Midgut bacterial microbiota of 12 fish species from a marine protected area in the Aegean Sea (Greece)

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ABSTRACT

Fish microbiome science is progressing fast, but it is biased toward farmed or labora-tory fish species against natural fish populations, which remain considerably underinvestigated. We analysed the midgut bacterial microbiota of 45 specimens of 12 fish species collected from the Gyaros Island marine protected area (Aegean Sea, Greece). The species belong to seven taxonomic families and are either herbivores or omnivores. Mucosa midgut bacterial diversity was assessed by amplicon metabarcoding of the 16S rRNA V3–V4 gene region. A total of 854 operational tax-onomic units were identified, of which 28 were shared among all samples. The majority of the dominating bacterial taxa have been reported to occur both in wild and farmed fish populations. The midgut bacterial communities were different among the 12 fish, except for *Pagrus pagrus* and *Pagellus erythrinus*, which belong to the Sparidae family. No differentiation of the midgut mi-crobiota was found based on feeding habits: omnivorous vs. carnivorous. Comparing wild and farmed *P. pagrus* midgut microbiota revealed considerable variation between them. Our results expand the gut microbiota of wild fish and also support the host phylogeny as the prime factor shaping intestinal microbiota.

Keywords: host-microbe associations; fish; microbiota; microbiome; intestinal; midgut; symbiosis; fisheries; Mediterranean

INTRODUCTION

Research on fish microbiomes is currently transitioning from a rather observational, i.e. structural diversity, to a more interventionist phase, i.e., looking for ways to ma-nipulate specific microbiomes of animals of human interest to benefit the host and the environment [Luna et al. 2022]. Such manipulations are extremely restricted, if not im-possible, for animals living in the wild. In addition, farmed animals experience a major deviation in their growth and environmental conditions compared with their natural counterparts, and this is expected to affect their microbiomes [Legrand et al. 2020].

The benefits of knowing animal-associated microbiota stretch further than just en-hancing our knowledge of microbial diversity and host-microbe interactions. It can also be considered a novel contribution to conservation practices, as host-associated microbiomes are now considered to be good indicators or even biosensors of environmental health or disturbance [Zolti et al. 2020]. As microbial communities are highly responsive to en-vironmental changes, an animal microbiota presents features which are indicative of environmental disturbance [Coyte et al. 2015]. Regarding intestinal microbiota, dysbiosis — an imbalanced microbiome with no or little beneficial traits for its host—can be related to disease or decreased well-being of the animals. In various environments, it is known that the risk of animal disease increases in degraded or disturbed environments [Evariste et al 2019]. As microbiota analysis is fast becoming more precise and less costly, it can be considered as a rather proactive, as opposed to reactive, conservation-assisting practice, which is more appropriate for the stable, long-term monitoring of ecosystem health [e.g., Mootapally et al. 2017, Glasl et al. 2019]. Recently, microbiome science has been credited with having predictive potency for evolutionary processes of macro-organisms [Simonet et al 2021].

To date, most fish microbiome research is focused on farmed species [Perry et al. 2020] or on a very small number of laboratory animals, which prevents us from knowing even approximate upper limits of natural microbiome variation in wild fish; consequently, there is an imperative need for the microbial profiling of natural populations [Givens et al. 2015, Uren Webster et al. 2018, Kim et al. 2021, Burtseva et al. 2021), especially the core microbiota and microbiome [Risely 2020]. The core microbiome can provide beneficial adaptations to its hosts [Wong et al. 2013; Romero et al. 2014; Risely 2020], while in-ter-individual microbiome variability might act as a selective pressure mechanism for host adaptation, fitness, and evolution [Suzuki 2017].

In this paper, we present a comparative analysis of the midgut bacterial community structure of 12 marine fish species from the Gyaros Island marine protected area (MPA), Aegean Sea, Greece, to identify the dominant and core microbiota members and depict similarities between midgut microbiota structure of closely related fish species.

MATERIALS AND METHODS

Study area. Sampling took place in the marine waters around Gyaros Island (Figure 1), central Aegean Sea, Greece, which has been an MPA since 2019 [Damalas et al. 2022]. According to the Greek Ministerial Decree 389/4.7.2019, spatiotemporal access for small-scale fishing is allowed and specific exploitation activities are permitted and regulated on the island. Gyaros, also locally known as Gioura, is an unpopulated island of 23 km^2 in the northern Cyclades complex 9 nautical miles (nmi) from the closest island, Syros. Historically, Gyaros has served as a place of exile during the Roman era and the 20th century, and after World War II until 1974, it was a concentration camp for displaced political prisoners. Afterwards, it was converted to a firing range for the Greek Navy. As a result, access to other human activity was limited or restricted, and Gyaros has been under this 'protected' status for more than five decades. In 2011, Gyaros and the surrounding marine area of 3 nmi was included in the list of European Natura 2000 Network sites.

Fish sampling procedure. Sampling was carried out on board chartered commercial fishing vessels in five fixed stations around Gyaros Island. The sites were selected by applying NOAA's Sampling Design Tool (https://coastalscience.noaa.gov/project/sampling-design-tool-arcgis/) and taking into account the depth strata and bottom substrate types (Figure 1, Table 1). Sampling was conducted as an experimental fishing survey from July 2018 to June 2019. Fishing gear consisted of static trammel nets with a mesh size greater than 32 mm, length from 500 to 1000 m and a height of around 2 m. The nets were cast in the late afternoon and retrieved early the next morning at sunrise. Depths over which fishing took place varied between 17 and 98 m.

Biological data acquisition

Immediately after the trammel nets were hauled back to the vessels, entangled fish were extracted from the netting and identified down to the lowest possible taxonomic level. In order to avoid erroneous misidentification on board the fishing vessel, all specimens were transferred to the Hellenic Centre for Marine Research (HCMR premises), where they were thoroughly examined and identified at the species level by using specialised fish taxonomic keys. Fish dissection took place in aseptic conditions on board fishing vessels, by using sterile

gloves and scissors and scalpel were sterilized with 70% ethanol before each dissection. The entire digestive tract was excised from 45 specimens and placed in individual sterile Falcon tubes (50 mL). Both the gut samples and the remaining bodies were labelled properly and stored in the vessels' freezers. After landing, the samples were transferred to laboratory facilities, where gut samples were stored at −80 °C and whole bodies were frozen at −20 °C for further processing.

