relative abundance of 1.46%, compared to the control groups.

In Group A, the most abundant species were *Acinetobacter schindleri* (6.47%), *Pseudomonas aeruginosa* (6.11%), and *Acinetobacter venetianus* (5.1%). Notably, *A. schindleri* and *A. venetianus* were absent in the CI and CF groups, while *P. aeruginosa* was found at a relative abundance of 0.35% in the CI group

Table 4. Fish gut species diversity according to Shannon and Simpsons values

	Species Level Diversity	
Sample	Shannon Index (H) / (H / LN (N))	Simpsons Index (D-1)
Experimental group 21st day (A)	4.503/0.7389	0.9769
Control group 21st day (CF)	4.933/0.7383	0.9762
Control group initial (CI)	4.158/0.6648	0.9309

*Indicates the species diversity in the samples. The Simpsons index takes a value between 0-1. 1 indicates diversity and 0 indicates no diversity. The Shannon index usually takes a value between 1.5-3.5 and the higher this index, the higher the diversity.

and 0.37% in the CF group. *Cutibacterium acnes* was present in Group A at 2.9%, whereas it was found at 0.06% in the CI group and 1.8% in the CF group. *Klebsiella pneumoniae*, which had a relative abundance of 4.1% in the CI group, decreased to 1.56% in the CF group and was not detected in Group A. *Nordella sp.* was found at 1.39% in the CI group and increased to 3.44% in the CF group. *Iamiaceae bacterium SCSIO 58843*, *Paracoccus kondratievae*, *Rhodobacter capsulatus*, and *Photorhabdus laumondii* were detected only in the CF group, while *Vibrio cholerae*, *Photorhabdus asymbiotica*, *Shigella flexneri*, and *Streptomyces peucetius* were found exclusively in the CI group. *Faecalibacterium prausnitzii* was found at a relative abundance of 2.91% in the CI group, which increased to 4.72% in the CF group % (Figure 4c).

Discussion

Macroalgae are rich sources of bioactive secondary metabolites, particularly phenolic and flavonoid compounds, which are known to possess antioxidant, anti-inflammatory, and immunomodulatory properties. These compounds have been reported to be effective not only in preventing oxidative stress but also in the treatment of protozoal, viral, fungal, and bacterial infections, as well as chronic diseases such as cancer and diabetes (De La Rosa et al., 2019). Some phenolics show strong antibacterial activity against both Gram-positive and Gram-negative bacteria (Alves et al., 2013). Furthermore, due to the adverse effects of synthetic antioxidants—including liver toxicity and carcinogenic risks—interest in natural alternatives has significantly increased. Phenolic-rich macroalgal extracts are being explored as dietary supplements that can protect the human body against free radical-induced damage (Kalasariya et al., 2021; Gür and Polat, 2023).

Assuming similar bioactivity in other organisms, this study initially aimed to screen macroalgal species with high antioxidant activity. Among the tested species, *S. vulgaris* and *P. pavonica* exhibited strong antioxidant potential, with *S. schimperi* showing the highest activity.

In various studies, extracts obtained from brown algae have been shown to exhibit higher antioxidant potential compared to red and green algae (Balboa et al., 2013; Dang et al., 2018). In our study, *S. schimperi*, a

