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An insight into gill microbiome of Eastern Mediterranean wild fish by applying next generation sequencing

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Bacterial diseases of marine fish inflict significant economic damage to fisheries and aquaculture and pose an increasing risk to public health. When addressing fish disease, an accumulating body of research suggests adding another factor to the classic epidemiological triangle of host-environment-pathogen: the microbiome. The gills, being a gateway into the fish body and bearing an important role in fish homeostasis, have been found to be a proxy of the gut microbiota as well as reflecting the microbial communities of surrounding water. In this study, 16S rRNA amplicons of bacterial DNA extracted from the gills of 89 asymptomatic specimens of three wild fish species (*Pagrus caeruleostictus*, *Scomber colias* and *Saurida lessepsianus*) were sequenced using Next Generation Sequencing methodology (NGS). Data analyses revealed the presence of 41 potentially pathogenic species, including several zoonotic agents. Five genera known to include widespread and potentially pathogenic species were chosen for further investigation: *Photobacterium, Shewanella, Staphylococcus, Streptococcus* and *Vibrio*. Of these, *Photobacterium* and *Shewanella* proved the most prevalent and abundant, making up 30.2% and 11.3% of the Bluespotted seabream (*P. caeruleostictus*) gill microbiome alone. *Photobacterium damselae* and *Shewanella baltica* were most common at the species level. The remaining genera - *Vibrio, Staphylococcus* and *Streptococcus* – were less prevalent, and at a species level were comprised of only 1–4% potentially pathogenic representatives. Gill microbiomes exhibited host species specificity, with strong correlations between certain bacterial taxonomic groups. No definite obligatory pathogenic bacteria were found in this study, and it was suggested that pathogenic species are present as either covert pathobionts or as opportunists of the fish found to host them.

KEYWORDS

Photobacterium, *Shewanella*, *Staphylococcus*, *Streptococcus*, *Vibrio*, Marine fish, Wild fish pathogens, *Gill microbiome*

Introduction

In many areas of the world, one of the most common horizontal transmission routes of pathogens into wild fish is the rapidly growing mariculture cage-farm industry (Arechavala-Lopez et al., 2013; Barrett et al., 2019; Shea et al., 2020). A reverse pattern of pathogen transmission can also be observed from wild fish to farm stocks (Arechavala-Lopez et al., 2013). Fish cage farms have become ecological hotspots, releasing a steady source of residual uneaten feed and providing a refuge for small-bodied fish species in an otherwise unsheltered open sea habitat. The farms also present an opportunity for replenishment for migratory fish (Shea et al., 2020) and become an attraction for predators (Papastamatiou et al., 2010; Piroddi et al., 2011; Barash et al., 2018). Today, over a quarter of globally farmed fish species are non-native to their rearing environment (Atalah and Sanchez-jerez, 2020), which means in addition to competing with native wild populations over local resources, mariculture escapees pose a risk for introduction of alien pathogens to naïve hosts. The effects of mariculture are coupled with many other anthropogenic factors that increase risk of disease outbreaks in wild fish, including increases in sea surface temperature, pollution and structural alterations to ecosystems through development and industry (Harvell et al., 1999; Halpern et al., 2008; Lejeusne et al., 2010; Nguyen and Liou, 2019). Human-driven changes in the ocean environment directly impact the health of fish. When faced with physiochemical conditions outside of their optimal range, fish may become stressed and immunosuppressed, lowering their defenses against agents of infectious disease (Johnson et al., 1992; Conte, 2004). The epidemiological triangle, describing such interactions between a host, a pathogen, and their environment (King et al., 2019), forms the basis of research aimed at understanding the effects of disease on marine animals (Andrade et al., 2017; Elarabany et al., 2017; Wang et al., 2018; Genin et al., 2020; Zarantoniello et al., 2021).

In recent years, there has been great interest in adding the contribution of the microbiome to this complex interplay, applying the concept of the holobiont, a host with all of its associated microorganisms, to disease research. The intimate partnership between hosts and their symbiotic microbiota plays a significant role in host maintenance and well-being, contributing to metabolism, immune system maturation, and additional defenses against pathogenic invaders (Aschenbrenner et al., 2016; Ramsey et al., 2016; Apprill, 2017; Vorburger and Perlman, 2018). Shifts in this microbial composition due to external pressures from natural or anthropogenic changes in the environment (Halpern et al., 2008; Pérez-Ruzafa et al., 2018; Nguyen and Liou, 2019), or internal physiological pressures (Yildirimer and Brown, 2018), may lead to substantial consequences for the host, and maintaining the balance of the microbiome has been shown to be of importance for maintenance of fish health (Llewellyn et al., 2014). Microbiota composition shifts may serve as early-warning bioindicators, enabling assessment of the host's health even before clinical signs become visible. Combined with data on shifts in pathogen prevalence, these aspects become key factors in understanding the intricacies of host-pathogen relations.