For each specimen, the total length (L_T; mm) was measured from the tip of the snout to the tip of the upper caudal fin. Eviscerated mass (M_E; g) was weighed after the removal of the remaining organs from the body cavity. In osteichthyans, sex was determined by macroscopic observation of the gonads, and the sexual maturity stage was assessed according to Nikolsky's scale [Nikolsky 1963]. In chondrichthyans, sex was determined by direct examination for the presence or absence of claspers and sexual maturity was assessed following the MEDITS maturity scale (MEDITS, 2017). Gonad mass (M_G) and liver mass (M_L) were weighed to the nearest gram (g) when possible. The 45 fish specimens used for this study were clustered in seven families and 12 species. Details regarding standard biometric measurements of the specimens with corresponding metadata can be found in the supplementary material (Table S1).

Microbiota analysis. After the midgut samples were thawed on ice, each one was collected and its digesta was mechanically squeezed out using forceps. Tissue samples (ca. 0.25 g) were rinsed three times with sterile, particle-free sea water. Total DNA was isolated using the QIAGEN QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol "DNA Purification from Tissues". From the extracted total DNA, bacterial DNA was amplified with the primer pair S-D-Bact-0341-b-S-17 and S-D-Bact-1150785-a-A-21 [Klindworth et al. 2012] targeting the V3–V4 regions of the 16S rRNA gene. The amplicons were sequenced on a MiSeq Illumina instrument (2x300 bp) at the MRDNA Ltd. (Shallowater, TX, USA) sequencing facilities.

Data analysis. Raw DNA sequences can be found in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) under BioProject PRJNA835803. The raw 16S rRNA sequencing data were processed using the MOTHUR standard operating procedure (v.1.45.3) [Schloss et al., 2009, 2011] and the operational taxonomic units (OTUs) were classified with the SILVA database release 138 [Quast et al., 2013; Yilmaz et al., 2014]. Identification of the closest relatives of the resulting OTUs was performed using a Nucleotide Blast (http://blast.ncbi.nlm.nih.gov). Statistical analysis—including cluster analysis based on the unweighted pair group method with arithmetic mean Bray-–Curtis similarity, –permutational

multivariate analysis of variance (PERMANOVA), and graphic illustrations were performed using Palaeontological STudies (PAST) software (Hammer et al., 2001) and R Studio platform Version 1.1.419 [RStudio Team, 2020] with 3.4.3 R version.

In our study, most of the investigated fish individuals were of similar age (Table S1). However, due to the low number of individuals per fish species, we pooled individual samples from the same fish species as they had 9% to 25 % overlapping OTUs (Figure S1), to provide a more inclusive view of the core midgut bacterial diversity for each of the 12 fish species.

RESULTS

From the amplicon sequencing of the 16S rRNA gene V3–V4 region, a total of 3,475,413 sequences were obtained after quality filtering and chimera removal from the 45 gut samples, ranging between 160,531 and 23,517 sequences/sample. These sequences were assigned to 854 unique OTUs at a cut-off level of 0.03. Sequencing data were rarefied to be equal to the smallest number (23,517) of sequences per sample.

As core microbiota are considered functional when they are abundant [Risely 2020], we present the core microbiota of the most dominant OTUs across the 12 fish species, comprising ≥70% of the cumulative relative abundance, which consists of 28 bacterial OTUs corresponding to 23 known genera and two unclassified genera (Table S2). Rank abundance curves (RACs, Figure 2) showed that only a small number of dominant OTUs were found in the midgut bacterial communities, while a much larger number of OTUs were present in low and very low relative abundance. In each species, between 2 and 18 OTUs were the most abundant OTUs (cumulative relative dominance ≥70%) (Table 2). The OTU richness (Table 2) was higher in *Diplodus annularis*, and according to PERMANOVA analysis with the Bray-Curtis distance matrix, this difference was reported to be significant compared to *Pagrus pagrus* (p = 0.029), *Pagellus erythrinus* (p = 0.028), *S. cantharus* (p = 0.031), *S. scrofa* (p = 0.029), *Scyliorhinus canicula* (p = 0.026), and *Sparisoma cretense* (p = 0.029). The Simpson 1-D diversity index ranged between 0.25 ± 0.26 (*P. pagrus*) and 0.83 ± 0.12 (*Scorpaena scrofa*) (Table 2). The midgut bacterial diversity had statistically significant differences between *P. pagrus* and eight fish species (PERMANOVA, p_{range} = 0.027 - 0.030) and between *Spondyliosoma cantharus* and five fish species (PERMANOVA p_{range} = 0.028–0.030).

There was no clear clustering pattern for species of the same family (five species in the Sparidae family and two in the Scorpaenidae family), except for *P. pagrus* and *P. erythrinus* of the Sparidae family (Figure 3). Similarly, no clustering was observed regarding

feeding habits (carnivore/omnivore, see Table S3). However, regarding the presence of shared family-level OTUs, the five Sparidae species shared 61 of 142 (42.9%) OTUs, while the two Scorpaenidae species shared 62.2% of their total OTUs (Figure 4).

Fish midgut microbiota at the phylum level were characterized by the predominance of Proteobacteria (61.1% relative abundance). Of the rest of the 24 detected phyla in the whole dataset, only seven (Firmicutes, Bacteroidota, Actinobacteriota, Patescibacteria, Fusobacteriota, Planctomycetota, and Dependentiae) were found to occur with ≥1% relative abundance in at least one of the 12 species. Regarding the taxonomic differences in the midgut microbiota, OTUs comprising ≥70% of the relative abundance in each fish species were assigned to 31 and 2 bacterial families and orders, respectively (Figure 5). Even the dominant OTUs were assigned to different and variable taxonomic families, except for *P. pagrus* and *P. erythrinus*.

DISCUSSION

The definition of the core microbiota is based on shared microorganisms among comparable habitats [Shade & Handelsman 2012], and for this, a large number of replicate samples was needed to overcome the effect of individual variability [Panteli et al. 2020]. Indeed, such prerequisites are achievable under experimental conditions [e.g., Uren Webster et al. 2018, Panteli et al. 2021] or in the case of farmed fish populations [e.g., Nikouli et al. 2018, Le et al. 2020, Nikouli et al. 2021]. However, when core microbiota of fish from natural populations are investigated by experimental fishing in the open sea, collecting adequate replicates of specimens of the same age cannot be secured, so scientists have to rely on what they catch.