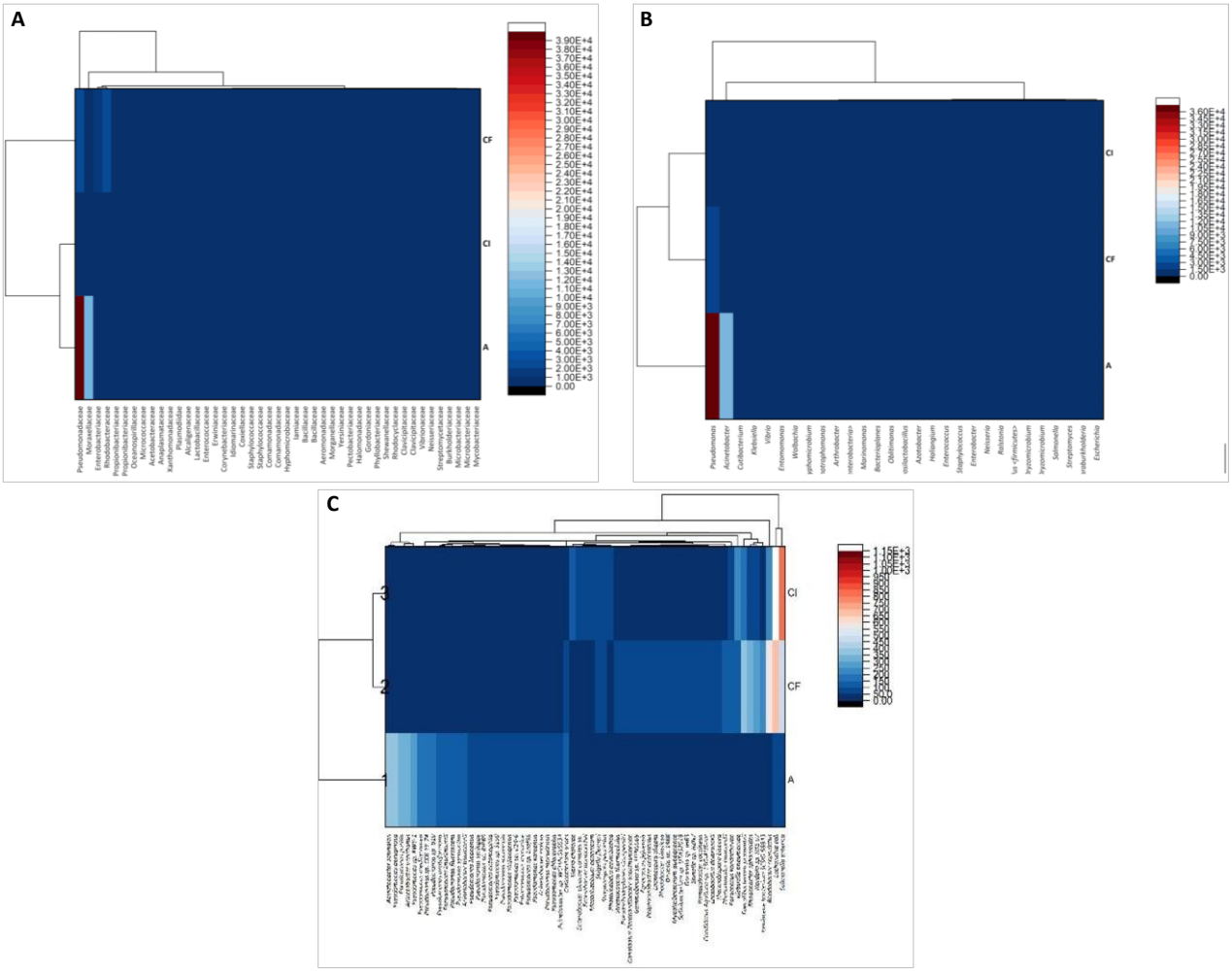

Figure 4. A. Hierarchical cluster analysis (HCA) and Heatmaps plot of the highest bacterial family of all gut microbiota samples; **B.** Hierarchical cluster analysis (HCA) and Heatmaps plot of the highest bacterial genus of all gut microbiota samples; **C.** Hierarchical cluster analysis (HCA) and Heatmaps plot of the highest bacterial species of all gut microbiota samples (Experimental group day 21 (A), Control group day 21 (CF), Control group baseline (CI).

brown alga, also demonstrated high antioxidant activity. Therefore, *S. schimperi* was selected for dietary trials in fish. It is believed that the antioxidant activity of *S. schimperi* is due to the presence of ascorbic acid, flavonoids, and phenolic compounds in its structure.

