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Distinct Structuring Mechanisms of Extrinsic and Intrinsic Factors on the Wild Marine Fish Gut Microbiome

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Abstract

The fish gut microbiome (GM) plays a critical role in nutrient absorption, immune homeostasis, and host physiology. Although shaped by extrinsic and intrinsic factors, the distinct mechanisms driving GM structure (e.g., whether through shifts in microbial abundance or changes in phylogenetic community composition) remain unclear. In this study, we characterized the GM of 21 wild marine fish species using 16S rRNA amplicon sequencing and examined how season, trophic level, and fish species differentially structure GM. Season and trophic level were primarily associated with shifts in microbial abundance, whereas fish species showed phylogenetic similarity among GMs, as evidenced by the phylogenetic clustering of fish species-core amplicon sequence variants (ASVs). This suggests that host-specific filtering drives the phylogenetic structure of GM within fish species. In addition, the predicted functional pathway profiles were stratified by season. Collectively, our findings reveal that extrinsic and intrinsic factors differentially structure fish GM through distinct processes.

Introduction

The term “microbiome” refers to the microbial community in a reasonably well-defined habitat with distinct physicochemical properties as its theatre of activity[1]. Theatre of activity includes the entire spectrum of molecules produced by microorganisms, such as structural elements (nucleic acids, proteins, lipids, and polysaccharides), metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules produced by interactions with symbiotic hosts or surrounding environments[2]. The microbial community interacts with hosts and environments, contributing to host adaptation to environmental change and ecosystem shaping. For instance, although the gut microbial composition of the Gilthead seabream (*Sparus aurata*) changes owing to perturbation, its metabolic capacity is maintained[3]. This finding suggests that symbiotic microorganisms aid host adaptation to environmental changes by maintaining their functions.

Fish are the most phylogenetically diverse vertebrates and play essential roles in the aquatic ecosystem through predation and nutrient flux mediation[4,5]. In addition, fish are an economically critical source of animal proteins for humans. To optimize fish productivity and welfare, fish gut-derived probiotics enhance disease resistance, stress tolerance, and reproductive performance[6,7]. However, such applications require a comprehensive understanding of the fish gut microbiome (GM) as a baseline.

The fish GM is influenced by various extrinsic and intrinsic factors. Extrinsic factors, including water temperature, salinity, and diet, vary seasonally[8,9]. Seasonal fluctuations in water temperature and salinity are attributed to variations in air temperature and rainfall, respectively[9]. Seasonal dietary changes in fish are driven by prey availability, energy requirements for reproduction, and habitat changes[8]. Given that fish are poikilotherms and that season is an integrated factor, we predicted that seasonal changes would strongly affect the fish GM. Previous studies have shown that fish GM differs significantly by season, and seasonal changes affect the dominant bacteria in the gut at the phylum level[10,11]. Further, diet

significantly influences fish GM[12,13]. However, inferring the diets of wild fish is challenging, especially when seasons change because seasonal fluctuations can alter fish diets. To overcome this challenge, the trophic level has been used, which refers to the position of an organism in the food web[14]. Trophic level and gut microbial diversity is negatively correlated, with trophic level being identified as a significant contributor to GM variation[12]. Moreover, the predicted gene distribution in the GM is influenced by the fish trophic levels[15]. Intrinsic host factors, such as species, genetics, immunity, and physiology, influence the fish GM, and fish species identity has been consistently reported as an important driver of community composition. In our previous study, we found that the interspecific dissimilarity in GMs among seven wild fish species was significantly greater than the intraspecific dissimilarity[16], suggesting that genetic factors in fish influence GM, regardless of dietary variation.

To date, most fish GM studies have focused on factors that influence GM. These studies generally reported a single metric, such as taxonomic composition, alpha diversity, or beta diversity, to demonstrate that a given factor shapes the GM. Consequently, these findings demonstrate that the GM responds to various extrinsic and intrinsic factors, but do not fully address whether these factors act similarly or differently. For instance, one factor may primarily reorganize the dominant taxa without phylogenetic similarity in the GM, whereas another may shape phylogenetic similarity without distinctly shifting microbial composition. Without comparing factors within the same dataset and across multiple aspects of the GM, it remains unclear how and to what extent each factor differentially shapes fish GM. Understanding how each factor acts is essential for predicting fish GM responses to environmental changes and for designing probiotics that account for the main drivers of GM variation in fish. Here, the contributions of extrinsic and intrinsic factors to fish GM composition were compared using 16S rRNA V3-V4 amplicon sequencing. We further characterized the distinct patterns by which extrinsic and intrinsic factors differentially affect fish GM composition.

Results

Seasonal and trophic level changes influence the bacterial abundance in fish GM

When the BC dissimilarity was applied, the GM composition was separated by season in the NMDS ordination (Fig. 1a). In summer, *Catenococcus* served as an indicator taxon of fish GM, whereas *Aliivibrio* and *Shewanella* were characteristic taxa in winter. Season significantly influenced GM composition across all three dissimilarity metrics (BC, Unweighted UniFrac, and Weighted UniFrac; Fig. 1b and Supplementary Table S1a). However, the dispersion between seasons based on the Weighted UniFrac was significantly heterogeneous, which reduced the reliability of this metric for between-group comparisons. Among the three metrics, BC yielded the highest ANOSIM R-value, indicating that seasonal differences in the GM composition were most evident based on taxon abundance. No significant seasonal differences in the microbial diversity were observed (Supplementary Fig. S1a). However, seasonal changes in the gut bacterial composition and relative abundance were observed at the ASV level (Fig. 1c). ASVs affiliated with *Cetobacterium*, *Enterovibrio*, *Photobacterium*, *Vibiro*, *Catenonoccus*, and

Vibrionaceae were characteristic of the fish GM in summer, whereas those affiliated with *Ureaplasma*, *Brevinema*, *Arcobacteraceae_uncultured*, *Holosporaceae_uncultured*, *Propionogenium*, *Photobacterium*, and *Aliivibrio* were characteristic of winter. ASVs belonging to *Photobacterium* (ASV2165 and ASV2171), *Catenococcus* (ASV2722 and ASV2699), *Vibrionaceae* (ASV2791, ASV2789, ASV2779, and ASV2705), *Vibrio* (ASV2535, ASV2666, ASV2517, ASV2480, and ASV2627), *Enterovibrio* (ASV2260), and *Nautella* (ASV1333) were significantly enriched in summer (Fig. 1d). In contrast, ASVs belonging to *Aliivibrio* (ASV2594, ASV2620, ASV2580, ASV2595, ASV2560, and ASV2655), *Photobacterium* (ASV2368, ASV2370, ASV2337, and ASV2329), *Shewanella* (ASV2089 and ASV2080), *Vibrio* (ASV2746, ASV2744, and ASV2454) were enriched in the winter. Collectively, *Catenococcus* and *Aliivibrio* were identified as the most consistent seasonal indicators of fish GM enriched in summer and winter, respectively.

The GM composition varied with fish trophic level based on BC dissimilarity (Fig. 2a). At the genus level, *Vibrio* was an indicator taxon for fish with low trophic levels (< 3.4), whereas *Photobacterium* was an indicator taxon for fish with high trophic levels (> 3.4). The GM was significantly influenced by trophic level across all three dissimilarity metrics (Fig. 2b and Supplementary Table S1b). However, dispersions among trophic level groups were significantly heterogeneous across all three metrics, which may be attributed to the treatment of trophic level as a categorical variable, with 18 categories in ANOSIM and PERMANOVA. To address this, the Mantel test was used to assess the correlation between trophic level as a continuous variable and GM dissimilarity. Pairwise differences in fish trophic levels were correlated with BC dissimilarity in the GM (Fig. 2c; Mantel's R = 0.1305, p = 0.001), whereas correlations based on Unweighted and Weighted UniFrac were not significant (Supplementary Table S2). No significant differences in gut microbial diversity across trophic levels were observed using Simpson, Shannon, and PD indices, and no association between trophic level and microbial diversity was detected (Supplementary Fig. S1b and Supplementary Table S3). Among the ASVs that significantly correlated with fish trophic level, 26 ASVs were clustered by trophic level in the heatmap (Fig. 2d and Supplementary Fig. S2). ASVs affiliated with *Photobacterium* (ASV2368, ASV2370, ASV2381, ASV2382, ASV2352, ASV2384, ASV2350, ASV2329, ASV2379, ASV2375, ASV2345, and ASV2337) showed characteristically high log-transformed relative abundance in fish with high trophic levels, whereas ASVs affiliated with *Vibrio* (ASV2766, ASV2666, and ASV2667), *SAR11_Clade_α* (ASV1575), *Propionigenium* (ASV1514 and ASV1517), *Shewanella* (ASV2072), and *Pseudoalteromonas* (ASV2011 and ASV2012) were characteristic of fish with low trophic levels. These results suggest that *Photobacterium* and *Vibrio* are key microbial indicators of fish trophic levels in the GM.

