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Abundance-based and presence-based signals distinguish season, trophic level and host species effects on the wild marine fish gut microbiome

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Abstract

Wild fish gut microbiomes are shaped by extrinsic and intrinsic factors, but whether these factors act on taxon abundance or on lineage composition remains unclear. We profiled the gut microbiome of 21 wild marine fish species (n = 115) by 16S rRNA V3–V4 sequencing and compared Bray-Curtis (taxon abundance), Weighted UniFrac (abundance weighted by phylogenetic branch length) and Unweighted UniFrac (presence or absence of phylogenetic lineages) dissimilarities. Season was more strongly associated with Bray–Curtis than with Unweighted UniFrac dissimilarity (R^2 = 0.191 versus 0.034), with no significant dispersion difference between seasons for either metric. Trophic level as a categorical factor showed heterogeneous dispersion in all three metrics; as a continuous variable at the species level, distance-based redundancy analysis (dbRDA) associated it only with Bray–Curtis (adjusted R^2 = 0.047, p = 0.019). For host species, dispersion was heterogeneous for Bray–Curtis and Weighted UniFrac but not Unweighted UniFrac, and species-core ASVs clustered phylogenetically within 10 of 16 species, compatible with host filtering. Predicted functional profiles separated more clearly by season (AUPRC = 0.857) than by trophic category (0.579). Season and trophic level were thus associated mainly with taxon abundance, whereas host species was associated with phylogenetic structure.

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 aquatic ecosystems 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 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 are 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 fish trophic levels[15]. Intrinsic host factors, such as species, genetics, immunity, and physiology, influence the fish GM, and fish species identity has consistently been 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 identified factors that influence the GM using a single metric, such as taxonomic composition, alpha diversity, or beta diversity. These studies show that the GM responds to many extrinsic and intrinsic factors, but not whether these factors act in the same way. A factor may change the relative abundance of taxa that are already present in the GM. Alternatively, it may determine which taxa are present. β-diversity metrics differ in their sensitivity to these two types of change. Quantitative metrics such as Bray-Curtis (BC) dissimilarity respond to shifts in relative abundance, whereas qualitative metrics such as Unweighted UniFrac consider only which lineages are present and are most informative when communities differ in which taxa can persist. Comparing the effect of each factor across these metrics within the same dataset can therefore indicate how that factor structures the GM. We hypothesized that extrinsic factors, which vary over short time scales, act mainly by shifting the relative abundance of shared taxa, whereas host species acts mainly by determining which lineages are present, consistent with host filtering. To test this hypothesis, we characterized the GM of 21 wild marine fish species using 16S rRNA V3–V4 amplicon sequencing and compared the effects of season, trophic level, and fish species across abundance-based and presence-based metrics at the levels of β-diversity, α-diversity, and individual taxa.

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 non-metric multidimensional scaling (NMDS) ordination (stress = 0.166; 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). The test for homogeneity of

multivariate dispersions (PERMDISP) showed no significant difference in dispersion between seasons for BC ($p = 0.279$) or Unweighted UniFrac ($p = 0.231$), whereas dispersion differed significantly for Weighted UniFrac ($p = 0.0014$; Supplementary Table S1a). Under heterogeneous dispersion, a significant PERMANOVA or ANOSIM result can reflect differences in within-group spread rather than in group centroids, which reduced the reliability of this metric for between-group comparisons. Among the three metrics, BC yielded the highest ANOSIM R (0.871) and PERMANOVA R^2 (0.191), 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). Among the 30 most abundant ASVs with significant seasonal differences in MaAsLin2, those affiliated with *Cetobacterium*, *Enterovibrio*, *Vibrio*, *Catenococcus*, unclassified *Vibrionaceae*, and *Photobacterium* were characteristic of summer, whereas those affiliated with *Ureaplasma*, *Brevinema*, uncultured *Arcobacteraceae*, uncultured *Holosporaceae*, *Propionigenium*, *Aliivibrio*, and other *Photobacterium* ASVs were characteristic of winter (Fig. 1c). Among ASVs with MaAsLin2 coefficients greater than 3 or less than − 3, *Photobacterium* (ASV2165 and ASV2171), *Catenococcus* (ASV2699 and ASV2722), unclassified *Vibrionaceae* (ASV2705, ASV2779, ASV2789, and ASV2791), *Vibrio* (ASV2480, ASV2517, ASV2535, ASV2627, and ASV2666), *Enterovibrio* (ASV2260), and *Nautella* (ASV1333) ASVs were enriched in summer (Fig. 1d). In contrast, *Aliivibrio* (ASV2560, ASV2580, ASV2594, ASV2595, ASV2620, and ASV2655), *Photobacterium* (ASV2329, ASV2337, ASV2368, and ASV2370), Shewanella (ASV2080 and ASV2089), and *Vibrio* (ASV2454, ASV2744, and ASV2746) ASVs were enriched in winter. Thus, all seasonally enriched *Catenococcus* and *Enterovibrio* ASVs were enriched in summer and all *Aliivibrio* ASVs in winter, whereas *Photobacterium* and *Vibrio* contained ASVs enriched in each season.