The core microbiota across different animal hosts is considered to be a kind of genetic link among these species since these microorganisms hold the essential core genes for different hosts [Zilber-Rosenberg & Rosenberg 2021]. Following [Hamady & Knight 2009], we present the core midgut bacterial microbiota of the most abundant OTUs of 12 marine fish species from the Gyaros Island marine protected area in the Aegean Sea, Greece. For 11 of these species, there were no available data on gut microbiota and microbiomes, except for *Pagrus pagrus* because its gut microbiota has been studied in a farmed population [Nikouli et al. 2021]. The core microbiota of these 12 species consisted of 23 known and two yet-unaffiliated (from the Clostridiaceae and Vibrionaceae families) genera (Table S2). The majority of these genera are known to occur in the intestinal systems of healthy wild and

farmed fish species [Llewellyn et al. 2014; Tarnecki et al. 2017; Legrand et al. 2020], thereby expanding their keystone and fundamental ecophysiological role across several fish species.

There were multiple differences in midgut microbiota structure among the 12 species. At first, OTU richness was highly variable, as illustrated by both the range (Table 2) and the RACs (Figure 2). The latter revealed only a small number of dominant OTUs; however, these bacteria were taxonomically different. Such a microbiota structure, i.e., a few dominant OTUs with a large "rare biosphere" [Sogin et al. 2006], has been reported in other comparative studies of fish gut microbiota [Givens et al. 2015, Kim et al. 2021]. In a comparison of wild and farmed populations of the same species, higher gut bacterial diversity in animal hosts is thought to be beneficial [Reese & Dunn 2018] because it sustains a functional microbiota in an environment with a fluctuating food supply [Givens et al. 2015]. The same has been proposed for other animals in the wild [Levin et al. 2021]. Due to functional redundancy of bacteria, taxonomic diversity does not necessarily reflect metabolic diversity [Shade et al. 2012]; however, the extent of functional diversity of wild fish microbiota [Luna et al. 2022] remains to be studied.

In closely related taxa or species with similar intestinal system development [Youngblut et al. 2019], host genetics is not an important distinguishing microbiome factor (e.g., Bledsoe et al. 2018). In the present study, only two pairs of fish species had more than 70% similarity in their midgut microbiota (Figure 4). Despite this structural similarity, PERMANOVA for the first pair, *E. alleteratus* and *S. cantharus*, which belong to different taxonomic families, showed that their midgut microbiota was statistically different (PERMANOVA $p < 0.001$, F = 9.71). The second pair, *P. pagrus* and *P. erythrinus*, of the Sparidae family, are more closely related (Figure S2), as no statistically significant difference was found (PERMANOVA $p = 0.889$, F = 0.768). Both fish pairs consisted of one omnivorous and one carnivorous species (Table S3). Our dataset included two pairs of fish species from the same genus, *D. annularis*/*D. vulgaris* and *S. porcus/S. scrofa*; however, their in-between midgut microbiota structure was very distant (Figure 4). Although host phylogeny and diet remain the two major shaping factors of animal gut microbiota [Youngblut et al 2019], the above pairwise comparisons suggest that host genetics can shape the gut microbiota rather than diet or feeding habit of wild marine fish. Even in co-farmed species reared under the same environmental and dietary conditions, each fish species was found to host its own distinct gut microbiota [Nikouli et al. 2021]. However, gut microbiota is susceptible to manipulations at least for experimental or commercial rearing purposes [Luna et al. 2022]. In a recent study, host habitat, i.e. freshwaters vs. marine waters, was found to be

the prime factor for converging microbiota structure in multiple wild fish species [Kim et al. 2021]. In the present study, all fish were caught around a small island in a marine protected area from similar habitats and substrates (Figure 1, Table S3), so habitat variability was not expected to have a large effect on the midgut microbiota.

The dominant bacterial phyla found in the current study were the expected ones based on what is known about intestinal microbiota of wild [Kim et al. 2021] and farmed marine fish [Legrand et al. 2020]. *P. pagrus* and *P. erythrinus*, the only two species with the highest similarity in midgut microbiota compared to the other species, were dominated by OTUs belonging to the families of Xanthobacteriaceae and Comamonadaceae. More specifically, for both species the dominant OTU was affiliated with the genus *Bradyrhizobium*, which was also dominant in *Mullus surmuletus*, *Scorpaena scrofa* and *Uranoscopus scaber* (Table 2). *Bradyrhizobium* has been found to co-dominate with known probiotic bacteria in the midgut of the olive flounder *Paralichthys olivaceus* under experimental conditions [Niu et al. 2020]. In the gilthead seabream, *Bradyrhizobium*, along with the known probiotic *Weissella*, was favoured after a transition from a high- to low-lipid diet [Pelusio et al. 2021]. *Bradyrhizobium* has not been reported to be associated with fish disease, but it has been found to be sensitive to *Vibrio harveyi* infection [Deng et al. 2020]. Thus, it seems to be a fundamental, i.e., highly abundant, bacterial resident in the *P. pagrus* and *P. erythrinus* gut microbiota with potentially beneficial role to its hosts.

Most of the other dominant OTUs were affiliated with multiple bacterial genera (Table 2) that had a proven beneficial, or at least neutral, effect on their hosts. *Pseudoalteromonas* genus is often found among dominant OTUs in the healthy intestines of several wild fish species [Burtseva et al 2021], so it is considered to be a beneficial microorganism for fish hosts [Wesseling et al., 2015, Mladineo et al. 2016] even in the early life stages [Nikouli et al. 2019, Jiang et al. 2020]. Furthermore, we recently found that it dominates the fertilized eggs of *Seriola dumerili* in commercial hatcheries [Kormas et al. unpublished]. Fish-associated *Diaphorobacter*-related OTUs have been reported only once and found to prevail in the wild and farmed gilthead seabream midguts [Kormas et al. 2014]. *Mycoplasma* dominated in the wild but not in the farmed populations of red-cusk eels [Romero et al. 2022], Atlantic salmon [Villasante et al. 2018, Heys et al. 2020], rainbow trout [Brown et al. 2019] and a few other wild-caught fish species [Burtseva et al. 2021]. *Clostridium* spp. are favoured in the gut of herbivorous fish [Legrand et al. 2020] and are well-established probiotics in the aquaculture industry [Ringø et al. 2020]. A *Microbulbifer* sp. strain was recently isolated from the intestine of the herbivorous teleost *Girella melanichthys* and was found to degrade cellulose

[Tanaka et al. 2021], a rather useful trait for the omnivorous *D. vulgaris* midgut in the current study. *Thaumasiovibrio* is a recently described genus [Amin et al. 2017] and has not yet been reported to be associated with fish.