Fish species shape microbial genetic similarity within GM

Distinct species-specific clustering of GM was not apparent across the 21 fish species in the PCoA ordination (Fig. 3a). The GM varied within fish species, and clustering overlapped among species (Fig.3a; Supplementary Fig. S3). Accordingly, the five taxa identified as potential indicators (*Brevinema*, *Fimbriimonadaceae*, *env.OPS_17*, *Candidatus_Obscuribacter*, and *SAR11_Clade_*) could not be unambiguously assigned as species-specific indicators. However, the fish species-specificity of the GM, as determined by Unweighted UniFrac dissimilarity, was significant, with homogenous dispersion among

the fish species (Fig. 3b and Supplementary Table S1c). No significant differences in microbial diversity among the 21 fish species were observed using Simpson or Shannon indices (Supplementary Fig. S1c); however, significant differences were observed in the PD index (Supplementary Fig. S1c; Py vs. Cs, Py vs. Ok, Tm vs. Ok, Sm vs. Pm, Py vs. Sm, and Tm vs. Sm). Unlike season and trophic level, which showed the strongest signal in BC dissimilarity, fish species-specificity was most pronounced in the Unweighted UniFrac dissimilarity. Given that Unweighted UniFrac is calculated based on phylogenetic distance and ASV presence/absence, fish species-specific ASVs may exhibit phylogenetic similarity within the host species regardless of relative abundance. Fish species-specificity was further characterized by the consistent detection of ASVs within each host species (fish species-core ASVs), rather than through DAA. Phylogenetic similarities of fish species-core ASVs were detected for Tm, Ma, Pi, Sl, St, Pi, Pm, Ph, Cn, As, and Pp (Fig. 3c). Specifically, the Cn- and Pp-core ASVs showed phylogenetic similarities within their host species in the clade containing *Vibrio* and *Catenococcus* ASVs. The Ph- and Pm-core ASVs showed phylogenetic similarities within fish species in the *Enterovibrio* clade. ASVs belonging to *Photobacterium* diverged into two distinct clades, containing St-core and Pi-core ASVs. When samples from both seasons were combined, core ASVs were identified in 16 fish species, whereas core ASVs were not detected in five fish species (Cs, Ok, Sc, Si, and Sm) (Supplementary Table S4). However, the core ASVs of seven fish species (Cs, Ok, Ps, Pse, Sc, Si, and Sm) were detected within a single season (Supplementary Table S5) and showed phylogenetic similarities within each fish species (Supplementary Fig. S4a-b). In summer, the core ASVs of Ps, Pse, Si, and Sm showed phylogenetic similarity within fish species, whereas in winter, Ok, Ps, Pse, Sc, and Si showed phylogenetic similarity.

Season is crucial to determine the fish gut microbial functions

Season, trophic level, and fish species significantly influenced the predicted functional pathway profiles as measured by Aitchison dissimilarity (Supplementary Table S6). However, the dispersion among groups within each factor was significantly heterogeneous (Supplementary Table S6), limiting the interpretability of these results. To evaluate the classification accuracy of the functional pathway profile for each factor, independent of dispersion issues, a random forest classifier was applied. As the number of samples per group was imbalanced across factors, the area under the precision-recall curve (AUPRC) was used as the performance metric to account for unequal group sizes. The model performance was assessed using 5-fold cross validation, repeated 10 times. Furthermore, fish species and trophic levels could not be evaluated using the random forest classifier because of insufficient sample sizes per group. Therefore, the trophic level was categorized into high (> 3.5) and low (< 3.5) to enable machine learning analysis. The random forest classifier showed robust predictive performance for the season (AUPRC = 0.8571), whereas the lower AUPRC for the trophic category (0.5791) indicated that the functional pathway profiles were less clearly partitioned by trophic category (Fig. 4a and Supplementary Fig. S5a-b). Given that seasonal variation most strongly structured the predicted functional profiles, subsequent analyses of pathway-level differences focused on season. Most of the predicted functional genes were related to metabolism (47.7%), followed by organismal systems (14.8%), human diseases (13.7%), genetic information processing (7.4%), environmental information processing (7%), and cellular processes (6.3%). Based on the feature importance of the random forest model, the top 10 KO pathways

with the strongest seasonal signals were selected (Supplementary Fig. S6 and Supplementary Fig. S7). Pathways related to styrene degradation, polycylclic aromatic hydrocarbon (PAH) degradation, and carbohydrate digestion and absorption of GM were enriched in summer, whereas pathways related to protein digestion and absorption, atrazine degradation, and steroid biosynthesis were enriched in winter (Fig. 4b).

Discussion

The GM is affected by seasonal changes[10,17-19]. BC dissimilarity has commonly been used for β-diversity analysis and typically reinforces that the dominant bacteria in the GM varies by season. Because BC dissimilarity is calculated from the difference in abundance of shared taxa between samples, this seasonal variation in β-diversity indicates that season alters bacterial abundance in the gut[20]. In the gut of the red-spotted grouper (*Epinephelus akaara*), the abundances of *Vibrio*, *Enterovibrio*, *Aliivibrio*, and *Photobacterium* are strongly associated with seasonal changes[18]. However, in the present study, the seasonal patterns of *Vibrio* and *Photobacterium* in fish guts were unclear at the genus level, as ASVs belonging to both genera were significantly detected in both summer and winter (Fig. 1c-d; Supplementary Fig. S8). The effect of season on *Shewanella* abundance is similarly unclear at the genus level, as this genus is commonly found in the gut of both warm- and cold-water fish species, and its abundance also increases in the gut microbiota of rainbow trout (*Oncorhynchus mykiss*) under acute heat stress[21-23]. Consistent with the lack of a clear genus-level pattern, *Shewanella* ASVs were detected in both seasons in the present study (Supplementary Fig. S8). These results indicated that the seasonal patterns of *Vibrio*, *Photobacterium*, and *Shewanella* in the fish gut were resolved only at a taxonomic rank below the genus level. In contrast, *Catenococcus*, *Enterovibrio*, and *Aliivibrio* exhibited clear seasonality (Supplementary Fig. S8). *Catenococcus* is mainly found in the gut of fish inhabiting water with temperatures that exceed 25 ^{o}C, and its abundance increases during recovery from prolonged heat stress in the pyloric caeca of the Malabar grouper (*Epinephelus malabaricus*)[24,25]. *Enterovibrio* abundance is similarly associated with thermal stress in the GM and has been detected in the stomachs of coral reef fish in tropical regions[24,26]. The association of both genera with warm water conditions and post-stress recovery was consistent with the high abundances of *Catenococcus* and *Enterovibrio* observed in summer. Conversely, the relative abundance of *Aliivibrio* in the gut of red-spotted orange grouper (*Epinephelus akaara*) was higher in winter and spring than in summer and autumn[18], which is consistent with our finding that *Aliivibrio* characterized the winter gut microbiota.