GM composition varied with trophic level in the NMDS ordination based on BC dissimilarity (Fig. 2a). Among the genera correlated with the ordination, *Vibrio* was oriented toward fish with lower trophic levels (≤ 3.4) and *Photobacterium* toward fish with higher trophic levels (> 3.4). When trophic level was treated as a categorical factor, it was significantly different in GM composition across all three dissimilarity metrics. However, the test for homogeneity of PERMDISP showed that dispersion differed significantly among trophic level groups across all three metrics ($p < 0.01$; Fig. 2b and Supplementary Table S1b). Because these groups largely corresponded to individual host species (Supplementary Table S2), we tested the association between trophic level, treated as a continuous variable, and species-level GM composition using distance-based redundancy analysis (dbRDA). Trophic level was significantly associated with BC-based GM composition among the 21 species ($R^2 = 0.0945$, adjusted $R^2 = 0.0469$, $p = 0.019$; Fig. 2c), whereas no significant association was detected with Unweighted or Weighted UniFrac distances (Supplementary Table S3). The BC-based association remained significant when summer (n = 15 species; adjusted $R^2 = 0.1066$, $p = 0.005$) and winter (n = 13 species; adjusted $R^2 = 0.1233$, $p = 0.0091$) samples were analyzed separately (Supplementary Fig. S2a,b). These results indicate that trophic level is associated with differences in the relative abundance of GM taxa rather than with their phylogenetic composition, and that this association is not attributable to seasonal differences alone. Gut microbial diversity (Simpson, Shannon, and Faith's PD) did not differ significantly among trophic level groups and

was not correlated with trophic level (Supplementary Fig. S1b and Supplementary Table S4). MaAsLin2 identified 26 ASVs significantly associated with trophic level ($q < 0.05$; Fig. 2d and Supplementary Fig. S3). Twelve *Photobacterium* ASVs (ASV2329, ASV2337, ASV2345, ASV2350, ASV2352, ASV2368, ASV2370, ASV2375, ASV2379, ASV2381, ASV2382, and ASV2384) were positively associated with trophic level, whereas ASVs affiliated with *Vibrio* (ASV2666, ASV2667, and ASV2766), SAR11 clade Ia (ASV1575), *Propionigenium* (ASV1514 and ASV1517), *Shewanella* (ASV2072), and *Pseudoalteromonas* (ASV2011 and ASV2012) were negatively associated with trophic level. *Photobacterium* and *Vibrio* were therefore the genera most consistently associated with high and low trophic levels, respectively.