Previous fish microbiome studies focused mostly on skin and intestinal microbiota (Ni et al., 2013; Liu et al., 2016; Egerton et al., 2018; Tarnecki et al., 2019; Krotman et al., 2020), and occasionally on other organs, such as kidneys and liver (Sevellec et al., 2014; Meron et al., 2020). These studies found that gut, kidney microbiota are deeply influenced by fish trophic levels and diversity of their prey, while skin microbiota is both highly adaptive and affected by qualities of the ambient water. Gills, however, are receiving increasing attention, as they may be sampled non-destructively from live fish (Merrifield and Rodiles, 2015; Mohammed and Arias, 2015). In addition to their role in gas and waste exchange (Evans et al., 2005), gills are a gateway into the fish body and an important site of mucosal immunity (Salinas, 2015), constantly in direct interaction with the aquatic environment and its associated microbes. To a certain extent, the gill microbiome reflects the microbial composition of the water, including pathogens present (Kuang et al., 2020). Understanding the mechanisms of pathogen adherence to and entry through the gill mucosal barrier, and the potential impact of the microbiome and surrounding environment on this process, is an ongoing challenge in aquaculture. To date, only a handful of studies aimed at farmed (Brown et al., 2019; Rosado et al., 2019; Minich et al., 2020) or wild fish (Hess et al., 2015; Minich et al., 2020) have been published on the gill microbiome. Pratte et al., (2018) studied reef fish and found that gill and intestinal microbiomes from the same individual showed greater similarity than respective gill or intestinal microbiomes from different individuals, and these authors concluded the presence of a core microbiome amidst the intra and inter-species variances. The gill microbial community, then, could be a representative metric for the total fish microbiome.

Due to its cost-effectiveness and relative accuracy (Caporaso et al., 2011; Vayssier-Taussat et al., 2013; Walters et al., 2015), the

use of 16S rRNA amplicon sequencing is considered common practice in such studies aiming to elucidate the composition of bacterial communities (Sevellec et al., 2014; Mohammed and Arias, 2015; Pratte et al., 2018; Krotman et al., 2020; Meron et al., 2020; Minich et al., 2020). A certain tradeoff exists between accuracy at the species and subspecies level and the ability to comprehensively screen bacterial communities (Ghyselinck et al., 2013; Martínez-porchas et al., 2016). Improvements in primer design and bioinformatics tools have helped bridge that gap, and enabled both higher reliability of NGS screening and the additional benefit of discovering novel species (Al-Hebshi et al., 2015; Johnston et al., 2017; Abu Fanas et al., 2021; Greay et al., 2021). In the present study, we use 16S rRNA NGS screening to provide an analysis of the gill microbiome of three fish species, in order to assess community composition and the presence of potential pathogens. Furthermore, this study aims to show that this method is useful in detecting multiple fish pathogens in parallel and finding correlations between pathogenic species residing together.

Materials and methods

Fish collection

All fish samples used were collected during a trawler survey conducted during May–June 2020, at depths of 20–80m, as part of a biannual survey, conducted by the Israeli Oceanographic and Limnological Research center (IOLR), in Haifa, Israel. The surveys are carried out at constant locations southwest of Ashdod, and eight kilometers away from cultured fish cages. Fish were immediately placed on ice and transferred to the lab. Eighty-nine fish individuals were collected: Atlantic chub mackerel (*Scomber colias*; n = 40), Bluespotted seabream (*Pagrus caeruleostictus*; n = 25) and Lessepsian lizardfish (*Saurida lessepsianus*; n = 24). Some of the samples were dissected fresh while others were frozen at −20°C to be later thawed and necropsied.

Tissue sampling

Frozen specimens were gradually thawed in small batches, weighed and measured, and were then dissected aseptically according to an established fish necropsy protocol (Yanong, 2003). Gills tissue samples were gently removed and placed in predesignated test tubes, then frozen at a temperature of −80°C until undergoing DNA extraction. *P. caeruleostictus* samples ranged in length between 11.4-20.4cm and in weight between 23.4-153.4g. *S. lessepsianus* samples ranged in length between 15.2-30.2cm and in weight between 21.7-230.7g. *S. colias* samples ranged in length between 13.2-19.5cm and in weight between 18.6-71.8g.

DNA extraction

Extractions of DNA were done using the GeneMATRIX Soil DNA Purification Kit (EURx, Gdańsk, Poland), following the manufacturer instructions for tissue lysates, with an additional two hour incubation at 55°C following suspension of the sample tissue in the kit-provided lysis buffer. DNA quality was examined by NanoDrop spectrophotometry analysis and agarose gel-electrophoresis.

PCR amplification and amplicon sequencing

Total DNA extracts were used as template for amplification of partial 16S rRNA gene sequences, at the V4 hypervariable region. Amplicons were generated using a two-stage PCR amplification protocol as described previously (Naqib et al., 2018). Each of the first stage PCR reactions consisted of a total of 50µl in volume and included: 25µl of GoTaq Green Master mix (Promega, Fitchburg, WI, USA), 2µl of mixed forward and reverse primers (in a concentration of 1nM each), 2µl of bovine serum albumin (BSA), 18µl of ultra-purified water (UPW) and 3µl of 80ng/µl template DNA. The primers contained 5' common sequence tags (known as common sequence 1 and 2, CS1 and CS2) compatible with Access Array™ primers for Illumina sequencers (Fluidigm, South San Francisco, CA, USA) (Caporaso et al., 2012). The primers used for amplification were (linker sequences in **bold**): CS1_518F: 5' – **ACACTGACGACATGGTTCTACA**CCAGCAGCCGCGG TAATACG – 3' (Nakasaki et al., 2009) and CS2_806Rc: 5' – **TACGGTAGCAGAGACTTGGTCT**GGACTACNVGGG TWTCT – 3' (Walters et al., 2015).