According to FishBase, fish at trophic level ³ 2.8 mainly consume animals[27]. Given that the trophic levels of fish used in this study ranged narrowly between 2.84–4.23, the divergence in feeding habits among these fish was likely limited. Therefore, we focused on the significant correlation between fish GM and trophic level, rather than on the differences among trophic-level groups. In the gut of carnivorous fish, *Photobacterium* has been frequently detected in high proportions, which is consistent with the results of the present study[12,28]. Specifically, *Photobacterium* is a characteristic of the GM of piscivores compared to other feeding groups (herbivores/omnivores and zooplanktivores/zoobenthivores), and

Photobacterium isolated from the fish digestive tract degrades chitin in the scales and exoskeletons of crustaceans[12,29-31]. *Photobacterium* aids in the host digestion of animal prey and serves as a key indicator of GM in fish at high trophic levels. In contrast, *Vibrio* abundance increased as fish trophic levels decreased in this study, in contrast to previous reports of *Vibrio* enrichment in the guts of carnivorous fish at high trophic levels[28]. We attribute this difference to the diet composition. Although fish at trophic level ³ 2.8 are generally classified as primarily carnivores, this classification does not preclude plant material in the diet. Among the fish examined here, *Stephanolepis cirrhifer* (2.84), *Pseudolabrus sieboldi* (3.14), and *Acanthopagrus schlegelii* (3.24), which had the lowest trophic levels, consume seaweed in addition to aquatic animals (such as zooplankton and marine vertebrates), indicating a partially herbivorous diet despite their classification as carnivores[32-34]. In contrast, fish at high trophic levels (*Okamejei kenojei* [3.99], *Sebastes inermis* [4.02], and *Platycephalus indicus* [4.23]) consume only aquatic animals[35-37]. Because seaweed contains complex polysaccharides that require microbial degradation, the GM of these low-trophic-level fish may be enriched in *Vibrio* to support digestion across a broader range of dietary substrates: *Vibrio* strains from the fish digestive tract produce amylase, protease, lipase, and chitinase[31], and *Vibrio alginolyticus* isolated from omnivorous and herbivorous fish carried genes encoding alginate lyase and chitinase[38]. Accordingly, *Vibrio* is more abundant in the gut of the omnivorous Stellar rockskipper *(Entomacrodus stellifer*) than in that of the herbivorous rockskipper (*Andamia tetradactylus*)[38]. These results indicate that *Vibrio* may support digestion in omnivorous and herbivorous fish by degrading a broad range of nutrients including carbohydrates, proteins, and lipids.

Fish species-specificity of the GM has been reported in several studies using BC and UniFrac dissimilarity measures[15,39,40]. In this study, we used fish species-core ASVs to assess the species-specificity of GM, as measured by the Unweighted UniFrac. The cladogram based on fish species-core ASVs clustered within fish species, although this pattern was not observed in all species (Fig. 3c). This phylogenetic clustering likely reflects host filtering, a process in which host traits (e.g., gut pH and gut structure) select against microbes poorly suited to the host environment over evolutionary timescales[41], generating host-specific microbial habitats. Consistent with this, *Aliivibrio* oligotypes from the gut of *Harpagifer bispinis* are phylogenetically distinct from those of other *Harpagifer* species[42]. Although host traits were not directly measured here, the consistent detection of core ASVs within each fish species suggests that these ASVs reflect host filtering and are, therefore, genetically conserved within species. In contrast, fish species-core ASVs identified across combined summer and winter samples were largely undetectable: among the seven species sampled in both seasons (Cs, Ok, Ps, Pse, Sc, Si, and Sm), only Ps and Pse retained a small number of core ASVs when seasons were combined (Supplementary Table S4). We attribute this loss of detectable core ASVs to the temporary effect of season on gut microbial composition, which alters the specific ASVs classified as core ASVs, even though the underlying host-fish relationship remains unchanged. A similar pattern has been reported in invasive carp species (*Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix*, and *Cyprinus carpio*), whose core microbiomes in the wild and laboratory settings shared only five operational taxonomic units (OTUs)[43]. Notably, when

we examined the phylogenetic relationships of the core ASVs identified from each season (summer and winter, respectively) dataset for these seven fish species, the resulting cladograms showed phylogenetic clustering within each fish species (Supplementary Fig. S4). This indicates that, despite the seasonal loss of detectable core ASVs, the phylogenetic similarity among the remaining core ASVs within each fish species, which is shaped by host filtering, remains conserved.

A random forest model revealed that the predicted functional profiles were distinctly structured by season, indicating that environmental changes alter fish GM and its functions. Specifically, seasonal changes showed functional differences of fish gut microbes in the organismal systems, xenobiotics biodegradation and metabolism, and antimicrobial activity. In the organismal systems, carbohydrate digestion and absorption were enriched in fish GM collected in summer, whereas protein digestion and absorption were enriched in those collected in winter. Seasonal differences in carbohydrate digestion and absorption may be attributed to environmental changes. For example, seaweed biomass in the subtidal zone is higher in spring and summer than in winter[44]. Because *Stephanolepis cirrhifer*, *Pseudolabrus sieboldi*, and *Acanthopagrus schlegelii* consume seaweed[32-34], carbohydrate digestion in these species may be more active during summer than in winter. Moreover, the carbohydrate digestibility of warm-water fish is higher than that of cold-water fish owing to the high amylase activity of warm-water fish[45]. In contrast, intestinal protease activity in yellowtail kingfish (*Seriola lalandi*) at winter water temperatures (12.6 ± 0.1 °C) is significantly higher than that in fish at summer water temperatures (20.8 ± 0.4 °C), because intestinal transit time is slower in winter[46]. Collectively, seasonal changes in feeding habits and host physiology are likely to shape the digestive functions of fish GM. In the xenobiotics biodegradation and metabolism category, the pathway for atrazine degradation was enriched in fish GM collected in winter. Atrazine is a widely used herbicide that contaminates aquatic environments via runoff[47]. We attribute this enrichment to rainfall rather than to seasonal changes. Atrazine flows into the ocean via rainfall[48]. Goseong-gun and Namhae-gun, the agricultural regions adjacent to the South Sea collection site, are subject to such runoff, and rainfall occurred the day before fish collection in winter, but not in summer, although this association is based on a single sampling event and warrants further verification with replicated sampling across multiple rainfall conditions. In contrast, the pathway for PAH degradation was enriched in fish GM collected in the summer. PAH concentrations in seawater around Oki Island, located along the same current pathway as the South Sea, peaked in summer and autumn (July-October) as the PAH-rich continental shelf from the East China Sea entered through the Tsushima Strait after passing through the South Sea[49]. Therefore, fish GM collected in the South Sea during summer may be enriched in PAH detoxification pathways. Pathogenic bacteria, particularly *Vibrio* species, are more abundant in the ocean during summer than in winter[50]. Consistently, antibacterial pathways, including phosphonate and phosphinate metabolism and biosynthesis of siderophore group non-ribosomal peptides, were enriched in fish GM collected in summer. Phosphonates exhibit a broad range of biological activities, including antibacterial, antiviral, and antifungal properties; fosfomycin, a clinically used phosphonate antibiotic, inhibits bacterial cell wall synthesis[51]. Siderophores act as iron chelators that deprive pathogenic microorganisms of iron and can also serve as carriers that deliver

antimicrobial compounds into bacterial cell (i.e., Trojan Horse strategy)[52]. Taken together, these results suggest that the antibacterial function of fish GM is more active in summer than in winter, coinciding with the increased proliferation of pathogenic bacteria during this season.

Extrinsic factors, such as season and trophic level, altered the relative abundance of bacterial taxa, as captured by Bray–Curtis dissimilarity: *Aliivibrio* ASVs were enriched in winter, whereas *Catenococcus* and *Enterovibrio* ASVs were enriched in summer; *Vibrio* and *Photobacterium* ASVs indicated low and high trophic levels, reflecting differences in the digestion of animal- and plant-derived prey. In contrast, the intrinsic factors of fish species shaped the GM through phylogenetic selection rather than abundance shifts, as captured by Unweighted UniFrac dissimilarity: fish species-core ASVs clustered phylogenetically within host species, a pattern likely reflecting host filtering that remained conserved even when the season altered which ASVs were detected as core. Among these factors, the season also most strongly structured the predicted functional profile of GM (AUPRC = 0.857 versus 0.579 for the trophic category), with carbohydrate digestion and antibacterial pathways enriched in summer and protein digestion enriched in winter. As host traits underlying host filtering were not directly measured and functional pathways were inferred rather than experimentally validated, further studies incorporating host physiological measurements and direct functional assays are required to confirm these mechanisms. Despite these limitations, our study provides insights into the distinct mechanisms structuring fish GM in response to extrinsic and intrinsic factors, as well as seasonal effects on its functions.

Methods

Sample collection and processing

In July 2021, 15 wild fish species (*Acanthopagrus schlegelii*, As; *Pagrus major*, Pm; *Seriola lalandi*, Sl; *Parajulis poecilepterus*, Pp; *Hexagrammos agrammus*, Ha; *Pleuronectes herzensteini*, Ph; *Chromis notata*, Cn; *Platycephalus indicus*, Pi; *Chelidonichthys spinosus*, Cs; *Sebastiscus marmoratus*, Sm; *Parapercis sexfasciata*, Pse; *Sebastes inermis*, Si; *Pseudolabrus sieboldi*, Ps; *Stephanolepis cirrhifer*, Sc; *Okamejei kenojei*, Ok) were acquired from Docheon Port Market, Tongyeong, South Korea. Thirteen wild fish species (Cs; Sm; Pse; Si; Psi; Sc; Ok; *Sebastes thompsoni*, St; *Thamnaconus modestus*, Tm; *Hexagrammos otakii*, Ho; *Pleuronectes yokohamae*, Py; *Microstomus achne*, Ma; *Lycodes tanakae*, Lt) were acquired from Docheon Port Market in March 2022. Seven species (Cs, Ok, Ps, Pse, Sc, Si, and Sm) were collected at both sampling times, resulting in 21 unique wild fish species. Fish were transported to the Fisheries Resource Research Institute of Gyeongsangnam-do in aerated containers to minimize handling stress and maintained in fiberglass tanks supplied with local seawater and continuous aeration until sampling.