Phlorotannins, which are the most abundant and synthesized compounds in brown algae, have antifouling, antimicrobial, anti-inflammatory, antioxidant, and anticancer activities (Liu et al., 2017; Dang et al., 2018; Kumar et al., 2022; Ashokan et al., 2025). Phenolic acids and flavonoids are also used in functional foods and nutritional enhancement applications (Bai et al., 2022). However, research on the use of phlorotannins in the nutrition of aquatic animals is limited. In a study conducted by Zhang et al., phlorotannins extracted from algae were added at three different concentrations, and they reported that the growth performance of sea cucumbers significantly increased. It was also determined that the addition reduced mortality rate, promoted higher weight gain, increased total antioxidant capacity, enhanced the immune system, and positively affected gut health in sea cucumbers.

In a study by Gür and Polat (2023), *S. schimperi* was found to be rich in crude protein and lipids and to have high flavonoid and phenolic compound content (Gür and Polat, 2023). In another study by Unal et al. (2023), high levels of protein, carbohydrates, ascorbic acid, and phenolic compounds were detected, and the values of P, Mg, K, Na, and Ca were found to be higher than other microelements (Unal et al., 2023). In our study as well, the use of *S. schimperi* as a feed additive was found to cause increases in specific growth rates (SGR) and changes in fish length. This effect of *S. schimperi* is thought to be due to its content of ascorbic acid, phenolic compounds, and microelements such as P, Mg, K, Na, and Ca.

The antimicrobial activity of macroalgae has also been reported in previous studies (Tüney et al., 2006), and this property is particularly relevant when evaluating potential impacts on gut microbiota upon dietary inclusion. In our study, *S. schimperi* extract demonstrated low-level antibacterial and antifungal effects. Disk diffusion tests revealed inhibition against Gram-positive bacteria such as *B. subtilis* and *B. cereus*, but not *S. aureus*. Among Gram-negative bacteria, inhibition zones were observed for *E. coli* and *P. vulgaris*. Additionally, a zone of inhibition was detected for the fungal pathogen *A. parasiticus*. However, according to CLSI standards (2021), these inhibition zones were not within ranges considered effective for antimicrobial therapy. For instance, the effective inhibition zone diameter for carbapenem against *E. coli* is >19 mm, and 15–22 mm for ampicillin. These findings suggest that *S. schimperi* extract does not exert strong inhibitory effects on the tested pathogens. However, the data obtained are valid only for the strain(s) tested in this study, and antibiotic resistance/susceptibility can vary significantly among strains. The strain's origin, environment, or the transfer of different gene regions (such as resistance genes) may influence antibiotic susceptibility of strains.

To evaluate its influence on gut microbiota, fish were fed a diet supplemented with 5% algal for 21 days. Metagenomic analyses were conducted on intestinal samples from both treatment and control groups, including baseline (day 0) samples.

Previous studies have shown that antibiotics such as oxytetracycline (OTC) can alter gut and water microbiota in zebrafish. At low OTC concentrations, changes occurred in the water microbiota, whereas high doses led to gut microbiota alterations, including increased abundance of Alphaproteobacteria and Actinobacteria and decreased Gammaproteobacteria (Almeida et al., 2019). Similarly, exposure to OTC and sulfamethoxazole in zebrafish larvae caused a significant increase in *Flavobacterium* spp., suggesting that early-life antibiotic exposure can promote harmful bacterial growth (Yu et al., 2021).

In our study, Gammaproteobacteria were dominant in Group A (algae-fed fish), followed by Actinobacteria. In the control fish (CF), Alphaproteobacteria were dominant, with notable representation of both Gammaproteobacteria and Actinobacteria.

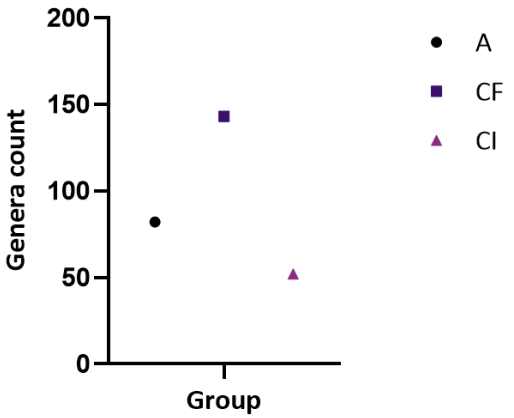

Figure 5. Numerical value of Proteobacteria diversity in groups.