The captivity conditions of wild animals [e.g., Clayton et al. 2016, San Juan et al.] and even urbanization of human populations [e.g., Schaan et al. 2021] have been shown to alter the gut microbiomes of these macroorganisms. Of the 12 fish species investigated in the present study, and according to the Hellenic Statistical Authority (https://www.statistics.gr/en/home/), only *Pagrus pagrus* is included in the list of farmed fish in Greece. The midgut microbiota of this farmed species was recently reported after being farmed sympatrically with four other fish species under the same environmental and dietary conditions [Nikouli et al. 2021]. It was found that the *P. pagrus* midgut hosted only 29 bacterial OTUs, which were dominated by *Hydrogenophilus*, followed by *Pseudomonas, Stenotrophomonas, Bradyrhizobium, Hydrogenophaga, Comamonas, Propionibacterium* and *Janibacter*. All these OTUs comprised ca. 81% of the total relative abundance of all midgut bacteria. In the present study, the wild *P. pagrus* midgut had 179 OTUs with just the *Bradyrhizobium* and *Pelomonas* OTUs comprising ca. 90.1% of the relative bacterial abundance. Thus, wild and farmed *P. pagrus* midgut microbiota have a different community structure, although specimens in the current study were smaller (Table S1) from those reported in [Nikouli et al 2021; their Table S1]. Such differences in the microbiota between wild and farmed populations of the same species have also been shown in targeted studies for several fish species such as gilthead seabream [Kormas et al. 2014], red-cusk eel [Romero et al. 2022], fine flounder [Ramirez & Romero 2017a], Atlantic salmon [Uren Webster et al. 2018], yellowtail amberjack [Ramirez & Romero 2017b], and large yellow croaker [Zhu et al. 2022].

This study investigated the amplicon metabarcoding of the midgut bacterial communities of 12 wild fish species collected from the Gyaros Island marine protected area in Aegean Sea, Greece. For 11 of them, it is the first time their gut microbiota are reported, while for the remaining one, only limited data exist on its farmed counterpart, but the wild and natural populations seem to host different midgut microbiota. There was a general pattern of diverging microbiota in closely related fish species, expect for *P. pagrus* and *P. erythrinus* of the Sparidae family, which had very similar midgut microbiota. The dominant core microbiota consisted of 28 different OTUs, with most of them being related to bacterial taxa reported in other healthy wild and farmed species, some of which have a beneficial effect on their hosts. Finally, as new conservation strategies are starting to include natural microbiomes

as part of ecosystem monitoring for conservation strategies, our results provide microbiota composition and structure, related mostly to the nutrition of higher trophic-level macro-organisms in a pristine and protected marine area.

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REFERENCES

Amin AKMR, Tanaka M, Al-saari N, Feng G, Mino S, Ogura Y, Hayashi T, Meirelles PM, Thompson FL, Gomez-Gil B, Sawabe T, Sawabe T (2017) Thaumasiovibrio occultus gen. nov. sp. nov. and Thaumasiovibrio subtropicus sp. nov. within the family Vibrionaceae, isolated from coral reef seawater off Ishigaki Island, Japan. Systematic and Applied Microbiology 40:290-296

Bledsoe JW, Waldbieser GC, Swanson KS, Peterson BC, Small BC (2018) Comparison of Channel Catfish and Blue Catfish Gut Microbiota Assemblages Shows Minimal Effects of Host Genetics on Microbial Structure and Inferred Function. Frontiers in Microbiology 9:1073

Brown RM, Wiens GD, Salinas I (2019) Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (Oncorhynchus mykiss). Fish & Shellfish Immunology 86:497-506

Burtseva O, Kublanovskaya A, Fedorenko T, Lobakova E, Chekanov K (2021) Gut microbiome of the White Sea fish revealed by 16S rRNA metabarcoding. Aquaculture 533:736175

Clayton JB, Vangay P, Huang H, Ward T, Hillmann BM, Al-Ghalith GA, Travis DA, Long HT, Tuan BV, Minh VV, Cabana F, Nadler T, Toddes B, Murphy T, Glander KE,

Johnson TJ, Knights D (2016) Captivity humanizes the primate microbiome. Proceedings of the National Academy of Sciences 113:10376-10381

Coyte KZ, Schluter J, Foster KR (2015) The ecology of the microbiome: Networks, competition, and stability. Science 350:663-666

Damalas D, Stamouli C, Fotiadis N, Kikeri M, Kousteni V, Mantopoulou-Palouka D (2022) The Gyaros island marine reserve: A biodiversity hotspot in the eastern Mediterranean Sea. PLOS ONE 17:e0262943

Deng Y, Zhang Y, Chen H, Xu L, Wang Q, Feng J (2020) Gut–Liver Immune Response and Gut Microbiota Profiling Reveal the Pathogenic Mechanisms of Vibrio harveyi in Pearl Gentian Grouper (Epinephelus lanceolatus♂ × E. fuscoguttatus♀). Frontiers in Immunology 11

Evariste L, Barret M, Mottier A, Mouchet F, Gauthier L, Pinelli E (2019) Gut microbiota of aquatic organisms: A key endpoint for ecotoxicological studies. Environmental Pollution 248:989-999

Givens C, Ransom B, Bano N, Hollibaugh J (2015) Comparison of the gut microbiomes of 12 bony fish and 3 shark species. Marine Ecology Progress Series 518:209-223

Glasl B, Bourne DG, Frade PR, Thomas T, Schaffelke B, Webster NS (2019) Microbial indicators of environmental perturbations in coral reef ecosystems. Microbiome 7:94

Hamady M, Knight R (2009) Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Research 19:1141-1152

Hammer Ø, Harper D, Ryan P (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electr 4:9

Heys C, Cheaib B, Busetti A, Kazlauskaite R, Maier L, Sloan WT, Ijaz UZ, Kaufmann J, McGinnity P, Llewellyn MS (2020) Neutral Processes Dominate Microbial Community Assembly in Atlantic Salmon, Salmo salar. Appl Environ Microbiol 86:e02283-02219

Jiang Y, Liu X, Xu Y, Shi B, Wang B (2020) Microbiota characteristics in Sebastes schlegelii intestine in early life stages. Journal of Oceanology and Limnology 38:275-287

Kim PS, Shin N-R, Lee J-B, Kim M-S, Whon TW, Hyun D-W, Yun J-H, Jung M-J, Kim JY, Bae J-W (2021) Host habitat is the major determinant of the gut microbiome of fish. Microbiome 9:166

Klindworth A, Pruesse E, Schweer T, Peplies Jr, Quast C, Horn M, Glöckner FO (2012) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 41:e1

Kormas KA, Meziti A, Mente E, Frentzos A (2014) Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). MicrobiologyOpen 3:718-728