The PCR conditions were as follows: 10 cycles of denaturation at 95°C for 15s, annealing at 60°C for 15s and elongation at 72°C for 30s; followed by 10 cycles of denaturation at 95°C (15s), annealing at 55°C (15s) and elongation at 72°C (30s); continued with 10 more cycles at 95°C (15s)/50°C (15s)/72°C (30s); and then 5 additional cycles with yet another change of annealing temperature, performed at 62°C. The PCR concluded with 2 minutes of incubation at 72°C, before being lowered to 4°C for one hour (or until samples were removed). Amplicons were sent to UIC Sequencing Core (Chicago, IL, USA), in which a second PCR amplification was performed in 10 microliter reactions in 96-well plates using MyTaq HS 2X mastermix (Bioline, Taunton, MA, USA). Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). One microliter of PCR product from the first stage amplification was used as template for the 2nd stage, without cleanup. Cycling conditions were 95°C for 5 minutes,

followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". Libraries were then pooled and sequenced with a 20% phiX spike-in on an Illumina Miniseq sequencer employing a mid-output flow cell (2x150 paired-end reads). Final library preparation, pooling, and sequencing were performed at the Genome Research Core (GRC) at the University of Illinois at Chicago (UIC).

Sequence data processing

Detailed information regarding the sequence data processing is provided in the Supplementary Information File. In brief, sequence data was analyzed using the Dada2 pipeline (Callahan et al., 2016) using R package 'dada2' (version 1.14.1). Error rate estimation was carried out in order to sample nucleotides and reads for model building randomly across all samples. The dada2 algorithm was implemented for error correction and a count table containing the amplicon sequence variants and counts per sample was produced. For each amplicon sequence variant (ASV), taxonomy (up to the species level) was inferred by alignment to the Silva non-redundant small subunit ribosomal RNA database (version 138) using dada2 commands 'assignTaxonomy' and "addSpecies" with minimum bootstrap value set to 80%.

Data analysis

All data filtering parameter settings are detailed in the Supplementary Information File. In short, for data analysis and generation of figures, the online tool MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml) was used (Dhariwal et al., 2017; Chong et al., 2020). Taxonomy labels were assigned using the SILVA taxonomic framework (https://www.arb-silva.de/documentation/silva-taxonomy/). Initial analyses provided 189 bacterial ASVs identified to the taxonomic level of species, with 177 unique values (i.e., species). All 177 species were searched in the literature using their species name separately and together with conjugations of 'Pathogen', 'Infection' or 'Disease', with and without reference to fish/humans. In addition, sequences belonging to "pathogenic" genera were run through BLAST. This enabled identifying four more species and raised the total number of species to 181. Forty-one species were categorized as pathogenic to marine animals and/or humans. A literature-based scale was built applying several categories for their range of pathogenicity: from 'Unknown' to 'Rarely', 'Pathobiont', 'Opportunistic', 'Yes' and 'Obligatory'. Species were labeled 'Unknown' whenever the literature provided no evidence of pathogenicity whatsoever. 'Rarely' is a term used in the literature almost solely in reference to human pathogens. The commonly used term 'Facultative' was split into 'Pathobiont' and 'Opportunistic', differentiating them by defining the former as a mutualistic symbiont becoming virulent under certain conditions, while the latter refers to a commensal symbiont of pathogenic capabilities, a 'hitchhiker' that usually does not provide useful services to the host – nor causes harm – but turns virulent when conditions are favorable of it. 'Yes' marks an uncertainty whether the pathogen should be categorized as 'Rarely', 'Pathobiont' or 'Opportunistic'. 'Obligatory' refers to obligatory pathogens, meaning they always express virulence. This is a rare attribute found in bacteria and no obligatory pathogens were found in this study.

Phylogenetic trees

A detailed account of the parameters used for creating trees is given in the Supplementary Information File. Briefly, sequences identified as belonging to the several genera chosen for deeper enquiry were uploaded to Silva (https://www.arb-silva.de/aligner/) for preparing phylogenetic files (Quast et al., 2013; Yilmaz et al., 2014; Oliver et al., 2017). The ACT (Alignment, Classification and Tree Service) tool was used (SINA v1.2.11) (Pruesse et al., 2012). Output TREE format files were extracted for visualization with the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

All of the 89 fish collected appeared healthy both externally and internally upon inspection and necropsy. The community structure of the gill samples differed between fish species (Figure 1). Atlantic chub mackerel (ACM; *Scomber colias*) exhibited a higher and richer composition (Simpson index average: 0.9) than the Lessepsian lizardfish (LLF; *Saurida lessepsianus*) and Bluespotted seabream (BSSB; *Pagrus caeruleostictus*), which displayed indexes of 0.75 and 0.81, respectively. The LLF had the highest variance between samples. In all three fish species, low-richness outliers did not display an unusual increase in pathogenic agents' presence. A comparison of compositions (Figure 2) shows a clustering of microbiomes among species, with ACM displaying a community structure least similar to the others, and BSSB sharing most of its microbiome with the two other species.