Fish were anesthetized using Tricaine-S (Syndel Laboratories Ltd, Fernadale, WA, USA), and the total length, body length, and weight were measured to confirm the maturity status. Each fish was photographed and identified at the species level based on established taxonomic references[53,54]. The fish were rinsed with phosphate-buffered saline (PBS) to remove surface contaminants prior to

dissection. A ventral incision was made from the abdominal cavity to the anus, and approximately 4–5 cm of the hindgut was excised. Gut contents were expelled into 2 mL microtubes (Axygen, USA), containing 500 µL PBS by gentle hindgut compression. Samples were temporarily stored on dry ice to inhibit bacterial growth and subsequently stored at -80 ^{o}C until further analysis.

DNA extraction and library construction

DNA was extracted from gut contents using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Samples with high viscosity owing to intestinal residues were diluted with PBS prior to extraction to facilitate pipetting. Library construction was performed as previously described[55]. Amplicon indexing, purification, and pooling were performed as previously described[16].

Sequencing and data preprocessing

High-throughput sequencing was performed on a Mi-seq platform (2 × 250 bp, Illumina) at an external facility (NICEM, Seoul, South Korea). Paired-end sequences were processed using the QIIME2[56] software package (version 2024.05) for microbiome analysis. Because demultiplexing was performed using NICEM, primer sequences were trimmed using the Cutadapt plugin, followed by quality filtering, denoising, and chimera removal using the DADA2 plugin within QIIME2. Sequence reads with a quality score below 25 were discarded, and a minimum merged sequence length of 480 bp was applied. Chimeric sequences were additionally filtered using the vsearch uchime-denovo method, as DADA2's default chimera removal may not detect all chimeric ASVs, particularly those with mismatches between the parent and child sequences. An ASV-based feature table was generated for all the samples. Taxonomic classification was performed using the classify-sklearn method against the SILVA (version 138.1) database[57]. ASVs belonging to "Unassigned" at the phylum level were removed. In addition, we constructed a phylogenetic tree based on ASV sequences. Subsequently, the QIIME2 output artifacts were imported into R studio (version 4.4.2) as phyloseq objects using the *qiime2R* package[58]. ASVs represented by only one or two reads across all samples were removed as singletons and doubletons, respectively.

Diversity and statistical analysis

To standardize the sampling depth, the samples were rarefied to a sequencing depth of 43,634 using *vegan* package, based on the plateau of the rarefaction curve for most samples[59] (Supplementary Fig. S9). Although rarefaction omits valid data, it controls for variations in sequencing depth when measuring alpha and beta diversity[60]. As three samples (win_Ma_01, sum_Si_09, and win_Tm_06) had fewer than 43,634 reads, they were excluded from the 118 gut samples representing 21 fish species, and 2,703 ASVs were retained for analysis. Shannon and Simpson diversity indices were calculated using *phyloseq* package[61], and Faith's phylogenetic diversity (PD) index was calculated using *picante* package[62]. Normality was assessed using the Shapiro–Wilk test, and significant differences among groups were

identified using the Wilcoxon rank-sum and Kruskal–Wallis tests. When the Kruskal–Wallis test indicated significant differences, Conover's post-hoc test was applied, with p-values corrected using the Benjamini-Hochberg (BH) method. Correlation analysis between trophic level and microbial diversity was conducted using the Spearman and Kendall methods.

In β-diversity analysis, the contribution of each factor to the GM was evaluated using three dissimilarity metrics: Bray–Curtis (BC), Unweighted UniFrac and Weighted UniFrac. The BC dissimilarity was calculated based on the differences in taxon abundances between the two samples relative to their total abundances[63]. Unweighted UniFrac is a qualitative measure that calculates the fraction of the phylogenetic tree branch length unique to each community[64]. The weighted UniFrac is a quantitative measure that incorporates both phylogenetic distance and taxon abundance [64]. Aitchison dissimilarity was applied to β-diversity analysis of predicted functional pathway data. β-diversity results were visualized using non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA), indicator taxa for each group were identified using envfit function in the *vegan* package[59]. Significant differences in β-diversity among groups were assessed using analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations. The homogeneity of multivariate dispersions among groups was assessed to obtain reliable PERMANOVA results[65].

Differential abundance analysis (DAA) was performed using a Microbiome Multivariate Association with Linear Models (MaAslin2) using the *MaAslin2* package[66]. The minimum abundance and prevalence thresholds were set at 0.0001 and 0.1, respectively. *P*-values were corrected using the BH method with a significance threshold of $q < 0.05$. Data were normalized using total sum scaling (TSS) and log-transformed. A linear model (LM) was applied to MaAsLin2. When the trophic level was included as a continuous fixed effect, z-score standardization was applied.

The trophic levels of the 21 fish species were obtained from the FishBase website (URL: fishbase.org; Supplementary Table S7). The trophic levels of 15 fish species (Sc, Ps, As, Py, Ph, Ma, Cn, Pm, Tm, Ho, Sl, Cs, Ok, Si, and Pi) were estimated from food items, whereas Ha and Pp were estimated from diet composition[67]. In addition, the trophic levels of Pse, Lt, Sm, and St were estimated based on the size and trophic levels of their phylogenetically nearest relatives[68]. The Mantel test, based on the Pearson's coefficient, was used to assess the association between the fish trophic level and GM dissimilarity.

To identify fish species-specific ASVs, ASVs consistently detected within each fish species (fish species-core ASVs) were identified using a prevalence threshold of 0.99 and a detection abundance threshold of 1E-05. Circular cladograms were constructed based on the phylogenetic tree generated during the sequencing data preprocessing step.

Functional pathway predication

Functional pathway profiles were predicted from non-rarefied 2,807 ASV sequences using PICRUSt2 (version 2.6.1)[69]. The ASV sequences were placed in the bacterial and archaeal reference phylogenies. During this process, 19 bacterial ASV sequences and 69 archaeal sequences that poorly aligned with the reference sequences were excluded. Hidden state prediction (HSP) was performed to estimate 16S copy numbers and calculate the nearest sequenced taxon index (NSTI) score per predicted genome. The most appropriate domain (bacteria and archaea) for each ASV sequence was determined based on the NSTI scores. Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog group (KO) abundance per predicted genome was obtained using HSP. The bacterial and archaeal KO abundance tables were merged into a single table for metagenomic prediction. The KO abundance in the samples was predicted by applying an NSTI cutoff score of 2. The KO abundances were converted to KEGG pathway abundances using the *ggpicrust2* package[70]. A random forest classifier was implemented using the *caret* and *randomForest* packages[71,72] to evaluate the classification accuracy of the functional pathway profiles for each factor, as assessed by the area under the precision-recall curve (AUPRC) with 5-fold cross-validation repeated 10 times.

Declarations

Data Availability

The 16S-rRNA gene sequences used in this study were submitted to NCBI Sequence Read Archive (SRA) under BioProject accession numbers PRJNA1356816.

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Authors contributions

Han and Kwon designed the research. Han, Yu, Kang, and Noh conducted the sampling of fish microbiome. Han and Park conducted experiments and analyzed data. Han, Kwon, and Park wrote the manuscript. All authors approved the final manuscript.

Competing Interests

All authors declare no financial competing interests.

Animal and Human Rights Statement

Fish specimens used in this study were wild-caught individuals obtained alive through standard commercial seafood distribution channels for human consumption. Because the fish were commercially obtained specimens intended for human consumption rather than animals raised or captured specifically

for research, formal approval from an animal ethics committee was not required for this study. Nonetheless, all procedures strictly followed the guidelines established by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Ocean Science and Technology.