Le D, Nguyen P, Nguyen D, Dierckens K, Boon N, Lacoere T, Kerckhof F-M, De Vrieze J, Vadstein O, Bossier P (2020) Gut Microbiota of Migrating Wild Rabbit Fish (Siganus guttatus) Larvae Have Low Spatial and Temporal Variability. Microbial Ecology 79:539-551

Legrand TPRA, Wynne JW, Weyrich LS, Oxley APA (2020) A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Reviews in Aquaculture 12:1101-1134

Levin D, Raab N, Pinto Y, Rothschild D, Zanir G, Godneva A, Mellul N, Futorian D, Gal D, Leviatan S, Zeevi D, Bachelet I, Segal E (2021) Diversity and functional landscapes in the microbiota of animals in the wild. Science 372:eabb5352

Llewellyn MS, Boutin S, Hoseinifar SH, Derome N (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology 5,207

Luna GM, Quero GM, Kokou F, Kormas K (2022) Time to integrate biotechnological approaches into fish gut microbiome research. Current Opinion in Biotechnology 73:121-127

MEDITS 2017 MEDITS-Handbook. Version n. 9, 2017, MEDITS Working Group : 106 pp. Available at: https://www.sibm.it/MEDITS%202011/principaledownload.htm

Mladineo I, Bušelić I, Hrabar J, Radonić I, Vrbatović A, Jozić S, Trumbić Ž (2016) Autochthonous Bacterial Isolates Successfully Stimulate In vitro Peripheral Blood Leukocytes of the European Sea Bass (Dicentrarchus labrax). Frontiers in Microbiology 7

Mootapally CS, Poriya P, Nathani NM, Venmathi Maran BA, Gadhvi IR (2017) Recent Advances in the Metagenomics of Marine Mammals Microbiome. In *Understanding Host-Microbiome Interactions - An Omics Approach: Omics of Host-Microbiome Association*, Singh, R.P., Kothari, R., Koringa, P.G., Singh, S.P., Eds. Springer Singapore: Singapore, pp. 327-336.

Nikolsky GV (1963) The Ecology of Fishes. New York, NY: Academic Press, 352 pp.

Nikouli E, Meziti A, Antonopoulou E, Mente E, Kormas K (2018) Gut bacterial communities in geographically distant populations of farmed sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). Microorganisms 6:92

Nikouli E, Meziti A, Antonopoulou E, Mente E, Kormas KA (2019) Host-associated bacterial succession during the early embryonic stages and first feeding in farmed gilthead sea bream (Sparus aurata). Genes 10:483

Nikouli E, Meziti A, Smeti E, Antonopoulou E, Mente E, Kormas KA (2021) Gut Microbiota of Five Sympatrically Farmed Marine Fish Species in the Aegean Sea. Microbial Ecology 81:460-470

Niu K-M, Lee B-J, Kothari D, Lee W-D, Hur S-W, Lim S-G, Kim K-W, Kim K-D, Kim N-N, Kim S-K (2020) Dietary effect of low fish meal aquafeed on gut microbiota in olive flounder (Paralichthys olivaceus) at different growth stages. MicrobiologyOpen 9:e992

Panteli N, Mastoraki M, Lazarina M, Chatzifotis S, Mente E, Kormas KA, Antonopoulou E (2021) Configuration of Gut Microbiota Structure and Potential Functionality in Two Teleosts under the Influence of Dietary Insect Meals. Microorganisms 9:699

Panteli N, Mastoraki M, Nikouli E, Lazarina M, Antonopoulou E, Kormas KA (2020) Imprinting statistically sound conclusions for gut microbiota in comparative animal studies: A case study with diet and teleost fishes. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 36:100738

Pelusio NF, Scicchitano D, Parma L, Dondi F, Brini E, D'Amico F, Candela M, Yúfera M, Gilannejad N, Moyano FJ, Gatta PP, Bonaldo A (2021) Interaction Between Dietary Lipid Level and Seasonal Temperature Changes in Gilthead Sea Bream Sparus aurata: Effects on Growth, Fat Deposition, Plasma Biochemistry, Digestive Enzyme Activity, and Gut Bacterial Community. Frontiers in Marine Science 8

Perry WB, Lindsay E, Payne CJ, Brodie C, Kazlauskaite R (2020) The role of the gut microbiome in sustainable teleost aquaculture. Proceedings of the Royal Society B: Biological Sciences 287:20200184

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41:D590-D596

Ramírez C, Romero J (2017) Fine Flounder (*Paralichthys adspersus*) Microbiome Showed Important Differences between Wild and Reared Specimens. Frontiers in Microbiology 8,271

Ramírez C, Romero J (2017) The microbiome of *Seriola lalandi* of wild and aquaculture origin reveals differences in composition and potential function. Frontiers in Microbiology 8,1844

Reese AT, Dunn RR (2018) Drivers of Microbiome Biodiversity: A Review of General Rules, Feces, and Ignorance. mBio 9:e01294-01218

Ringø E, Van Doan H, Lee SH, Soltani M, Hoseinifar SH, Harikrishnan R, Song SK (2020) Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. Journal of Applied Microbiology 129:116-136

Risely A (2020) Applying the core microbiome to understand host–microbe systems. Journal of Animal Ecology 89:1549-1558

Romero J, Díaz O, Miranda CD, Rojas R (2022) Red Cusk-Eel (Genypterus chilensis) Gut Microbiota Description of Wild and Aquaculture Specimens. Microorganisms 10:105

Romero J, Ringø E, Merrifield DL (2014)The Gut Microbiota of Fish. In *Aquaculture Nutrition*, Wiley-Blackwell, pp 75-100.

RStudio Team. RStudio: Integrated Development for R. RStudio, URL http://www.rstudio.com/. PBC, B., MA, Ed. 2020.