An interaction network (Figure 3), expressing the strength of ties between bacteria genera to each other and their tendency to be hosted by the different fish species, highlights three main cohorts: the '*Psychrobacter* cohort', the '*Photobacterium* cohort' and the '*Staphylococcus-Streptococcus* cohort'. The *Psychrobacter* cohort was the most diverse and contained few genera associated with pathogenicity. It was mostly associated with ACM. The '*Photobacterium* cohort' expressed correlation especially to BSSB

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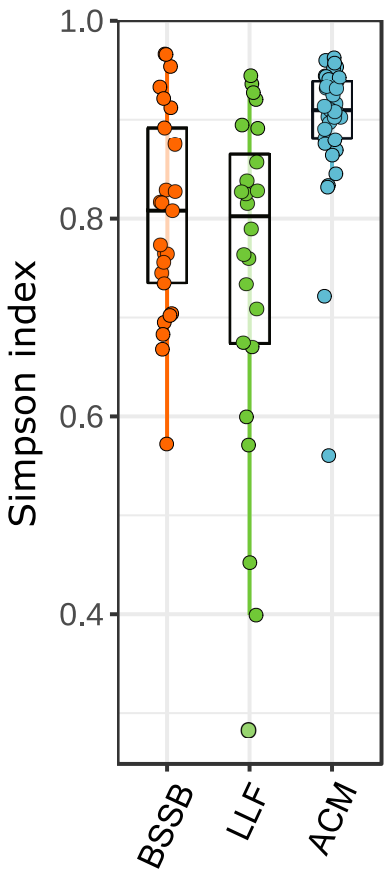

FIGURE 1
Community structure of fish gills samples. Data clustered by fish species. Kruskal-Wallis statistic: 24.928, $P<0.001$.

samples, and presented ties between the Gram-negative Gammaproteobacteria class members *Vibrio*, *Aliivibrio*, *Photobacterium* and *Shewanella*, together with *Cetobacterium* (class Fusobacteriia). The *'Staphylococcus-Streptococcus* cohort' was most strongly associated with LLF, and exhibited correlations between Gram-positive bacteria *Staphylococcus*, *Streptococcus* and *Gemella* (Bacilli) with *Actinomyces*, *Cutibacterium*, *Micrococcus* and *Rothia* (Actinobacteria). It also exhibited correlations with the Gram-negative *Cloacibacteria* (Bacteroidia) and *Enhydrobacter* (Gammaproteobacteria).

The relative abundance of each genus by fish type further supports species-specific microbial composition (Figure S2). The ACM gill microbiome was predominated by *Psychrobacter* (28.3%), and BSSB by *Photobacterium* (30.2%). These two genera were notably present in all three species, alongside *Shewanella*. The *Cetobacterium, Aliivibrio, Cutibacterium, Vibrio, Rothia, Staphylococcus* and *Streptococcus* genera were distributed in lower abundance between two or three species. Over 65% of the LLF, 27% of the ACM and 18% of the BSSB microbiome reads were classified as "Not-assigned" with an additional set of reads in each returned as "others", a summary of low count genera. The genera known to include many potential pathogenic species – *Photobacterium, Shewanella, Staphylococcus, Streptococcus*, and *Vibrio* – were also unevenly distributed between fish types (Figure 4). From this figure there are several observations to be made: (i) except for *Staphylococcus*, BSSB is host to a larger percentage of potentially pathogenic bacterial species than the other two fish species; (ii) error bars across all three fish species indicate that large variances occur amongst samples from each fish species, especially in the top quarter percentile of samples.

The NGS data analyses resulted in an output of 5,798 unique amplicon sequence variants (ASVs) of which 5,717 were identified as bacteria, 15 as archaea, five as eukaryotes and the rest unidentified. None of the ASVs appeared in 100% of the samples, nor in 100% of the samples of any specific fish species. Of those bacterial ASVs, 189 were initially identified to the taxonomic level of species, with 177 unique values (i.e., species). Of this list, 41 species were identified as bearing some pathogenic potential to humans and/or marine animals (Table S1): 36 had varying human clinical relevance or zoonotic potential. These were divided into the following taxonomic classes: Gammaproteobacteria (n = 15); Actinobacteria (n = 10); Bacilli (n = 8); Campylobacteria (n = 1); Alphaproteobacteria (n = 1); and Fusobacteriia (n = 1). The literature also revealed that of the 41 potentially pathogenic species, a total of 14 were known marine animal pathogens (meaning, some of those potentially pathogenic to humans may also cause disease in marine wildlife). These 14 species were of the taxonomic classes Gammaproteobacteria (n = 9); Bacilli (n = 3); and Bacteroidia (n = 2). Thirteen of the fish pathogens appeared in samples belonging to ACM, six in LLF and four in BSSB. The results are visualized in Figure 5. It shows *S. baltica* to be highly prevalent in these fish species (found in 95% of ACM, 46% of LLF and 92% of BSSB), and that *P. damselae* was also prevalent (73%, 38% and 84% of the ACM, LLF and BSSB samples, respectively). In contrast, pathogenic *Streptococcus*, *Staphylococcus* and *Vibrio* species were found less frequently.

Phylogenetic analysis of these five genera of interest demonstrated the most closely related reference species to the ASVs with pathogenic potential. The *P. damselae* clade (Figure S4) appears divided between ASVs associated with *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*, in which the former is predominant – both in number of ASVs and total number of reads. In total, *P. damselae* makes up >30% of the genus' ASV reads. The *Shewanella* phylogenetic tree (Figure S5) is comprised of numerous ASVs, as this genus is the most prevalent (though not most abundant) of all genera analyzed. The *S. baltica* associated ASVs were responsible for >70% of all *Shewanella* reads. The trees of *Staphylococcus*, *Streptococcus* and *Vibrio* (Figures S6–S8, respectively) are similar in terms of the pathogenic/non-pathogenic ratios they exhibit: between 1–4%. This data is summarized in Figure 6.