Fish species is associated with the presence of particular GM lineages

GM samples did not form distinct clusters by fish species in the NMDS ordination based on Unweighted UniFrac dissimilarity (stress = 0.044; Fig. 3a). Even after five outlier samples were excluded for visualization, GM composition varied within species and overlapped among species (Supplementary Fig. S4). Accordingly, the genera correlated with the ordination (*Lawsonella*, *Neptuniibacter*, *Photobacterium*, and *Sarcina*) could not be assigned to specific host species. Nevertheless, fish species significantly influenced the GM composition across all three dissimilarity metrics (Fig. 3b and Supplementary Table S1c). PERMDISP showed significant differences in dispersion among species for BC ($p < 0.001$) and Weighted UniFrac ($p = 0.0028$) but not for Unweighted UniFrac ($p = 0.057$; Supplementary Table S1c), which considers only which lineages are present, suggesting that species differences were associated with community membership rather than with the relative abundance of taxa. Consistent with this, Faith's PD differed significantly among species (Kruskal-Wallis test, $p = 0.0095$), whereas Simpson and Shannon diversity did not (Supplementary Fig. S1c). Significant pairwise differences in Faith's PD were found between Py and Cs, Py and Ok, Tm and Ok, Sm and Pm, Py and Sm, and Tm and Sm (Conover's test with BH correction). Because the species effect was expressed mainly in which ASVs were present, we characterized species specificity using ASVs consistently detected within each species (fish species-core ASVs) rather than differential abundance analysis (DAA). When samples from both seasons were combined, 145 core ASVs were identified in 16 species, whereas no core ASVs were detected in Cs, Ok, Sc, Si, or Sm (prevalence ≥ 0.99; Fig. 3c and Supplementary Table S5). In the cladogram, core ASVs clustered within host species for As, Cn, Ma, Ph, Pi, Pm, Pp, Sl, St, and Tm (Fig. 3c). For example, the core ASVs of Cn and Pp clustered in the clade containing *Vibrio* and *Catenococcus*, those of Ph and Sl in the *Enterovibrio* clade, and *Photobacterium* core ASVs formed two clades, one containing St-core ASVs and the other 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 S5). However, the core ASVs of seven fish species (Cs, Ok, Ps, Pse, Sc, Si, and Sm) were detected within a single season (Supplementary Table S6) and showed phylogenetic similarities within each fish species (Supplementary Fig. S5a-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.

Discussion

Consistent with our hypothesis, season and trophic level were associated mainly with shifts in the relative abundance of taxa shared across groups, whereas fish species was associated with which lineages were present in the GM. Season had a much larger effect on BC than on Unweighted UniFrac dissimilarity, and trophic level was associated with species-level GM composition only in BC dissimilarity. In contrast, differences among fish species were interpretable only with Unweighted UniFrac and were accompanied by differences in Faith's PD but not in Shannon or Simpson diversity.

The fish gut microbiome 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 vary 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. S9). 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. S9). 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. S9). *Catenococcus* abundance increases during recovery from prolonged heat stress in the pyloric caeca of the Malabar grouper (*Epinephelus malabaricus*)[24]. *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[25,26]. 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.

Trophic level was significantly associated with species-level GM composition in BC but not in UniFrac dissimilarity, consistent with differences in the relative abundance of taxa rather than in which lineages were present. All 21 species had trophic levels of 2.8 or higher, which FishBase classifies as feeding mainly on animals[27], and the association was weak (Adjusted $R^2 = 0.047$; Fig. 2c). 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 ≥ 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. A recent study reported that the gut microbiomes of three-spine stickleback fish differ at the population level, despite sharing the same species and environmental conditions[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 S5). 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 5 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.

Season also separated the predicted functional profiles more clearly than trophic category did (AUPRC = 0.857 vs. 0.579; Fig. 4a), which may reflect the seasonal shifts in the relative abundance of taxa described above. Seasonal changes showed functional differences in 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. Pathogenic bacteria, particularly *Vibrio* species, are more abundant in the ocean during summer than in winter[47]. 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[48]. 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)[49]. The summer enrichment of these pathways may therefore indicate a higher antibacterial potential of the fish GM, or it may reflect the summer increase in *Vibrionaceae* (Fig. 1d).

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 purchased alive from Docheon Port Market, Tongyeong, South Korea. In March 2022, 13 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 purchased from the same market. Seven species (Cs, Ok, Ps, Pse, Sc, Si, and Sm) were collected at both sampling times, resulting in 21 unique species. All fish had been caught for human consumption, and no animals were captured, bred, or maintained for this study. To reduce transport stress, fish were transported in aerated containers to the Fisheries Resource Research Institute of Gyeongsangnam-do and held for approximately 3 h in fiberglass tanks supplied with local seawater and continuous aeration before euthanasia. No experimental procedures were applied before euthanasia. Because the study involved only euthanasia and tissue collection from commercially obtained fish, it fell outside the scope of review by the Institutional Animal Care and Use Committee of the Korea Institute of Ocean Science and Technology, and ethical approval was not required.