References

1. Whipps, J. M., Lewis, K. & Cooke, R. Mycoparasitism and plant disease control. *Fungi in biological control systems* **1988**, 161–187 (1988).
2. Berg, G. *et al.* Microbiome definition re-visited: old concepts and new challenges. *Microbiome* **8**, 103 (2020). https://doi.org/10.1186/s40168-020-00875-0
3. Moroni, F. *et al.* Beyond microbial variability: Disclosing the functional redundancy of the core gut microbiota of farmed gilthead sea bream from a Bayesian network perspective. *Microorganisms* **13**, 198 (2025). https://doi.org/10.3390/microorganisms13010198
4. Helfman, G. S., Collette, B. B., Facey, D. E. & Bowen, B. W. in *The diversity of fishes: biology, evolution, and ecology* (John Wiley & Sons, 2009).
5. Villéger, S., Brosse, S., Mouchet, M., Mouillot, D. & Vanni, M. J. Functional ecology of fish: current approaches and future challenges. *Aquatic Sciences* **79**, 783–801 (2017). https://doi.org/10.1007/s00027-017-0546-z
6. Rahayu, S. *et al.* Probiotics application in aquaculture: its potential effects, current status in China and future prospects. *Frontiers in Marine Science* **11**, 1455905 (2024). https://doi.org/10.3389/fmars.2024.1455905
7. Yukgehnaish, K. *et al.* Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. *Reviews in Aquaculture* **12**, 1903–1927 (2020). https://doi.org/10.1111/raq.12416
8. Berezina, N. A. *et al.* Seasonal diet changes and trophic links of cold-water fish (Coregonus albula) within a northern lake ecosystem. *Animals* **14**, 394 (2024). https://doi.org/10.3390/ani14030394
9. Kang, J.-H., Lim, C. H., Cho, S.-g., Kwoun, C.-H. & Kim, S.-W. Seasonal variation of water temperature and salinity in the coastal waters around Ulsan new port. *Journal of Environmental Science International* **34**, 697–709 (2025). https://doi.org/10.5322/JESI.2025.34.11.697
10. Savard, P., Fernandes, T., Dao, A., McMeans, B. & Lazar, C. S. Seasons influence the native gut microbiome of lake trout salvelinus namaycush. *Applied Microbiology* **3**, 276–287 (2023). https://doi.org/10.3390/applmicrobiol3010019
11. Xu, L. *et al.* Deterministic processes dominate microbial assembly mechanisms in the gut microbiota of cold-water fish between summer and winter. *Frontiers in Microbiology* **15**, 1415931 (2024). https://doi.org/10.3389/fmicb.2024.1415931
12. Huang, Q. *et al.* Diversity of gut microbiomes in marine fishes is shaped by host-related factors. *Molecular ecology* **29**, 5019–5034 (2020). https://doi.org/10.1111/mec.15699

13. Ruiz, A., Gisbert, E. & Andree, K. B. Impact of the diet in the gut microbiota after an inter-species microbial transplantation in fish. *Scientific Reports* **14**, 4007 (2024). https://doi.org/10.1038/s41598-024-54519-6
14. Elliott, M. *et al. Links between Fish and Other Trophic Levels*. Vol. 1 124-216 (Fishes in Estuaries, 2002).
15. Soh, M. *et al.* The intestinal digesta microbiota of tropical marine fish is largely uncultured and distinct from surrounding water microbiota. *NPJ biofilms and microbiomes* **10**, 11 (2024). https://doi.org/10.1038/s41522-024-00484-x
16. Han, G. H. *et al.* Phylosymbiosis in seven wild fish species collected off the Southern Coast of korea: skin microbiome most strongly reflects evolutionary pressures. *Microbial Ecology* **87**, 153 (2024). https://doi.org/10.1007/s00248-024-02467-z
17. Liang, Y. *et al.* Variations and interseasonal changes in the gut microbial communities of seven wild fish species in a natural lake with limited water exchange during the closed fishing season. *Microorganisms* **12**, 800 (2024). https://doi.org/10.3390/microorganisms12040800
18. Sun, H. *et al.* Impact of captivity and natural habitats on gut microbiome in Epinephelus akaara across seasons. *BMC microbiology* **24**, 239 (2024). https://doi.org/10.1186/s12866-024-03398-y
19. Zarkasi, K. Z. *et al.* Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (Salmo salar L.) within a commercial mariculture system. *Journal of applied microbiology* **117**, 18–27 (2014). https://doi.org/10.1111/jam.12514
20. Klure, D. M. & Dearing, M. D. Seasonal restructuring facilitates compositional convergence of gut microbiota in free-ranging rodents. *FEMS Microbiology Ecology* **99**, fiad127 (2023). https://doi.org/10.1093/femsec/fiad127
21. Li, T. *et al.* Bacterial signatures of “red-operculum” disease in the gut of crucian carp (Carassius auratus). *Microbial ecology* **74**, 510–521 (2017). https://doi.org/10.1007/s00248-017-0967-1
22. Zhou, C. *et al.* Association of gut microbiota with metabolism in rainbow trout under acute heat stress. *Frontiers in Microbiology* **13**, 846336 (2022). https://doi.org/10.3389/fmicb.2022.846336
23. Liu, H. *et al.* The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Scientific reports* **6**, 24340 (2016). https://doi.org/10.1038/srep24340
24. Hassenrück, C., Reinwald, H., Kunzmann, A., Tiedemann, I. & Gärdes, A. Effects of thermal stress on the gut microbiome of juvenile milkfish (*Chanos chanos*). *Microorganisms* **9**, 5 (2021). https://doi.org/10.3390/microorganisms9010005
25. Huerlimann, R. *et al.* The gut microbiome and host molecular response of a grouper to acute and chronic heat stress. *Aquaculture* **599**, 742141 (2025). https://doi.org/10.1016/j.aquaculture.2025.742141
26. Hayes, C. *et al.* Stomach microbiome simplification of a coral reef fish at its novel cold-range edge under climate change. *Molecular Ecology* **34**, e17704 (2025). https://doi.org/10.1111/mec.17704. Epub 2025 Feb 22

27. Froese, R. & Pauly, D. *THE ECOLOGY Table*, <https://fishbase.se/manual/fishbasethe_ecology_table.htm> (2000).
28. Egerton, S., Culloty, S., Whooley, J., Stanton, C. & Ross, R. P. The gut microbiota of marine fish. *Frontiers in microbiology* **9**, 873 (2018). https://doi.org/10.3389/fmicb.2018.00873
29. Kumari, S. & Rath, P. K. Extraction and characterization of chitin and chitosan from (Labeo rohit) fish scales. *Procedia Materials Science* **6**, 482–489 (2014). https://doi.org/10.1016/j.mspro.2014.07.062
30. Ngasotter, S. *et al.* Crustacean shell waste derived chitin and chitin nanomaterials for application in agriculture, food, and health–A review. *Carbohydrate Polymer Technologies and Applications* **6**, 100349 (2023). https://doi.org/10.1016/j.carpta.2023.100349
31. Ray, A., Ghosh, K. & Ringø, E. Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture nutrition* **18**, 465–492 (2012). https://doi.org/10.1111/j.1365-2095.2012.00943.x
32. Kwak, S. N., Baeck, G. W. & Huh, S.-H. Feeding habits of Stephanolepis cirrhifer in a Zostera marina bed. *Korean Journal of Ichthyology* **15**, 219–223 (2003).
33. Matsumoto, K., Kohda, M. & Yanagisawa, Y. Size-dependent feeding association of two wrasses (genus Pseudolabrus) with the morwong, Goniistius zonatus. *Ichthyological Research* **46**, 57–65 (1999).
34. Sun, P., Ling, J., Zhang, H., Tang, B. & Jiang, Y. Diet composition and feeding habits of black sea bream (Acanthopagrus schlegelii) in Xiangshan Bay based on high-throughput sequencing. *Acta Ecologica Sinica* **41**, 1221–1228 (2021). https://doi.org/10.5846/stxb201911022308
35. Akeda, K., Yodo, T., Kai, Y. & Yoshioka, M. Feeding habits of the Sebastes inermis species complex in western Wakasa Bay, central Japan. *Aquaculture Science* **60**, 207–214 (2012). https://doi.org/10.11233/aquaculturesci.60.207
36. Jeong, J. M., Kim, H. J., Baeck, G. W., Ye, S. J. & Huh, S. H. Feeding habits of ocellate spot skate, Okamejei kenojei in the coastal waters of Gadoek-do, Korea. *J Kor Soc Fish Technol* **51**, 265–271 (2015). https://doi.org/10.3796/KSFT.2015.51.2.265
37. Kwak, S. N. & Huh, S.-H. Feeding habits of Platycephalus indicus in eelgrass (Zostera marina) beds in Kwangyang Bay. *Korean Journal of Ichthyology* **14**, 29–35 (2002).
38. Yoshida, M.-a., Tanabe, T., Akiyoshi, H. & Kawamukai, M. Gut microbiota analysis of Blenniidae fishes including an algae-eating fish and clear boundary formation among isolated Vibrio strains. *Scientific reports* **12**, 4642 (2022). https://doi.org/10.1038/s41598-022-08511-7
39. Kim, P. S. *et al.* Host habitat is the major determinant of the gut microbiome of fish. *Microbiome* **9**, 166 (2021). https://doi.org/10.1186/s40168-021-01113-x
40. Sadeghi, J., Chaganti, S. R., Johnson, T. B. & Heath, D. D. Host species and habitat shape fish-associated bacterial communities: phylosymbiosis between fish and their microbiome. *Microbiome* **11**, 258 (2023). https://doi.org/10.1186/s40168-023-01697-6
41. Mazel, F. *et al.* Is host filtering the main driver of phylosymbiosis across the tree of life? *Msystems* **3**, 10.1128/msystems. 00097–00018 (2018). https://doi.org/10.1128/msystems.00097-18