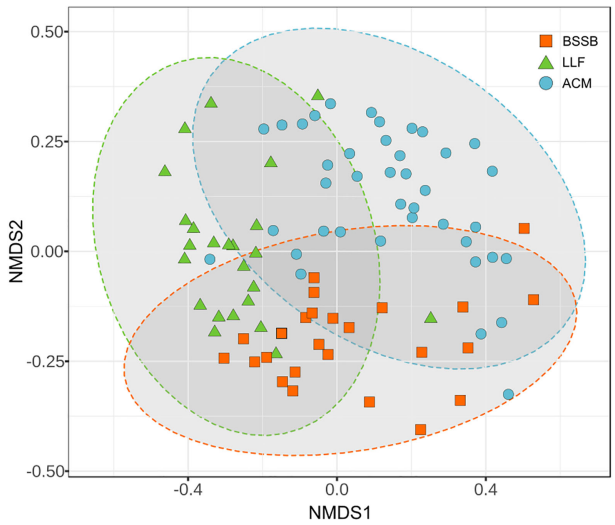

FIGURE 2
Comparison of compositions of the gills' microbiome between the sampled fish species. PERMANOVA: F: 7.7605, R^2: 0.1529, P<0.001, NMDS stress = 0.1740.

Discussion

The use of fish gill microbiomes to assess pathogen prevalence is gaining importance and becoming increasingly routine (Hess et al., 2015; Pratte et al., 2018; Brown et al., 2019; Rosado et al., 2019; Minich et al., 2020). However, to the best of our knowledge, this is the first study to be carried out in the Eastern Mediterranean. In previous studies, the presence of pathogens was inferred from taxonomic levels higher than species (Pratte et al., 2018; Rosado et al., 2019), sometimes supported by data on shifts in microbiome composition between treatment and control groups (Hess et al., 2015; Mohammed and Arias, 2015; Brown et al., 2019; Minich et al., 2020) or spatio-temporal differences (Minich et al., 2020). In the current study, we based our findings regarding pathogens only on ASVs that we could identify to the species level at a high degree of certainty. The data show that Atlantic chub mackerel (ACM), which was found to host the richest microbial community of the three fish species, also shows the largest total number of bacterial species with pathogenic potential. The microbiome of ACM included 35 out of the total of 41 pathogenic bacterial species found (and 13 of the 14 fish pathogens), while Lessepsian lizardfish (LLF) had 26 (6/14) and Bluespotted seabream (BSSB) just 6 (5/14 fish pathogens). A possible explanation for this result may be the pelagic-migratory nature of ACM, which means that it passes through diverse geographic zones, where it may accumulate a variety of bacteria on its gills. BSSB has a relatively high abundance of *Photobacterium*, and within the *Photobacterium* genus, ~30% of the reads belong to a specific and well-known pathogenic species. This entails that ~10% of BSSB gill microbiome is *P. damselae*. Had this putative pathogen been obligatory, many of the samples would have shown signs of infection. Since this was not the case, it raises the question whether the two subspecies of *P. damselae* (a multi-gene PCR array for sub-speciation was not performed in this study) are opportunistic or pathobionts. Previous studies suggest that a genetic diversity within *P. damselae* subsp. *damselae* strains means disease outbreaks in fish are most likely caused by multiclonal populations, containing several complementing virulence factors (Terceti et al., 2016). Cases in which *P. damselae* is highly prevalent in fish without causing disease may reflect the need for a few variants of this pathogen to 'join forces' to create effective infection (Terceti et al., 2016). This is supported by a study conducted on *P. damselae* subsp. *piscicida*, which found variability of virulence in different strains, governed by a protein exotoxin, AIP56 which is secreted by virulent *P. damselae* subsp. *piscicida* in large quantities but not by avirulent strains. This is a key factor responsible for apoptogenic activity targeting fish macrophages and neutrophils (do Vale et al., 2005).

The observed high correlations between Gammaproteobacteria of the *Photobacterium* cohort, and especially the Vibrionaceae members, *Vibrio*, *Aliivibrio* and *Photobacterium*, suggest that the ecological niche existing in the form of BSSB gills provides these genera with preferable conditions. A similar assumption can be made regarding the *Streptococcus-Staphylococcus* cohort and the gills of LLF. In reference to potentially pathogenic species, within the *Streptococcus-Staphylococcus* genera-related sequences, pathogenic species make up only a small percentage of the total reads, and these two genera are comparatively less dominant in terms of relative abundance. On the other hand, *S. baltica*, a bacterium that is not known to be pathogenic to marine animals,