Fish were euthanized by immersion in seawater containing 300 mg/L tricaine methane sulfonate (MS-222; Tricaine-S, Syndel Laboratories, Ferndale, WA, USA). Fish were considered dead after opercular movement ceased and no response to tactile stimulation was observed, and they were kept in the solution for approximately 10 min thereafter. No secondary physical method (e.g., exsanguination) was applied, to avoid additional host physiological responses that could alter the gut contents; otherwise, the procedure followed the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition[50]. Total length, body length, and weight were recorded for each fish. Each fish was photographed and identified at the species level based on established taxonomic references[51,52]. Fish were rinsed with phosphate-buffered saline (PBS) to remove surface contaminants before 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 by gentle hindgut compression into 2 mL microtubes (Axygen, USA) containing 500 µL PBS. Samples were temporarily stored on dry ice to inhibit bacterial growth and then stored at − 80°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[53]. Amplicon indexing, purification, and pooling were performed as previously described[16]. Negative controls were included during sample processing, comprising one extraction blank (PBS processed through the full extraction protocol) and one no-template PCR control. After amplification, neither control showed a visible band on gel electrophoresis, whereas the sample libraries processed in the same batch did. The control libraries did not meet the minimum concentration required by the sequencing facility and were therefore not sequenced.

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[54] 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[55]. 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[56]. 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[57] (Supplementary Fig. S9). Although rarefaction omits valid data, it controls for variations in sequencing depth when measuring

alpha and beta diversity[58]. 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[59], and Faith's phylogenetic diversity (PD) index was calculated using *picante* package[60]. 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[61]. Unweighted UniFrac is a qualitative measure that calculates the fraction of the phylogenetic tree branch length unique to each community[62]. The weighted UniFrac is a quantitative measure that incorporates both phylogenetic distance and taxon abundance [62]. Aitchison dissimilarity was applied to β-diversity analysis of predicted functional pathway data. β-diversity results were visualized using non-metric multidimensional scaling (NMDS), indicator taxa for each group were identified using envfit function in the *vegan* package[57]. 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[63].

Differential abundance analysis (DAA) was performed using Microbiome Multivariate Association with Linear Models (MaAslin2) using the *MaAslin2* package[64]. 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.

Trophic levels of the 21 fish species were obtained from FishBase (version 07/2026) (Supplementary Table S7). Trophic levels were estimated from diet composition data for Ha and Pp and from individual food items for 15 species (Sc, Ps, As, Py, Ph, Ma, Cn, Pm, Tm, Ho, Sl, Cs, Ok, Si, and Pi)[67]. For Pse, Lt, Sm, and St, for which no trophic level estimates based on food data were available, FishBase provides model-based estimates derived from the body size and trophic levels of taxonomically closest relatives[68]. Trophic level was analyzed as a continuous variable rather than as a categorical factor because the 21 species represented 18 distinct trophic level values, which produced small and unequal groups with heterogeneous multivariate dispersion. Because trophic level values were assigned per species, GM profiles were aggregated at the fish species level to avoid pseudo-replication. Species-level profiles were obtained by averaging the rarefied read counts of each ASV across individuals of the same species. BC,

unweighted UniFrac, and weighted UniFrac distances were calculated from the aggregated profiles, and the association between trophic level and GM composition was tested by dbRDA using the *dbrda* function in the vegan package[59] with Lingoes correction for negative eigenvalues[65]. Significance was assessed with 9,999 permutations.

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)[66]. 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[67]. 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[67,68]. A random forest classifier was implemented using the *caret* and *randomForest* packages[69,70] 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.