Dietary interventions also shape gut microbiota composition. For instance, a gluten-formulated diet in zebrafish increased the abundance of *Rhodobacter*, *Legionellales*, and *Rhizobiaceae* families (Koo et al., 2017). Another study showed that chitosan silver nanocomposites (CAgNCs) altered gut microbiota by increasing *Fusobacteria* and *Bacteroidetes*, while *Proteobacteria* remained dominant (Udayangani et al., 2017). Similarly, in our study, Proteobacteria was the most dominant phylum in Group A, while both Proteobacteria and Actinobacteria were abundant in the CF group.

Zheng et al. (2019) reported that graphene-family materials increased the abundance of *Fusobacteria*, *Cetobacterium*, and *Lactobacillus*, while decreasing *Firmicutes* and *Pseudomonas*. In contrast, our findings revealed *Pseudomonas* and *Acinetobacter* as the most dominant genera in Group A. In CF fish, *Rhodobacter* and *Pseudomonas* were prominent. The abundance of *Pseudomonas* and *Acinetobacter* in algae-fed fish is likely due to their capacity to degrade alginate—a major component of brown algae such as *Sargassum*. Notably, alginate is also a structural component of *P. aeruginosa* biofilms (Yang et al., 2011; Krell et al., 2024), which may explain the dominance of these genera in the gut microbiota of Group A.

The modulatory potential of gut commensals was demonstrated in a study using leafworm moths, where *Acinetobacter* spp. administration led to improved vitality and reduced inflammation following gut dysbiosis induced by dextran sulfate sodium (Pandey and Rajagopal, 2017). This supports our observation that algae extract-induced shifts in gut microbiota may have contributed to improved fish growth.

Although the use of macroalgae in functional feeds is increasing, algal species vary widely in their bioactive compound composition, and their host-specific effects must be thoroughly evaluated. Compared to other animal models, research on fish microbiomes remains relatively limited (Cezero et al., 2021; Siddik et al., 2023).

In aquaculture, *Solea senegalensis* fed a *Ulva ohnoi*-supplemented diet showed higher abundance of *Pseudomonas* in the anterior gut and *Vibrio* spp. in the posterior gut (Tapia-Paniagua et al., 2019). Similarly, Cezero et al. (2021) observed increased *Vibrio* and decreased *Stenotrophomonas* in fish fed with *Ulva*. In parallel, our study found *Pseudomonas* to be the dominant genus in algae-fed fish, suggesting that certain algal compounds selectively enrich this taxon.

In another study, *Ulva rigida* and *Ascophyllum nodosum* were added to Atlantic cod diets, leading to distinct microbial responses. PICRUSt2 analysis revealed the emergence of lysine metabolism and methane-related pathways at week 12, absent at baseline, highlighting the role of macroalgal supplementation in shaping gut microbial function (Keating et al., 2022).

Sargassum horneri has also been explored for its probiotic potential. Liyanage et al. (2022) found that it enhanced lactic acid bacteria (LAB) growth and displayed dose-dependent antibacterial effects. In vivo, co-treatment with LAB and S. horneri polysaccharides reduced mortality in zebrafish infected with *Streptococcus parauberis*. In our study, *S. schimperi* also showed inhibitory activity against *E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Rhodobacter capsulatus*, and *Vibrio cholerae*.

Conclusion

The gut microbiota is influenced not only by nutritional status but also by environmental conditions, age, gender, and the immune system. Changes in dietary regimes can lead to significant alterations in the gut microbiota. In fish fed with macroalgae-supplemented diets, a marked increase in the abundance of the phylum Proteobacteria was observed, along with a higher diversity compared to the control group. The various metabolites present in the algae inhibited certain pathogenic groups while promoting the growth of others. It is believed that, with proper dosage, the algae could have more beneficial effects on the gut microbiota. Additionally, by examining the effects of the algae on bacterial growth, it may be possible to promote the proliferation of specific bacterial groups.