42. Schwob, G. *et al.* Unveiling the co-phylogeny signal between plunderfish Harpagifer spp. and their gut microbiomes across the Southern Ocean. *Microbiology Spectrum* **12**, e03830–03823 (2024). https://doi.org/10.1128/spectrum.03830-23
43. Eichmiller, J. J., Hamilton, M. J., Staley, C., Sadowsky, M. J. & Sorensen, P. W. Environment shapes the fecal microbiome of invasive carp species. *Microbiome* **4**, 44 (2016). https://doi.org/10.1186/s40168-016-0190-1
44. Kang, J. C. & Kim, M. S. Seasonal variation in depth-stratified macroalgal assemblage patterns on Marado, Jeju Island, Korea. *Algae* **27**, 269–281 (2012). https://doi.org/10.4490/algae.2012.27.4.269
45. Wilson, R. á. Utilization of dietary carbohydrate by fish. *Aquaculture* **124**, 67–80 (1994).
46. Miegel, R., Pain, S., Van Wettere, W., Howarth, G. & Stone, D. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (Seriola lalandi). *Aquaculture* **308**, 145–151 (2010). https://doi.org/10.1016/j.aquaculture.2010.07.036
47. Graymore, M., Stagnitti, F. & Allinson, G. Impacts of atrazine in aquatic ecosystems. *Environment international* **26**, 483–495 (2001). https://doi.org/10.1016/S0160-4120(01)00031-9
48. Zhao, J. *et al.* Spatiotemporal distribution and fate of typical pesticides in the Bohai Sea and surrounding rivers, China. *Environmental Pollution* **371**, 125934 (2025). https://doi.org/10.1016/j.envpol.2025.125934
49. Matsunaka, T. *et al.* Seasonal variations in marine polycyclic aromatic hydrocarbons off Oki Island, Sea of Japan, during 2015–2019. *Marine pollution bulletin* **180**, 113749 (2022). https://doi.org/10.1016/j.marpolbul.2022.113749
50. Di, D. Y., Lee, A., Jang, J., Han, D. & Hur, H.-G. Season-specific occurrence of potentially pathogenic Vibrio spp. on the southern coast of South Korea. *Applied and Environmental Microbiology* **83**, e02680–02616 (2017). https://doi.org/10.1128/AEM.02680-16
51. Voráčová, M., Zore, M., Yli-Kauhaluoma, J. & Kiuru, P. Harvesting phosphorus-containing moieties for their antibacterial effects. *Bioorganic & Medicinal Chemistry* **96**, 117512 (2023). https://doi.org/10.1016/j.bmc.2023.117512
52. Ribeiro, M. & Simões, M. Advances in the antimicrobial and therapeutic potential of siderophores. *Environmental Chemistry Letters* **17**, 1485–1494 (2019). https://doi.org/10.1007/s10311-019-00887-9
53. Kim, I. S., Lee, C. R., Lee, Y. J. & Kim, B. J. *Illustrated Book of Korean Fishes*. (Kyohaksa, 2005).
54. Myeong, J. G. *The sea fishes of Korea*. (Yejowon, 2016).
55. Yu, J. *et al.* Comparison of intestine microbiota between wild and farmed Korean rockfish, Sebastes schlegelii. *Ocean Science Journal* **56**, 297–306 (2021). https://doi.org/10.1007/s12601-021-00022-2
56. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature biotechnology* **37**, 852–857 (2019). https://doi.org/10.1038/s41587-019-0209-9
57. McLaren, M. R. & Callahan, B. J. Silva 138.1 prokaryotic SSU taxonomic training data formatted for DADA2 [data sets]. *Zenodo* (2021). https://doi.org/10.5281/zenodo.4587954.

58. Bisanz, J. E. *Tutorial: Integrating QIIME2 and R for data visualization and analysis using qiime2R v0.99.6* (2021).
59. Oksanen, J. *et al. vegan: Community Ecology Package* (2026).
60. Schloss, P. D. Rarefaction is currently the best approach to control for uneven sequencing effort in amplicon sequence analyses. *Msphere* **9**, e00354–00323 (2024). https://doi.org/10.1128/msphere.00354-23
61. McMurdie, P. J. & Holmes, S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one* **8**, e61217 (2013). https://doi.org/10.1371/journal.pone.0061217
62. Kembel, S. W. *et al.* Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* **26**, 1463–1464 (2010). https://doi.org/10.1093/bioinformatics/btq166
63. Ricotta, C. & Podani, J. On some properties of the Bray-Curtis dissimilarity and their ecological meaning. *Ecological Complexity* **31**, 201–205 (2017). https://doi.org/10.1016/j.ecocom.2017.07.003
64. Lozupone, C. A., Hamady, M., Kelley, S. T. & Knight, R. Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and environmental microbiology* **73**, 1576–1585 (2007). https://doi.org/10.1128/AEM.01996-06
65. Anderson, M. J. & Walsh, D. C. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecological monographs* **83**, 557–574 (2013). https://doi.org/10.1890/12-2010.1
66. Mallick, H. *et al.* Multivariable association discovery in population-scale meta-omics studies. *PLoS computational biology* **17**, e1009442 (2021). https://doi.org/10.1371/journal.pcbi.1009442
67. Pauly, D. & Sa-a, P. *Estimating trophic levels from individual food items*, <https://www.fishbase.se/manual/English/FishbaseThe_FOOD_ITEMS_table.htm> (2000).
68. Pauly, D. & Palomares, M. L. *Preliminary estimation of trophic levels in fish species without food composition data*, <https://www.fishbase.se/manual/English/FishbaseThe_FOOD_ITEMS_table.htm> (2000).
69. Douglas, G. M. *et al.* PICRUSt2 for prediction of metagenome functions. *Nature biotechnology* **38**, 685–688 (2020). https://doi.org/10.1038/s41587-020-0548-6
70. Yang, C. *et al.* ggpicrust2: an R package for PICRUSt2 predicted functional profile analysis and visualization. *Bioinformatics* **39**, btad470 (2023). https://doi.org/10.1093/bioinformatics/btad470
71. Kuhn, M. Building predictive models in R using the caret package. *Journal of statistical software* **28**, 1–26 (2008). https://doi.org/10.18637/jss.v028.i05
72. Liaw, A. & Wiener, M. Classification and regression by randomForest. *R news* **2**, 18–22 (2002).