Ethics declaration

Fish were purchased alive from a commercial fish market, having been caught for human consumption, and no animals were captured, bred, or maintained specifically for this study. Because the fish were commercially obtained, formal approval from an animal ethics committee was not required, as confirmed by the Institutional Animal Care and Use Committee of the Korea Institute of Ocean Science and Technology. All procedures followed the guidelines of the Institutional Animal Care and Use Committee of the Korea Institute of Ocean Science and Technology and the Animal Protection Act of the Republic of Korea, were reported according to the ARRIVE guidelines 2.0, and were designed to minimize stress and suffering.

Declarations

Competing Interests

All authors declare no financial competing interests.

Author Contribution

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 reviewed the final manuscript.

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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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Figures

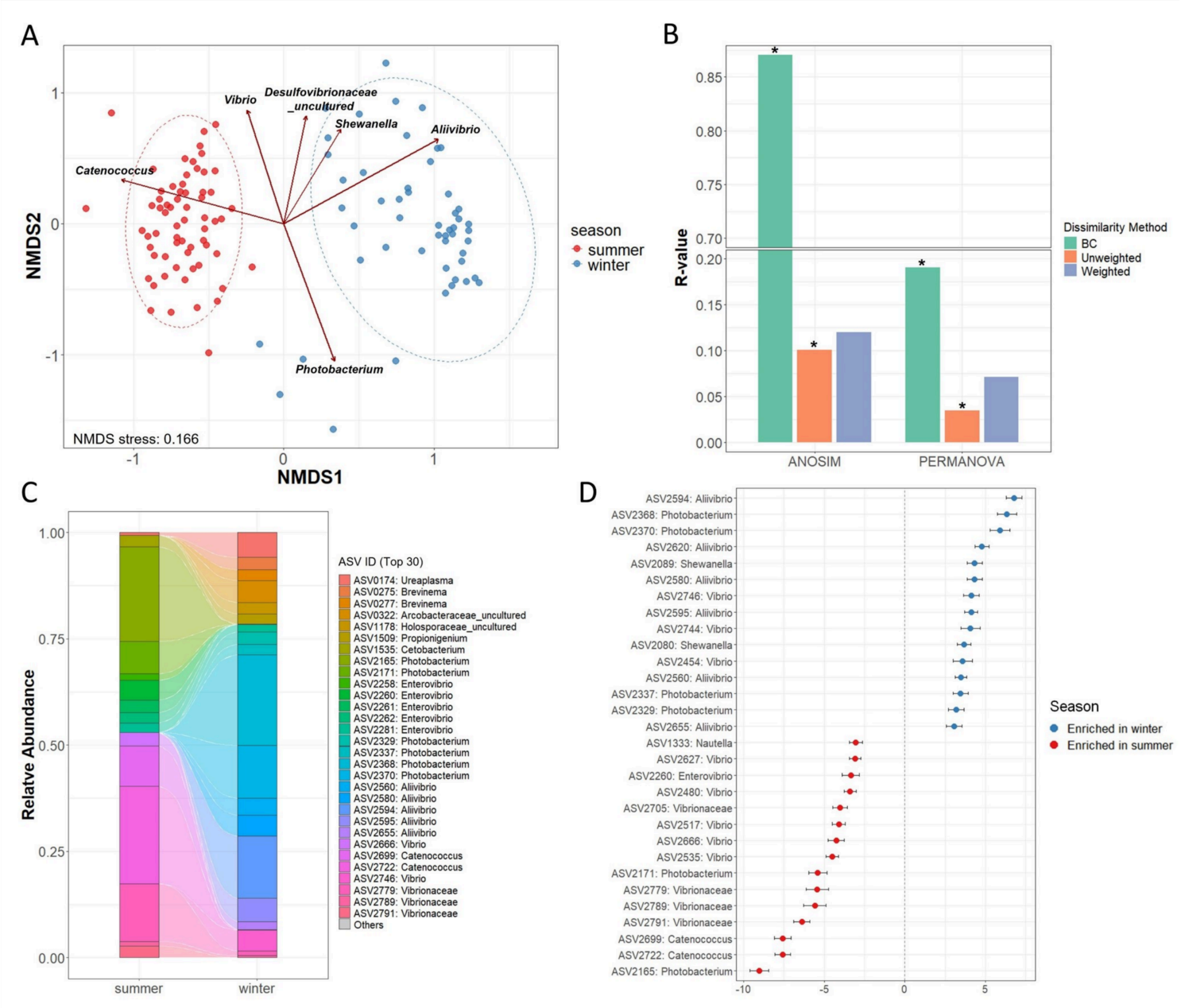


Figure 1

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.