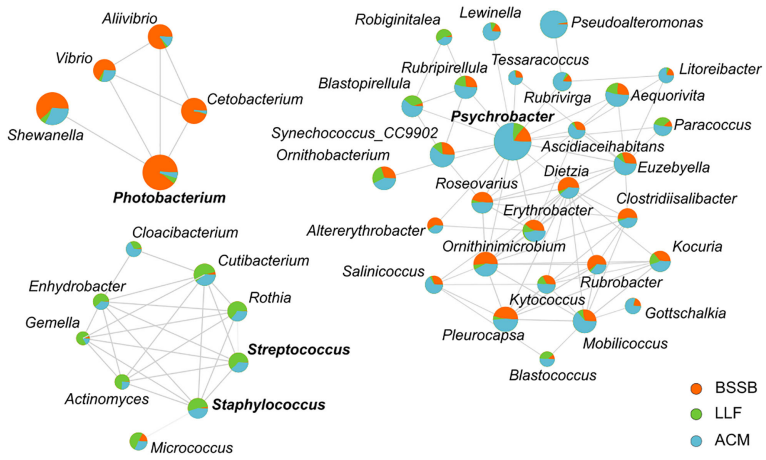

FIGURE 3
An interaction network between bacterial genera, in reference to fish species. Network calculation used Spearman's rank correlation coefficient and the threshold settings placed at: correlation > 0.5; $P<0.05$. Size of circles represents abundance and the coloring associates bacteria with host in regards to mean abundance. Names of 4 genera are emphasized by bold type, in order to identify them as those after which each cohort was named (i.e., *Photobacterium* cohort, *Psychrobacter* cohort and *Staphylococcus-Streptococcus* cohort).

but is rather a major cause of food spoilage (Zhang et al., 2020), dominates *Shewanella*-associated ASVs. A comparison of the pathogenic/non-pathogenic ratios per genus (Figure 1) summarizes these differences. According to our analyses, *Vibrio*, a genus found relatively much less abundant than *Photobacterium*, also comprised almost entirely of non-pathogenic species.

Nevertheless, it is important to remember that separating *Vibrionaceae* members by means of their 16S genes is problematic (Machado and Gram, 2015), therefore better identification may require lengthening the amplicon sequences by using a different set of primers (Morales and Holben, 2009; Martínez-porchas et al., 2016), or by complementing the identification using Multilocus Sequence Analysis (MLSA) – targeting protein-encoding genes such as *toxR* and *rpoD* (Pascual et al., 2010).

While using rRNA amplicon sequencing for the study of microbiomes has great advantages and is extremely useful, its limitations must be considered. In a highly diverse microbiome, different bacteria can display functional redundancy. Hence, a 'function over phylogeny' approach studying the functional characteristics of the microbial community – in addition to the species composition – may be advantageous and more informative (Moya and Ferrer, 2016; Gibbons, 2017; Louca et al., 2017). It was suggested by de Bruijn et al. (2018) (Louca et al., 2017) that the functional characteristics of greatest importance would be the protection of the fish against pathogens, and that this could be achieved by research of gene clusters involved in biosynthesis and production of proteins and metabolites, and studying these genes' frequency, diversity and transcription (Lynn and De Leenheer, 2019). Also, parallel investigation of both phylogeny and function is likely to help cut to a minimum the over- or underrepresentation of certain bacterial families, due to bias resulting from DNA extraction methods (Kashinskaya et al., 2017) and PCR, when relying solely on rRNA amplicon sequencing (Chakravorty et al., 2007; Sinclair et al., 2015; Stoddard et al., 2015).

The formation and function of the gill microbiome can be better understood when considering the much more investigated gut microbiome. During evolution, as the fish body structure developed and became increasingly complex, the adapting mucosa epithelia would have likely required additional resources, higher metabolic rates and improved metabolic capacities. In the ancient ocean, where microorganisms presumably flourished, interactions with fish inevitably formed the basis for creating early microbiomes (Gomez et al., 2013). According to Maynard et al. (2012), in the intestine and microbiome relationship, one counterpart receives a protected gut environment while the other benefits from the microbial highly adaptive metabolic engine that provides essential factors (e.g., vitamins) as well as an improved host ability to obtain nutrients from food. Gill et al., (2006) suggested that the human metabolism is the result of an amalgamation of microbial and human attributes. The microbiome can therefore be viewed as representing an evolutionary collection of species which is integral to the host's constant strive to augment the utilization of its food components (Maynard et al., 2012). In line with this hypothesis, i.e., the acceptance of commensal presence on the mucosal barriers, likely evolved in early vertebrates (e.g., fish), to profit from the ready-made microbial genetic material rather than independently, creating novel metabolic capabilities. It follows, that the greater the microbiome diversity, the better the chances of improving performance. The gill mucosa, as a