Figures

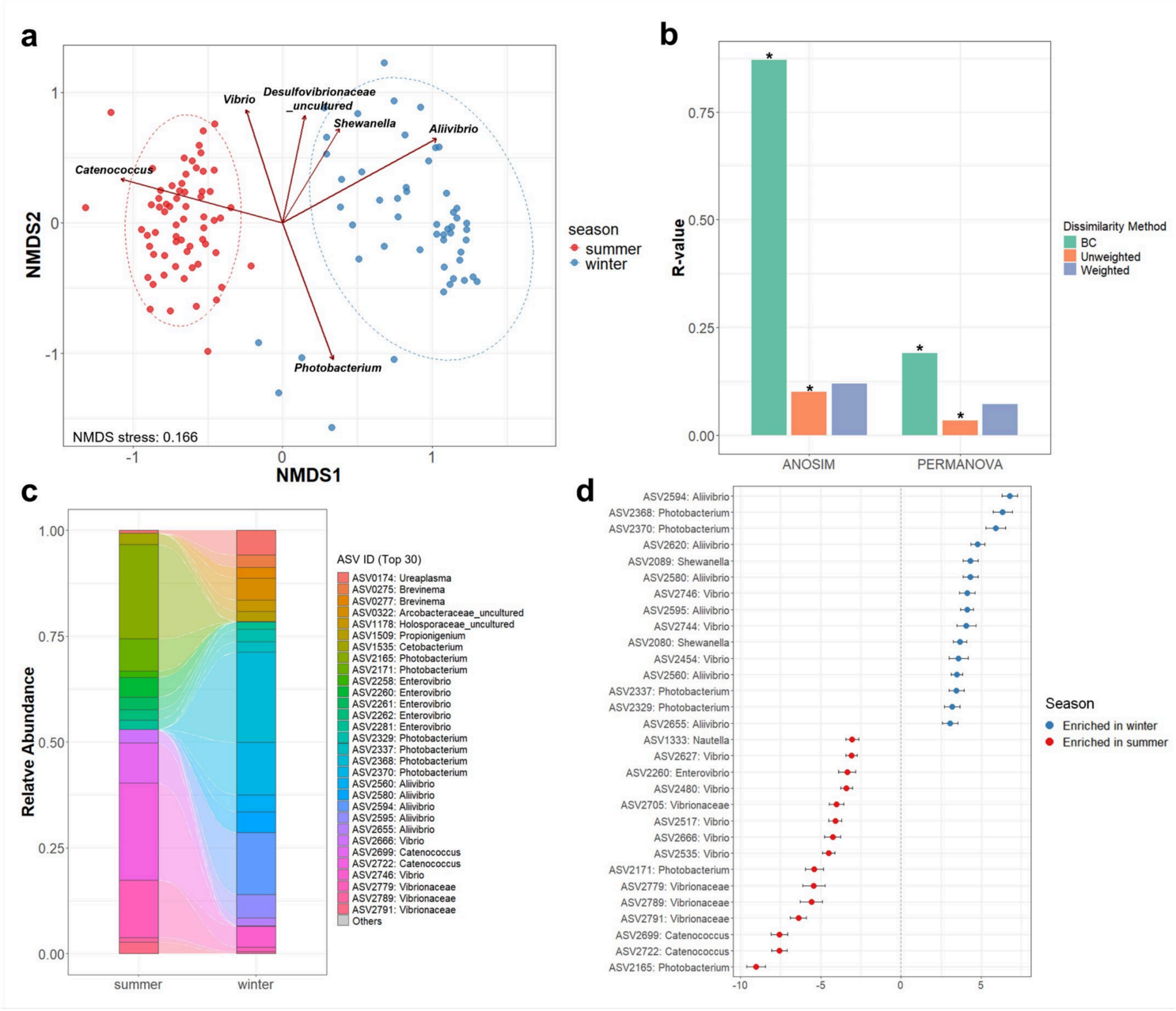


Figure 1

Comparison of fish gut microbial communities by season. **a** NMDS calculated based on Bray-Curtis dissimilarity at the ASV level (NMDS stress: 0.166). Ellipses represent the standard deviation (0.95) from each centroid (summer and winter). Brown arrows indicate the effects of six taxa (aggregated to genus) on the NMDS ordination. These taxa showed significant correlation with the NMDS ordination, and the top six R^2 values were selected. **b** Each R-value of ANOSIM and PERMANOVA by three dissimilarity measurements (BC, Bray-Curtis; Unweighted, Unweighted Unifrac; Weighted, Weighted Unifrac). All p-values were 0.0001. Asterisk marks (*) on the bar represent the homogeneity of dispersion of GM between summer and winter. **c** Seasonal flow diagram of GM according to relative abundance at the ASV level. This flow diagram was determined through seasonal aggregation of 116 samples. ASVs in the flow chart showed seasonality based on MaAsLin2 and represent the 30 most abundant ASVs across all

microbial communities. **d** Forest plots displaying MaAsLin2 coefficients and standard errors of seasonal ASVs. The seasonal ASVs were selected based on coefficients greater than 3 or less than -3.

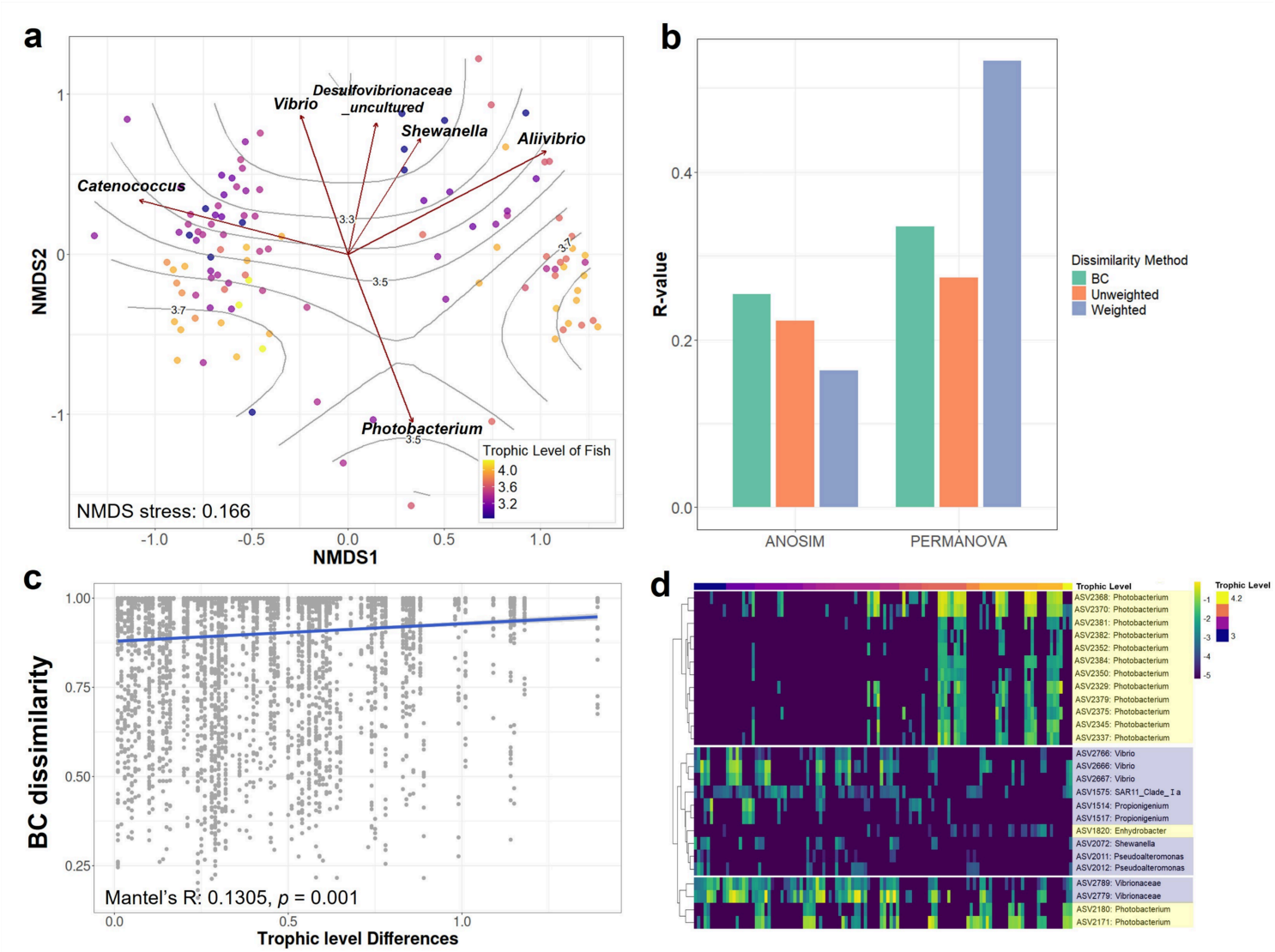


Figure 2

Comparison of fish gut microbial communities by trophic level. **a** NMDS calculated based on Bray-Curtis dissimilarity at the ASV level (NMDS stress: 0.166). Contour lines and colors represent the fish's trophic levels. Brown arrows indicate the effects of six taxa (aggregated to genus) on the NMDS ordination. These taxa showed significant correlation with the NMDS ordination, and the top six R^2 values were selected. **b** Each R-value of ANOSIM and PERMANOVA by three dissimilarity measurements (BC, Bray-Curtis; Unweighted, Unweighted Unifrac; Weighted, Weighted Unifrac). All p-values were 0.0001, whereas the dispersion between trophic levels significantly differed. **c** The association between the fish GM and the trophic level using Mantel's test (R = 0.1305, *p* = 0.001). The dissimilarity between GMs was measured by BC. **d** Heatmap for the log-transformed relative abundance of ASVs, which were significantly correlated with trophic level using MaAsLin2. The ASVs marked in yellow showed a positive correlation with trophic level, while those marked in navy showed a negative correlation.