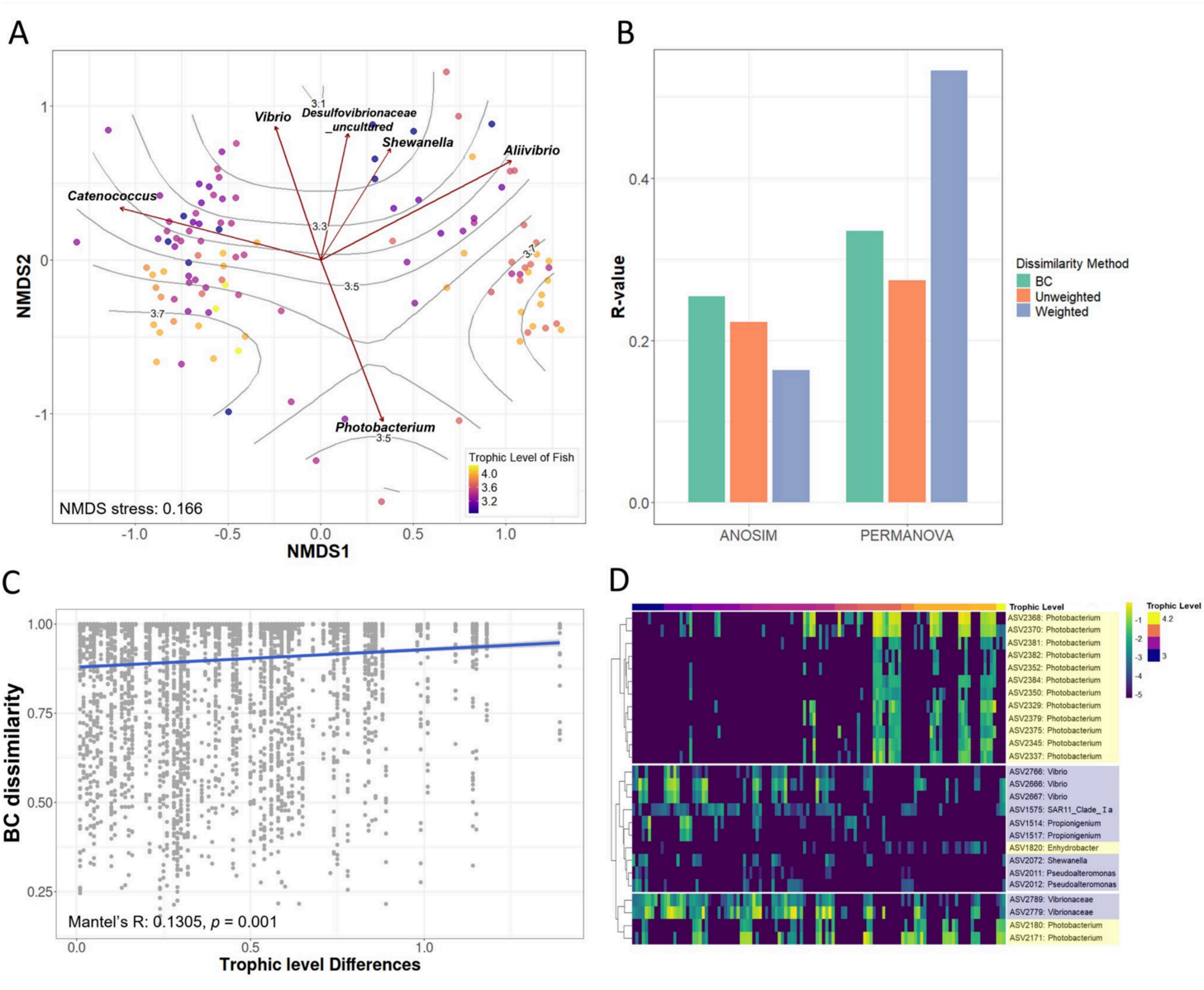


Figure 2

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 trophic level and fish species-level GM composition using BC-based RDA (R^2 = 0.0945, Adjusted R^2 = 0.0469, p = 0.019). **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