San Juan PA, Castro I, Dhami MK (2021) Captivity reduces diversity and shifts composition of the Brown Kiwi microbiome. Animal Microbiome 3:48

Schaan AP, Sarquis D, Cavalcante GC, Magalhães L, Sacuena ERP, Costa J, Fonseca D, Mello VJ, Guerreiro JF, Ribeiro-dos-Santos Â (2021) The structure of Brazilian Amazonian gut microbiomes in the process of urbanisation. npj Biofilms and Microbiomes 7:65

Schloss PD, Gevers D, Westcott SL (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS ONE 6:e27310

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541

Shade A, Handelsman J (2012) Beyond the Venn diagram: the hunt for a core microbiome. Environmental Microbiology 14:4-12

Shade A, Peter H, Allison S, Baho D, Berga M, Buergmann H, Huber D, Langenheder S, Lennon J, Martiny J, Matulich K, Schmidt T, Handelsman J (2012) Fundamentals of Microbial Community Resistance and Resilience. Frontiers in Microbiology 3:417

Simonet C, McNally L (2021) Kin selection explains the evolution of cooperation in the gut microbiota. Proceedings of the National Academy of Sciences 118:e2016046118

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences of the United States of America 103:12115-12120

Suzuki TA (2017) Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. Integrative and Comparative Biology 57:756-769

Tanaka D, Ohnishi KI, Watanabe S, Suzuki S (2021) Isolation of cellulase-producing Microbulbifer sp. from marine teleost blackfish (Girella melanichthys) intestine and the enzyme characterization. The Journal of general and applied microbiology 67:47-53

Tarnecki AM, Burgos FA, Ray CL, Arias CR (2017) Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. Journal of Applied Microbiology 123:2-17

Uren Webster TM, Consuegra S, Hitchings M, Garcia de Leaniz C (2018) Interpopulation variation in the atlantic salmon microbiome reflects environmental and genetic diversity. Appl Environ Microbiol 84,e00691-18

Villasante A, Ramirez C, Catalán N, Romero J (2018) First Report of Swim Bladder-Associated Microbiota in Rainbow Trout (Oncorhynchus mykiss). Microbes Environ 33:459-460

Wesseling W, Wittka S, Kroll S, Soltmann C, Kegler P, Kunzmann A, Riss HW, Lohmeyer M (2015) Functionalised ceramic spawning tiles with probiotic Pseudoalteromonas biofilms designed for clownfish aquaculture. Aquaculture 446:57-66

Wong S, Waldrop T, Summerfelt S, Davidson J, Barrows F, Kenney PB, Welch T, Wiens GD, Snekvik K, Rawls JF, Good C (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Appl Environ Microbiol 79:4974-4984

Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glöckner FO (2014) The SILVA and “All-species Living Tree Project (LTP)” taxonomic frameworks. Nucleic Acids Research 42:D643-D648

Youngblut ND, Reischer GH, Walters W, Schuster N, Walzer C, Stalder G, Ley RE, Farnleitner AH (2019) Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. Nature Communications 10:2200

Zhu J, Li H, Jing ZZ, Zheng W, Luo YR, Chen SX, Guo F (2022) Robust host source tracking building on the divergent and non-stochastic assembly of gut microbiomes in wild and farmed large yellow croaker. Microbiome 10:18

Zilber-Rosenberg I, Rosenberg E (2021) Microbial-driven genetic variation in holobionts. FEMS Microbiology Reviews 45:fuab022

Zolti A, Green SJ, Sela N, Hadar Y, Minz D (2020) The microbiome as a biosensor: functional profiles elucidate hidden stress in hosts. Microbiome 8:71

Table 1. Sampling locations off Gyaros Island, Greece, eastern Mediterranean Sea.

Station ID	Location	Coordinates	Depth (m)	Dominant substrate type
St. 1	Fyllada	37° 35.421’ N 24° 42.129’ E	18	*Posidonia*/Rocky
St. 2	Glaronissi	37° 34.860’ N 24° 45.015’ E	17	*Posidonia*/Rocky
St. 3	Fournaki	37° 37.486’ N 24° 45.117’ E	98	Maerl
St. 4	Colata	37° 37.323’ N 24° 40.998’ E	88	Maerl
St. 5	Fouis	37° 36.367’ N 24° 38.612’ E	47	Rocky

Table 2. Alpha diversity data of midgut bacterial communities of 12 fish species from the Aegean Sea. SD: standard deviation. OTU: operational taxonomic unit. Unique refers to the OTUs found only in the respective fish.

Fish species	**No. of OTUs ± SD (unique)**	**Simpson 1-D ± SD**	**Relative abundance of the most abundant OTU**	**No. of dominant OTUs***
Diplodus annularis (*n* = 4)	186 ± 60.8 (139)	0.66 ± 0.177	*Pseudoalteromonas* sp. 30.6 ± 31.6%	14
Diplodus vulgaris (*n* = 4)	160 ± 73.1 (86)	0.77 ± 0.139	*Microbulbifer* sp. 14.6 ± 29.2%	17
Euthynnus alleteratus (*n* = 4)	128 ± 26.3 (31)	0.83 ± 0.108	*Diaphorobacter* sp. 30.0 ± 16.7%	16
Mullus surmuletus (*n* = 4)	111 ± 38.3 (2)	0.67 ± 0.107	*Bradyrhizobium* sp. 20.3 ± 14.1%	7
Pagrus pagrus (*n* = 3)	101 ± 12.9 (3)	0.25 ± 0.257	*Bradyrhizobium* sp. 68.6 ± 45.0%	2
Pagellus erythrinus (*n* = 3)	101 ± 6.1 (3)	0.64 ± 0.168	*Bradyrhizobium* sp. 41.5 ± 14.7%	3
Spondyliosoma cantharus (*n* = 4)	104 ± 7.9 (5)	0.78 ± 0.063	*Diaphorobacter* sp. 25.2 ± 15.3%	11
Scyliorhinus canicula (*n* = 4)	120 ± 14.9 (19)	0.55 ± 0.110	*Mycoplasma* sp. 21.9 ± 37.1%	6
Sparisoma cretense (*n* = 4)	100 ± 10.9 (7)	0.83 ± 0.083	*Thaumasiovibrio* sp. 16.4 ± 22.8%	15
Scorpaena porcus (*n* = 4)	122 ± 23.9 (23)	0.82 ± 0.067	*Clostridium sensu stricto* (cluster I) 18.8 ± 21.5%	12
Scorpaena scrofa (*n* = 4)	115 ± 21.5 (12)	0.83 ± 0.122	*Bradyrhizobium* sp. 14.4 ± 22.8%	18

Uranoscopus scaber (*n* = 3)	114 ± 53.7 (20)	0.79 ± 0.106	*Bradyrhizobium* sp. 15.6 ± 26.2%	11