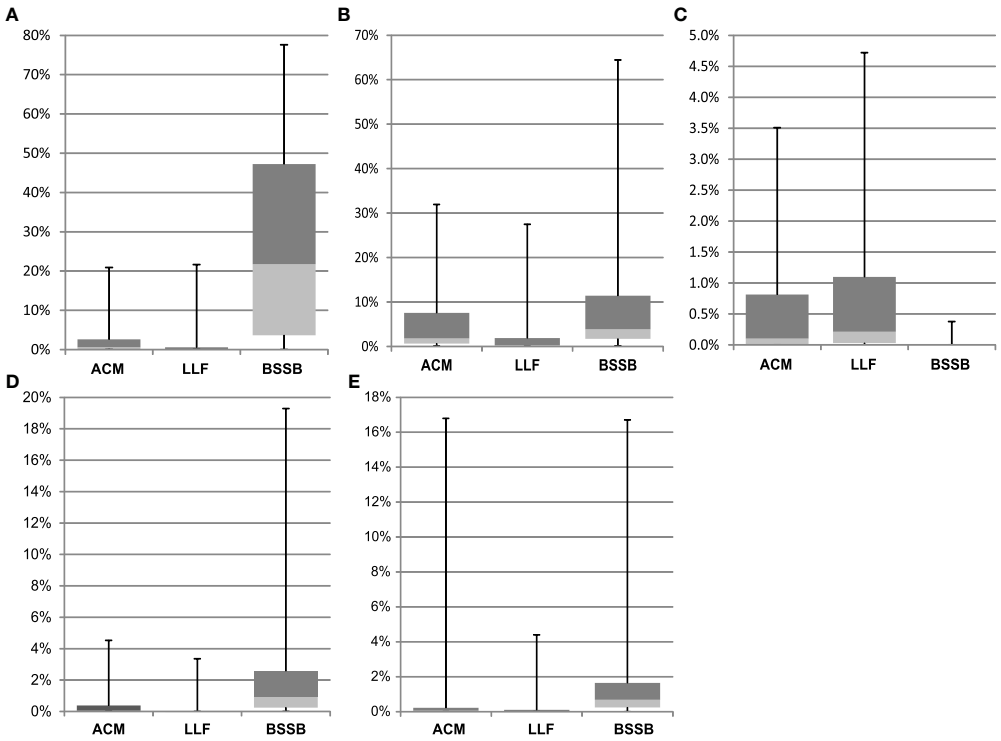

FIGURE 4
Relative abundance of five genera, clustered by fish species. Genera represented: *Photobacterium* **(A)**, *Shewanella* **(B)**, *Staphylococcus* **(C)**, *Streptococcus* **(D)**, and *Vibrio* **(E)**. Error bars depict quarterly percentiles, i.e., 25%, median, 75%, 100%.

semipermeable barrier between the fish and external milieu, would be faced with similar challenges (Koppang et al., 2015).

In the gills, similarly to the gut, the fish immune system must be able to identify commensal bacteria to avoid unnecessary inflammatory reactions triggered when pathogens threaten the intactness of the mucosal barrier (Gomez et al., 2013). The development of such immune tolerance to bacteria has been demonstrated in carp, where repeated anal administration of allogeneic cells has led to loss of allospecific cell-mediated immune response (Sato et al., 2005). Certainly, "false-alarm" inflammatory responses to innocuous bacteria, e.g., resulting in hyperplasia of the branchial epithelium, would not only place an unnecessary burden on the fish vital resources but also compromise the gas exchange processes of the respiratory epithelium.

Apart from host-pathogen relations, bacterial social interactions are another factor affecting virulence expression – both in terms of creating the conditions favoring virulence, and also in the ability to control virulence expression. Bacterial populations experience pressures of conflict and cooperation, which become a major factor in the organization and function of microbial communities (Asfahl and Schuster, 2017). Such interactions are found to be a stabilizing element widespread in *Vibrio* species, creating a protocooperation-based community, which shows increasing growth yield, while creating incentives to prevent a "Tragedy of the Commons" (Bruger and Waters, 2018). It was further shown that cooperative communities include members responsible for "policing", so that when challenged by cheaters, cooperative behavior can persist, provided that four conditions hold: (i) toxin-producers are present; (ii) the cost of toxin production surpasses that of public good production, meaning, policing becomes more expensive than cooperation; (iii) the toxin's harmful effects on the cooperator has to be sufficiently high – in order to counterweigh that policing is more costly than cooperation; and finally, (iv) the toxin's effects on the cheater must be even higher (Lynn and De Leenheer, 2019). An example coinciding with this theory is the pathogenic habits of *Vibrio harveyi*. In one study (Montánchez et al., 2019), this species was found to express virulent genes under heat stress, while cells of its community as a whole suffered extensive fatalities, demonstrating that disease outbreaks due to elevated sea surface temperatures, is but an escape route taken to avoid mortality. This means that pathogenicity in this species is not a display of offensive behavior, but rather a defensive mechanism. Some pathogens can survive for substantial periods in the open water, which explains how fish-cage originated secretions may travel many kilometers with the currents and still affect wildlife (or other cage farm stocks) downstream (Viau et al., 2011; Shapiro et al., 2013). Yet, pathogens are mostly prevalent in hotspots (e.g.,

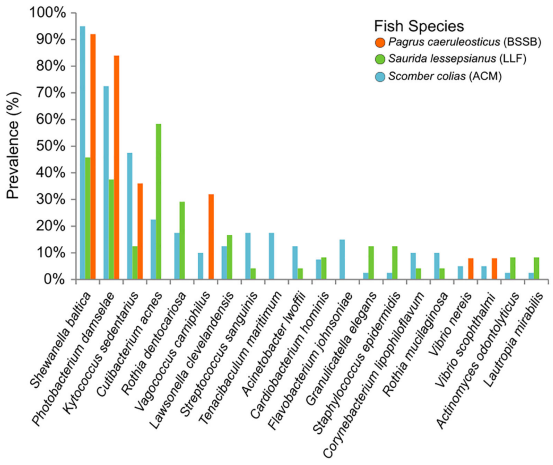

FIGURE 5
Prevalence of the 20 most commonly found pathogens in the gills of the fish sampled. The prevalence (y axis) refers to amount of samples per species found to contain these specific potential pathogens. Pathogens arranged (on the x axis) from the most (overall) prevalent (left) to the least (right).