Data Availability

All data generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. The raw sequence reads data were submitted to NCBI Sequence Read Archive (SRA), and the study was registered in NCBI BioProject and BioSample database (SubmissionID: SUB15318840; Accession No: PRJNA1262960).

Ethical Statement

All experimental protocols have been approved by, Animal Experiments Local Ethics Committee (No;2021/010) under the Ege University.

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Author Contribution

This paper has been compiled together with the roles and duties of each authors. (Dilek Ünal (DÜ), has conceptually investigated, designed, analyzed data, written, and reviewed; Fadime Özdemir (FÖ) has conceptually investigated, designed, analyzed data, written and reviewed, submitted a manuscript; Mustafa Koyun (MK) has funding acquisition, written and reviewed; İnci Tüney Kızılkaya (İTK) has collected sample, analyzed data, written and reviewed.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Genera	***A***	***CF***	***CI***
Pseudomonas	*37301*	*1947*	*873*
Acinetobacter	*11307*	*84*	*12*
Salmonella	*90*	*323*	*35*
Escherichia	*89*	*722*	*88*
Klebsiella	*65*	*144*	*17*
Vibrio	*64*	*122*	*22*
Entomomonas	*60*	*0*	*0*
Paraburkholderia	*35*	*318*	*160*
Kocuria	*27*	*9*	*0*
Wolbachia	*25*	*14*	*3*
Marinomonas	*24*	*1*	*0*
Ralstonia	*24*	*47*	*8*
Hyphomicrobium	*22*	*12*	*0*
Bacterioplanes	*21*	*0*	*0*
Oblitimonas	*21*	*1*	*2*
Oryzomicrobium	*21*	*86*	*55*
Enterobacter	*20*	*59*	*9*
Neisseria	*18*	*65*	*21*
Stenotrophomonas	*18*	*8*	*2*
Azotobacter	*14*	*0*	*0*
Haliangium	*14*	*1*	*0*
Buchnera <enterobacteria>	*14*	*11*	*2*
Idiomarina	*13*	*1*	*0*
Moraxella	*13*	*9*	*3*
Candidatus Ruthia	*13*	*0*	*1*
Spiribacter	*11*	*3*	*2*
Cellvibrio	*10*	*13*	*2*
Aquicella	*10*	*2*	*0*
Thalassospira	*10*	*2*	*1*
Orientia	*9*	*0*	*0*
Psychrobacter	*8*	*16*	*5*
Aeromonas	*8*	*47*	*6*
Terasakiella	*8*	*0*	*0*
Kangiella	*8*	*0*	*0*
Yersinia <enterobacteria>	*7*	*35*	*1*
Neptunomonas	*7*	*0*	*0*
Candidatus Methylospira	*7*	*1*	*0*
Hyphomonas	*6*	*1*	*0*
Sodalis	*6*	*29*	*1*
Comamonas	*5*	*3*	*3*
Edwardsiella <enterobacteria>	*5*	*13*	*0*
Coxiella <g-proteobacteria>	*5*	*1*	*0*
Commensalibacter	*5*	*1*	*0*
Thalassolituus	*5*	*5*	*1*
Cupriavidus	*4*	*10*	*0*
Candidatus Hodgkinia	*4*	*1*	*0*
Morganella <enterobacteria>	*4*	*7*	*1*
Moritella	*4*	*6*	*0*
Rhodobacter	*4*	*666*	*1*
Serpentinomonas	*3*	*25*	*12*
Paracoccus <a-proteobacteria>	*3*	*82*	*0*
Rhizobium	*3*	*8*	*1*
Legionella	*3*	*30*	*5*
Providencia	*3*	*4*	*0*
Acidovorax	*3*	*4*	*0*
Litoricola	*3*	*1*	*0*
Novosphingobium	*3*	*1*	*0*
Arabia	*3*	*5*	*0*
Oceanimonas	*3*	*0*	*0*
Halomonas	*3*	*75*	*1*
Marinobacterium	*3*	*0*	*0*
Delftia	*3*	*9*	*3*
Burkholderia	*3*	*25*	*7*
Pectobacterium	*3*	*14*	*4*
Cobetia	*3*	*0*	*0*
Lysobacter	*2*	*2*	*0*
Helicobacter	*2*	*19*	*6*
Francisella	*2*	*1*	*0*
Methylobacterium	*2*	*14*	*0*
Micavibrio	*2*	*0*	*0*
Serratia	*2*	*33*	*6*
Arsenophonus	*2*	*5*	*1*
Prevotella	*2*	*2*	*2*