518

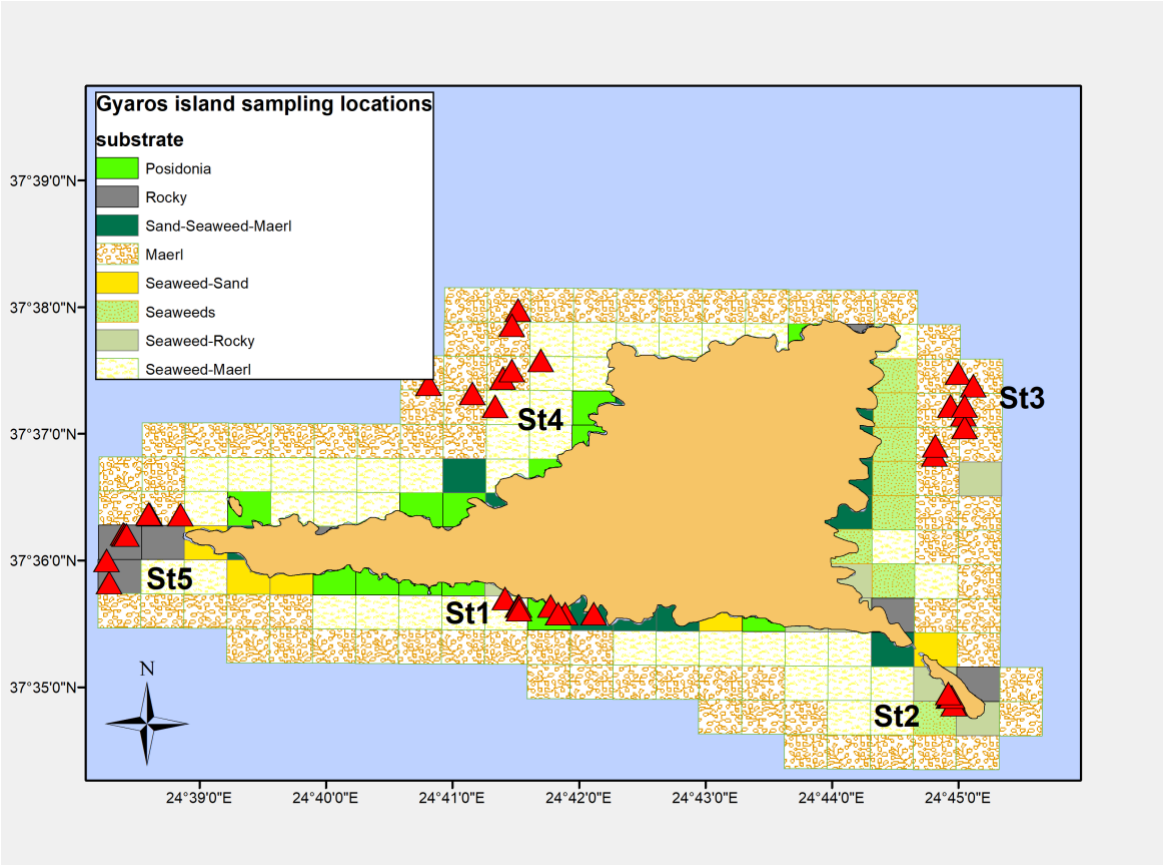

Figure 1. Map showing the sampling stations (St#) off Gyaros Island, Greece, in the eastern Mediterranean Sea. Red triangles indicate sampling locations.

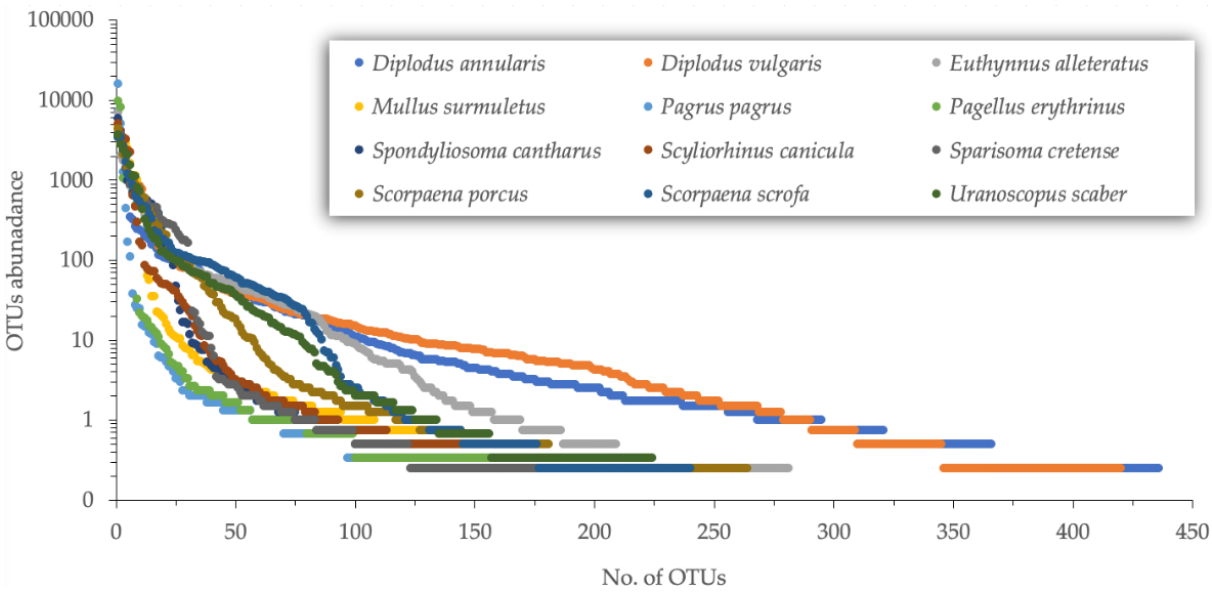

Figure 2. Rank abundance curves of the midgut bacterial operational taxonomic units (OTUs) abundance in 12 marine fish species from the Aegean Sea.