immunosuppressed animals, naïve hosts, or those associated with anthropogenic pollution-hit areas) (Lyons et al., 2010; Lobelle and Cunliffe, 2011). Therefore, perhaps *Pathogenicity*, being defined as "the quality of producing or the ability to produce pathologic changes or disease" (Shapiro-Ilan et al., 2005), is inherently a trait 'designed' to affect a host. Being host-oriented, the presence of the 'right' host cells is an essential (though not sufficient) prerequisite for expressing pathogenicity. Yet, variances in genotypic attributes between different fish species (even those sharing diets, trophic levels and habitats), will create different 'environmental' conditions within gills (and other fish organs, for that matter), meaning pathogens in these organs will face different microbial communities (Pratte et al., 2018), which in turn may have an effect on the expression of pathogenicity of a given pathogen. Such a mechanism may help explain why asserting whether some less known bacteria are pathogenic (and to which degree), may prove tricky: some pathogens are not necessarily the cause of a certain disease, but are rather secondary agents of it, helping progress it, or just gaining benefit from the change in conditions within the infected organ (Brink et al., 2019). It is known (Laanto et al., 2012) that some pathogens may develop into symbionts with opportunistic pathogenic capabilities, using this ability as a survival tool, which may hint why co-infection is common – an arrival of a non-symbiotic pathogen (due to injury), may cause the host to become immunosuppressed and create conditions favorable of pathogenesis for the symbiotic pathogen.

As a general rule, bacteria of all phyla are known to have certain 'preferable' physio-biochemical conditions in which they thrive, and a range of conditions that can be regarded as 'tolerable'. The Eastern Mediterranean water column displays stratification, with different physiochemical properties existing in different layers that affect the local microbial communities (Techtmann et al., 2015). It was also shown that Gorgonians (Octocorallia, Anthozoa, Cnidaria), which are at the heart of extremely biodiverse ecosystems second only to tropical coral reefs, exhibit great differences in their microbiomes relative to their surrounding waters (van de Water et al., 2017). It is therefore obvious that fish gills offer a niche with a unique set of conditions, inducing the formation of certain microbial communities that are different than those in the water the fishes swim in. However, pathogenesis expressed in fish gills may also be affected by: (i) changes in the gills' microbiome throughout the life-time of the fish (Nagelkerken and van der Velde, 2002; Wilson et al., 2010; Mercier et al., 2012); and (ii) the possible presence of protistan parasites (e.g., ciliates, flagellates, etc.), which frequently facilitate the establishment of secondary microbial species (Gibbons, 2017), as well as many other pathogenic agents – viruses, fungi (Bui et al., 2019), macro and micro-parasites such as helminths and myxosporea (Molnár, 2002; Liyanage et al., 2003; Nguyen et al., 2021).

Fish gills harbor species-specific microbiomes, exhibiting strong correlations between certain taxonomic groups. In the present study, we demonstrated some overlap between the three host species sampled, perhaps expressing a form of core microbiome. The genera which were the focus of the study, *Vibrio*, *Photobacterium*, *Shewanella*, *Staphylococcus* and *Streptococcus* are important members within these fish species gills microbiomes, and at least in the case of Bluespotted seabream, a substantial percent of its gills microbiome is populated by generalist pathogenic species, which are notorious marine pathogens. In conclusion, given that all fish sampled appeared healthy, and based on the notion that pathogenicity is

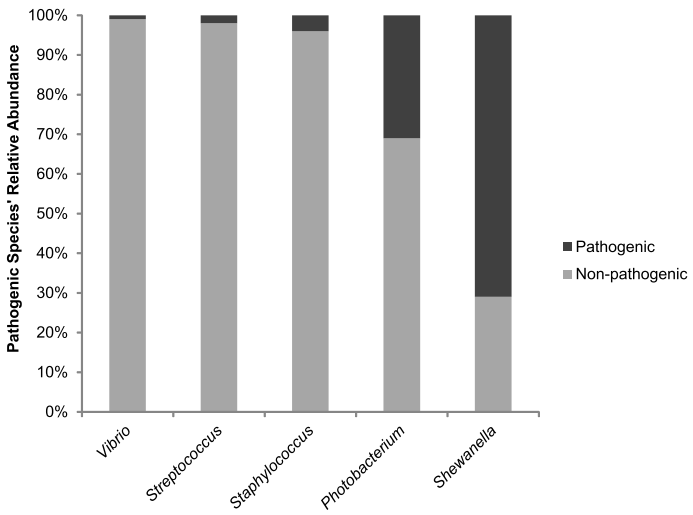

FIGURE 6
A comparison of the Pathogenic/Non-pathogenic ratios per genera of interest. Values represent relative abundance of potential pathogenic species identified out of all ASVs associated with a certain genus.

also influenced by environmental pressure against virulence (coming from microbial community interactions, carrying a strong preference for cooperation over cheating strategies), it can be inferred that pathogenesis is but one of many tools for survival and reproduction that bacteria are equipped with. This in turn explains the fact that pathogens are very rarely obligatory. What it also means is that healthy wildlife populations are not necessarily devoid of pathogens, but have a mix they coevolved with and which protect them from invasions of novel types.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and link to the data can be found below: DRYAD; https://doi.org/10.5061/dryad.wh70rxwr3.

Ethics statement

Ethical review and approval was not required for the animal study because the subjects of research were dead fish provided by another institute conducting an approved ecological study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1008103/full#supplementary-material

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