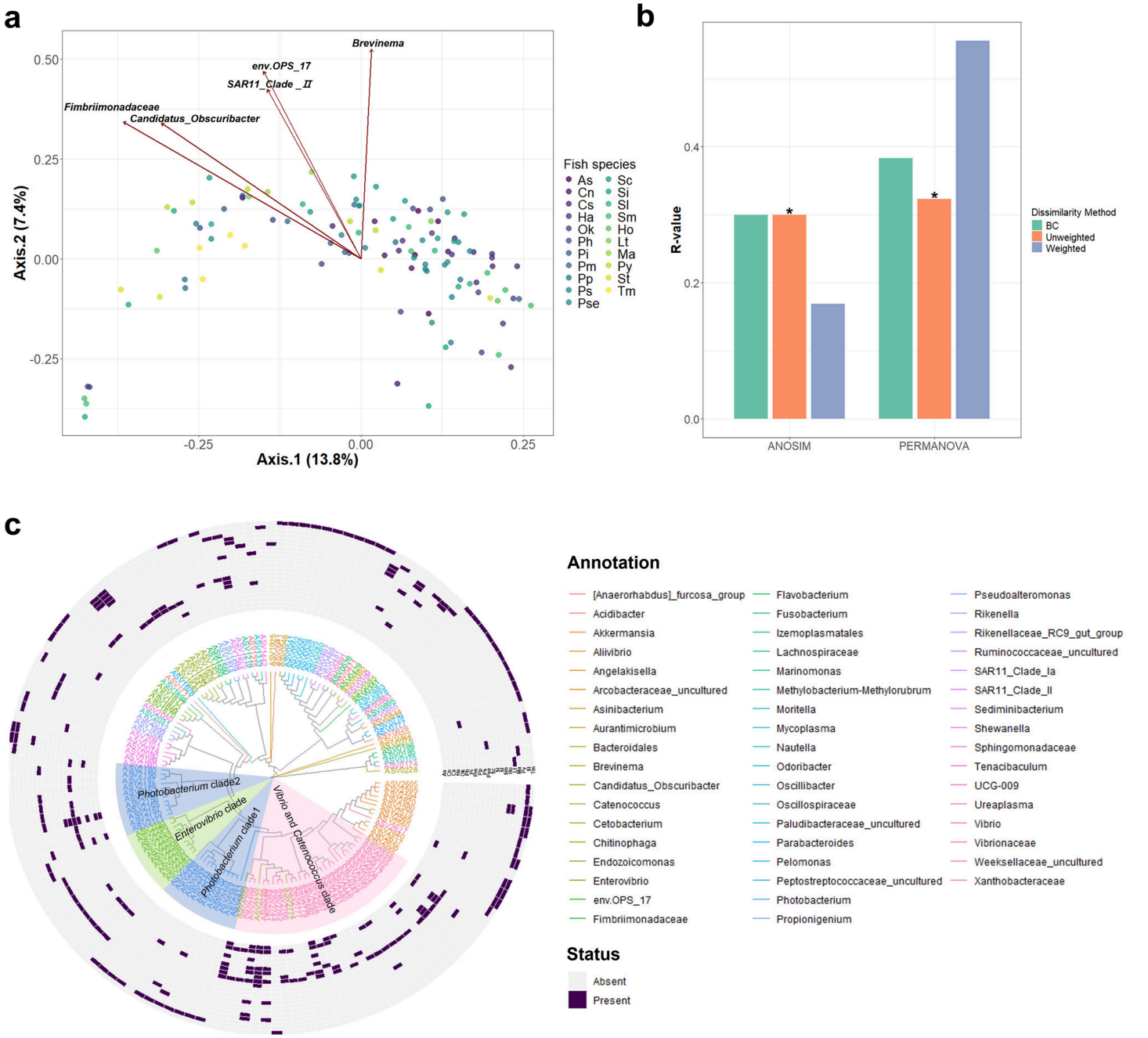


Figure 3

Comparison of fish gut microbial communities by fish species. **a** PCoA measured by Unweighted UniFrac dissimilarity at the ASV level. Brown arrows indicate the effects of five taxa (aggregated genus) on the NMDS ordination. These taxa showed significant correlation with the NMDS ordination, and the top five R^2 values were selected. **b** Each R-value of ANOSIM and PERMANOVA by three dissimilarity measurements (BC, Bray-Curtis; Unweighted, Unweighted Unifrac; Weighted, Weighted Unifrac). All p-values were 0.0001. Asterisk marks (*) on the bar represent the homogeneity of dispersion of GM among 21 fish species. **c** Circular cladogram reporting core ASVs by fish species (Fish species-core ASVs) in the

GM. Fish species-core ASVs were detected by each of the 16 fish species, and the total number of ASVs is 145 (threshold for prevalence (0.99) and detection abundance (1E-05)).

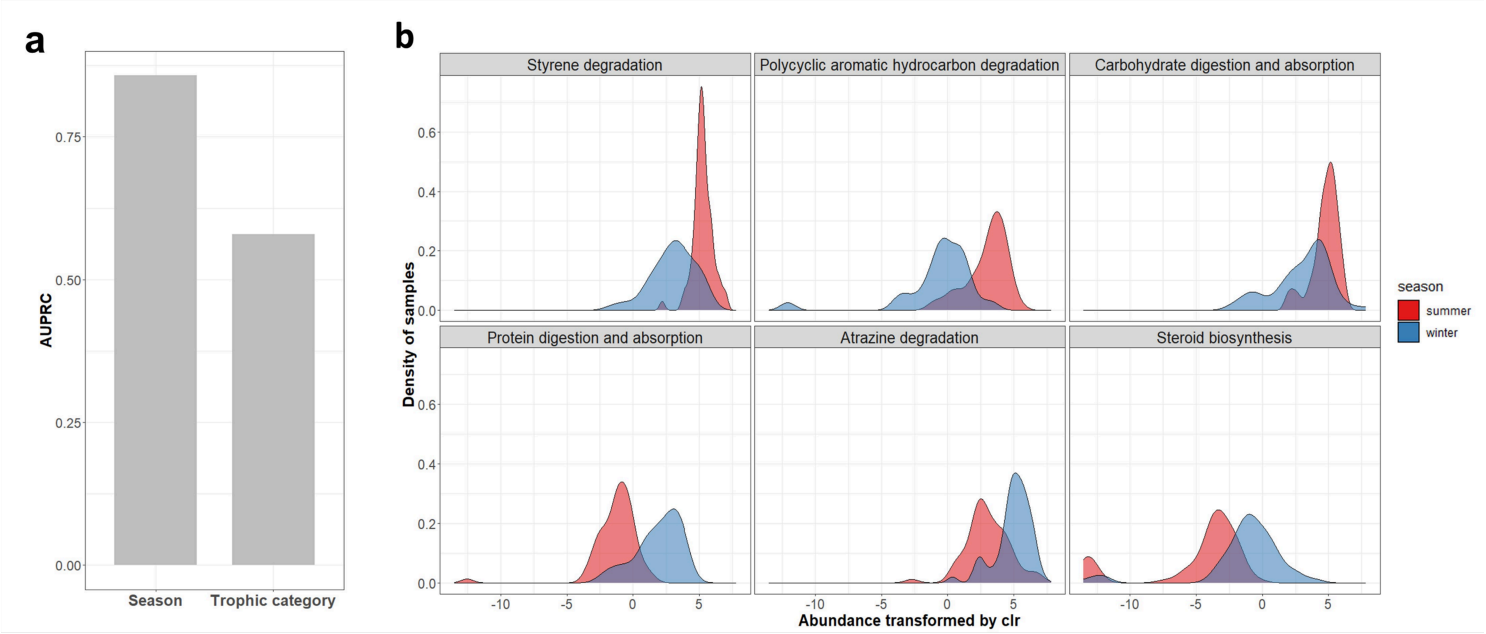


Figure 4

Seasonal effect on predicted functional gene distribution in the fish gut using PICRUSt2. **a** AUPRC for random forest classifiers for season and trophic category of functional gene composition in the fish gut. **b** Center-log ratio (CLR)-transformed abundance of discriminative KOs of the fish GM from summer and winter. We selected the top three KOs per season, based on feature importance in the random forest classification model.

Supplementary Files

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