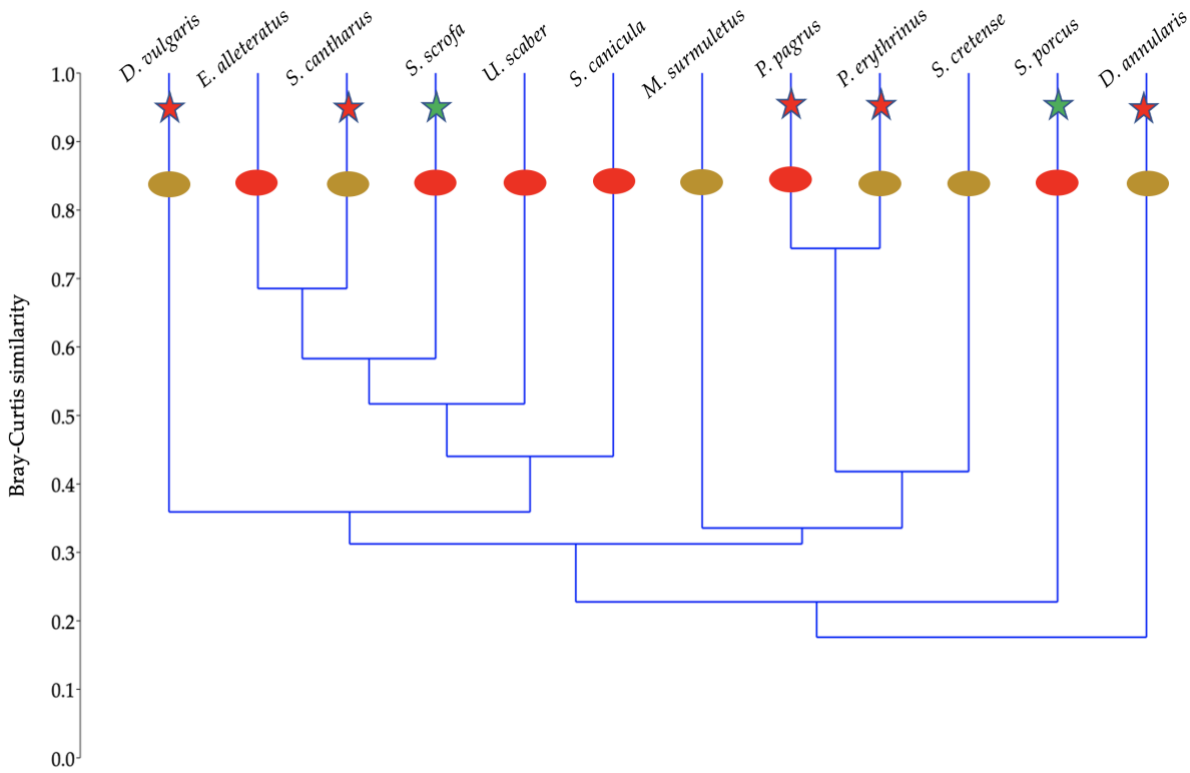

Figure 3. Cluster analysis of the 12 fish species' midgut microbiota: red star, Sparidae, green star, Scorpaenidae; red ellipse, carnivorous; brown ellipse, omnivorous.

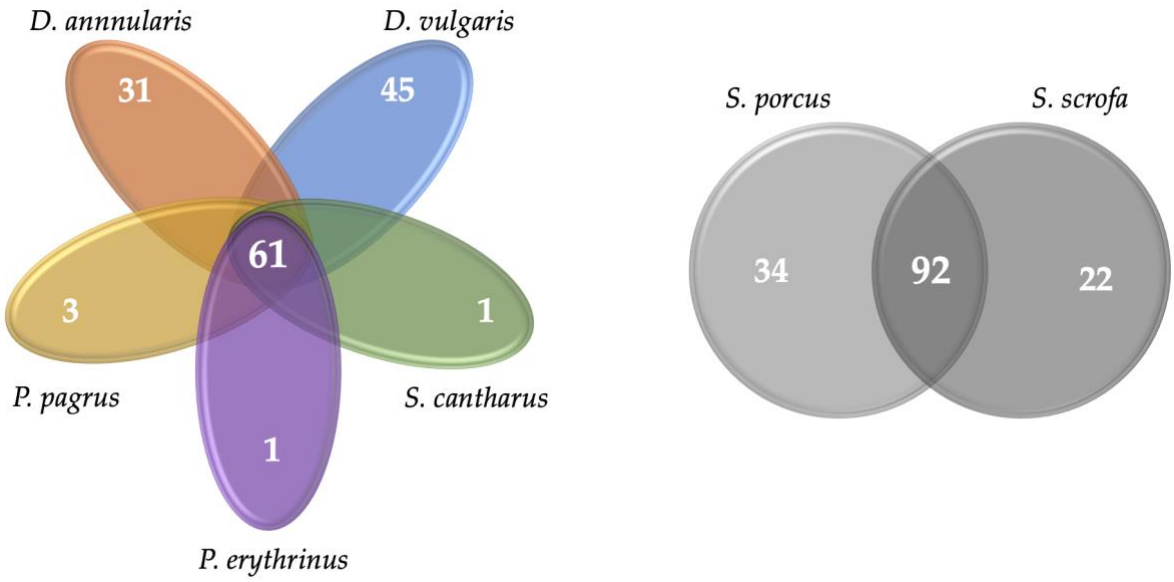

Figure 4. Shared and unique midgut bacterial (family -level) bacterial operational taxonomic units (OTUs) of the Sparidae (left) and Scorpaenidae (right) fish species.

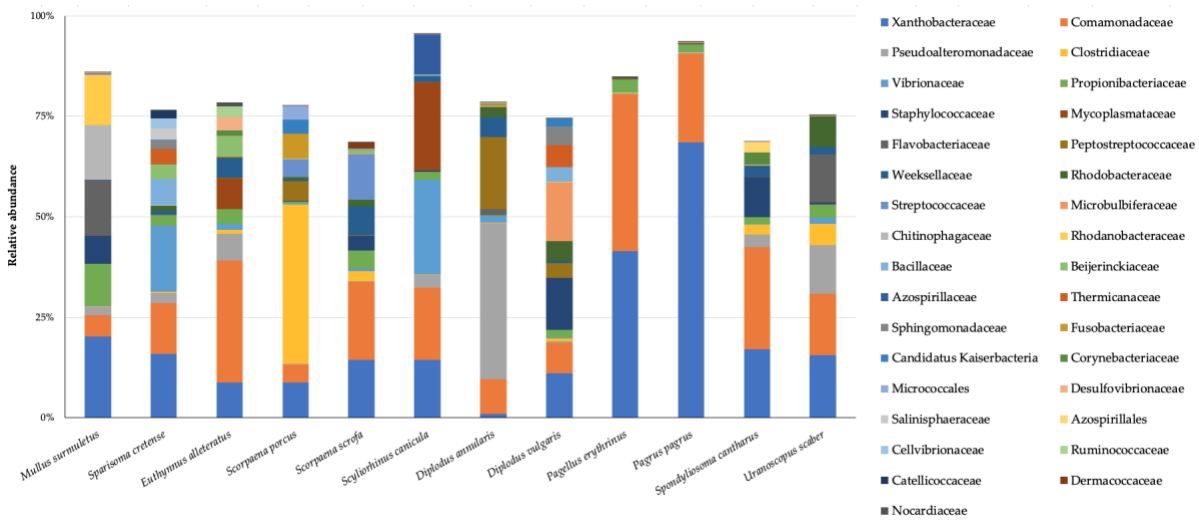

Figure 5. Taxonomic composition (31 families and two orders) of the most dominant (≥70% cumulative relative abundance) bacterial operational taxonomic units of the 12 fish species. Taxa in the legend are shown in decreasing total abundance in the whole dataset.