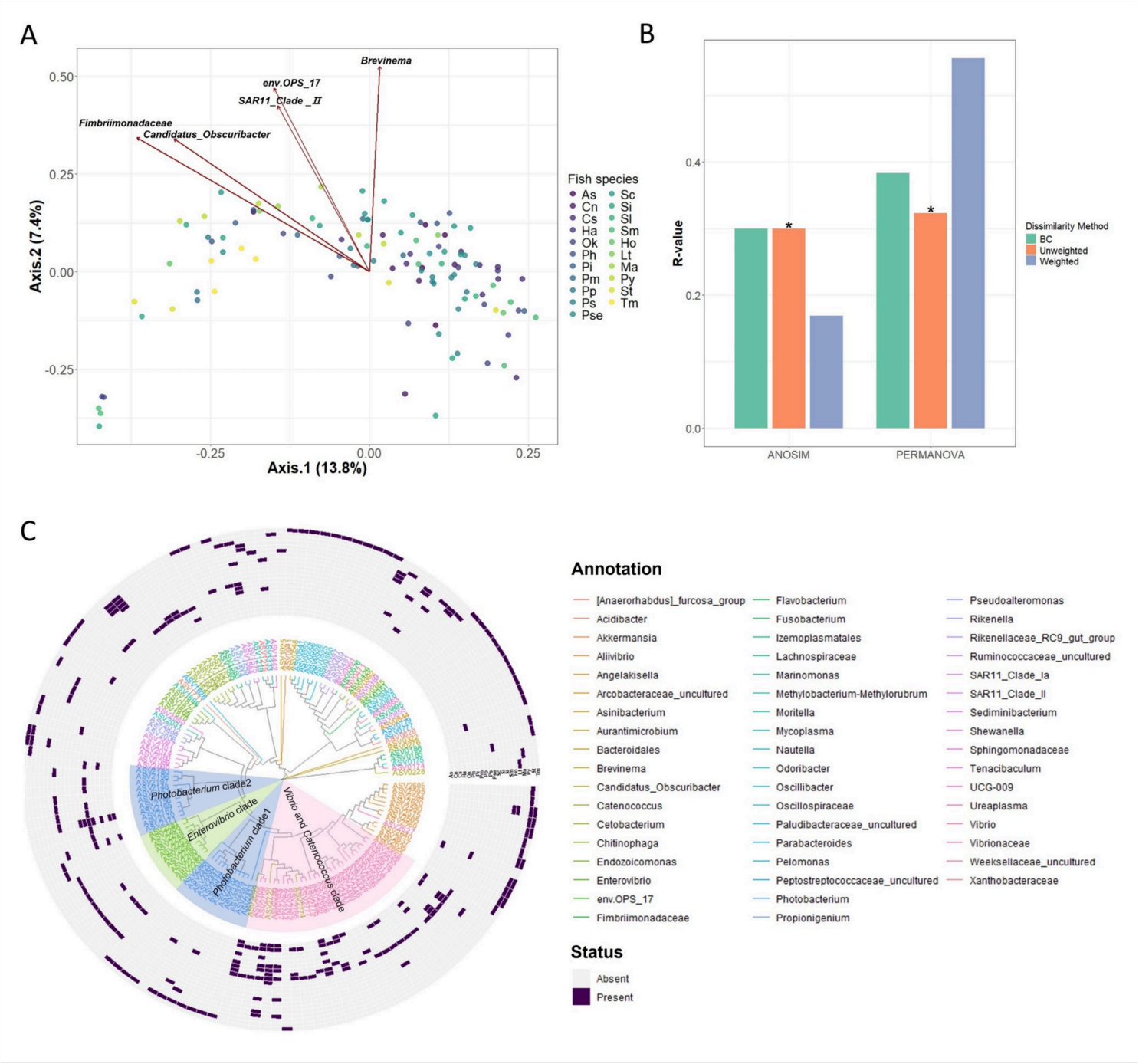


Figure 3

Comparison of fish gut microbial communities by fish species. **a** NMDS measured by Unweighted UniFrac dissimilarity at the ASV level (NMDS stress: 0.044). 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)).