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Genera	***A***	***CF***	***CI***
Raoultella	*2*	*4*	*0*
Shewanella	*1*	*40*	*6*
Caulobacter	*1*	*4*	*1*
Parashewanella	*1*	*4*	*3*
Achromobacter	*1*	*5*	*1*
Alteromonas	*1*	*15*	*1*
Candidatus Kinetoplastibacterium	*1*	*0*	*0*
Sulfurospirillum	*1*	*6*	*4*
Xanthomonas	*1*	*4*	*1*
Komagataeibacter	*1*	*10*	*1*
Erwinia	*1*	*9*	*1*
Xenorhabdus	*1*	*12*	*0*
Photobacterium	*1*	*7*	*2*
Pigmentiphaga	*1*	*3*	*1*
Bartonella	*1*	*5*	*0*
Acidithiobacillus	*1*	*3*	*1*
Liberibacter	*1*	*4*	*0*
Allofrancisella	*1*	*3*	*0*
Histophilus	*1*	*3*	*0*
Microvirga	*1*	*21*	*0*
Marinobacter	*1*	*6*	*0*
Pandoraea	*1*	*3*	*0*
Kosakonia	*1*	*4*	*1*
Oceanisphaera	*1*	*14*	*2*
Thiomicrorhabdus	*1*	*10*	*2*
Shinella	*0*	*12*	*2*
Vitreoscilla	*0*	*7*	*1*
Pantoea	*0*	*8*	*3*
Hydrogenovibrio	*0*	*7*	*2*
Brevundimonas	*0*	*23*	*0*
Acidiphilium	*0*	*5*	*0*
Citrobacter	*0*	*8*	*1*
Shigella	*0*	*52*	*5*
Azoarcus	*0*	*6*	*0*
Maritalea	*0*	*2*	*0*
Plesiomonas	*0*	*5*	*0*
Rhodoplanes	*0*	*13*	*0*
Photorhabdus	*0*	*33*	*2*
Herbaspirillum	*0*	*9*	*3*
Salaquimonas	*0*	*7*	*0*
Nordella	*0*	*74*	*4*
Pseudorhodobacter	*0*	*6*	*0*
Neorickettsia	*0*	*5*	*0*
Halovulum	*0*	*10*	*0*
Gilliamella	*0*	*4*	*0*
Silicimonas	*0*	*3*	*0*
Candidatus Paracaedibacter	*0*	*22*	*0*
Devosia	*0*	*52*	*1*
Methyloceanibacter	*0*	*11*	*3*
Massilia	*0*	*7*	*4*
Mannheimia	*0*	*5*	*1*
Martelella	*0*	*6*	*0*
Pseudorhodoplanes	*0*	*27*	*0*
Izhakiella	*0*	*7*	*0*
Candidatus Fukatsuia	*0*	*9*	*1*
Celeribacter	*0*	*1*	*0*
Mesorhizobium	*0*	*48*	*7*
Methylomicrobium	*0*	*3*	*0*
Labrenzia	*0*	*2*	*0*
Tolumonas	*0*	*5*	*0*
Scandinavium	*0*	*6*	*1*
Pseudolabrys	*0*	*6*	*0*
Dickeya	*0*	*32*	*1*
Bosea <a-proteobacteria>	*0*	*25*	*4*
Sinorhizobium	*0*	*6*	*0*
Paraglaciecola	*0*	*8*	*3*
Chelatococcus	*0*	*8*	*0*
Gemmobacter	*0*	*32*	*0*
Pseudoalteromonas	*0*	*2*	*5*