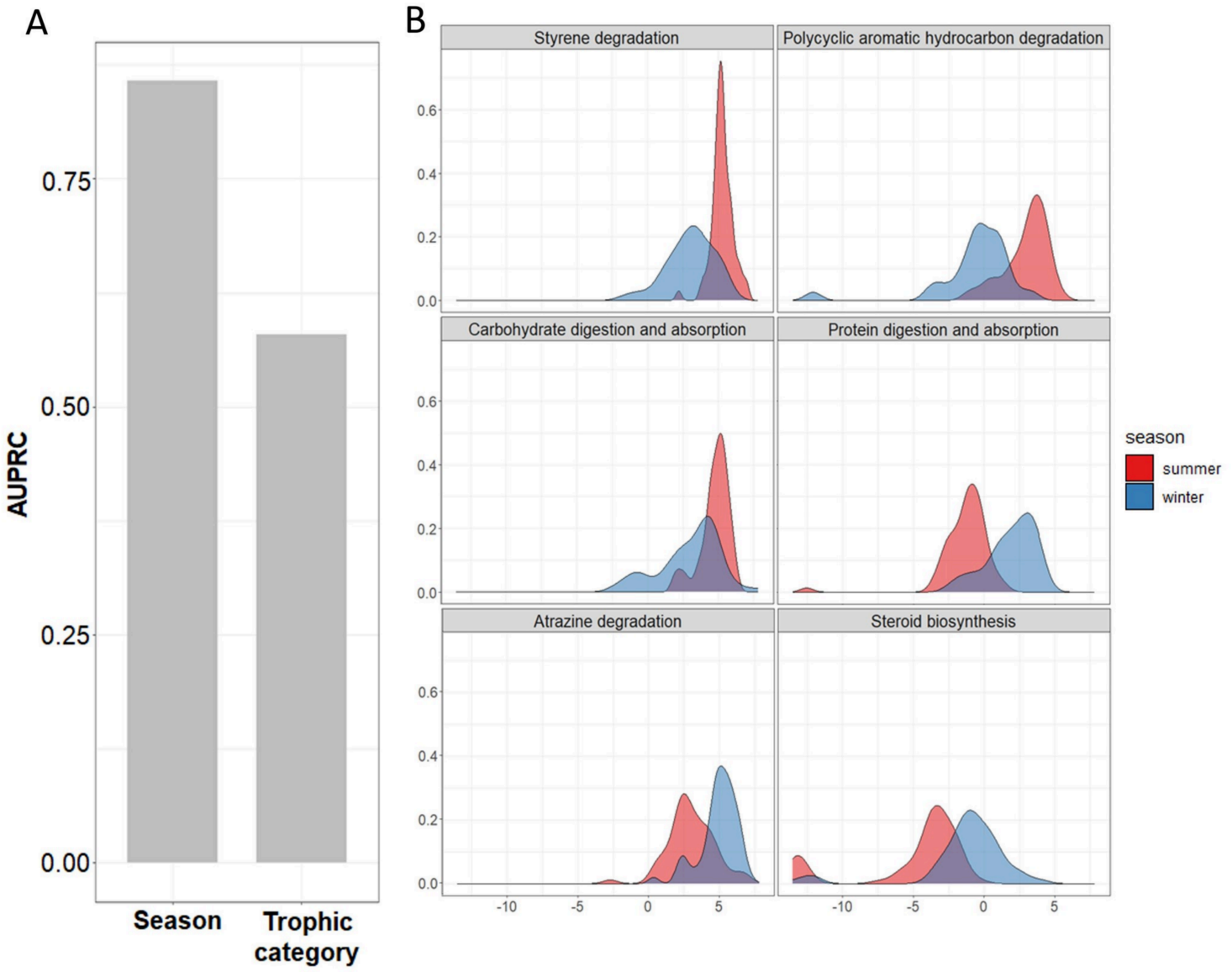


Figure 4

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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