Monitoring Fish Pathogens in Eastern Mediterranean Fish by Applying Next Generation Sequencing on Gills' Tissue

Peleg Itay

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER'S DEGREE

University of Haifa

Faculty of Natural Sciences

Leon H. Charney School of Marine Sciences

The Department of Marine Biology

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Abstract

Reports from around the globe suggest that in recent years, outbreaks of marine fish infectious diseases are becoming extremer and more frequent. Despite the fact that their effects are inflicting grave economic losses on fisheries and aquaculture and pose threats on public health, research of these pathogens' prevalence in many regions is still lacking, including in the Levantine basin. In this study, 89 apparently healthy specimens of three wild fish species, common in the region, were collected from open sea waters about 50km south west of Ashdod, Israel, and in relative proximity to fish farms. Bacterial DNA was extracted from the gills, and 16S rRNA amplicons were sequenced by means of Next Generation Sequencing (NGS). The data obtained was analyzed in search of differences in microbiome and pathogen prevalence. The analyses revealed the presence of many potential pathogenic species – some of which are zoonotic – though most in minute relative abundances. Nevertheless, out of the wide range of genera contributing to the microbial communities of these fishes' gills, five genera were chosen for deeper enquiry: *Photobacterium, Shewanella, Staphylococcus, Streptococcus* and *Vibrio* – all of which are widespread and include pathogenic species with a vast record of diseases inflicted on cultured and wild fish worldwide. Out of these five genera-of-interest, *Photobacterium* and *Shewanella* were found to be the most prevalent and abundant. This is especially true in the case of the Bluespotted seabream (*Pagrus caeruleostictus*), where 30.2% of its gills' microbiome belonged to *Photobacterium spp.* and 11.3% to *Shewanella spp.*, which were found to be dominated by *Photobacterium damselae* and *Shewanella baltica* (respectively). Other genera-of-interest were found in quantities of up to 1.4% (*Vibrio* in *P. caeruleostictus*) and shown to contain only 1-4% pathogenic species.

The data demonstrates that fish gills harbor species-specific microbiomes, exhibiting strong correlations between certain taxonomic groups, with some overlap between the three species sampled – perhaps expressing a core microbiome. Given that all fish sampled appeared healthy, it was suggested that pathogens are very rarely obligatory pathogenic and since pathogenicity is influenced by environmental pressure against virulence, coming from microbial community interactions carrying a strong preference for cooperation over cheating strategies, such species can flourish also without becoming virulent.

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Introduction

The ever growing global demand for high-protein content food, amidst steep declines in wild fish catch (due to overexploitation of fisheries), means growing fish and seafood on land and sea-based farms has become more necessary than ever (Campbell and Pauly, 2013). Marine aquaculture (Mariculture) is an industry on the rise, and in many countries is being developed in order to replace the more traditional freshwater fishpond systems, for economic and health reasons. These farms create water pollution by nutrient enrichment in the form of fish-excreted ammonia, phosphate, CO_2 and 'sludge' – a collective name for feces, excess nutrients and particulate organic matter (POM) – known bacterial hotspots, which often are the source of fish-toxic metabolites (Abreu et al., 2011, Shpigel et al., 2016). Advanced technologies have been administered in recent years to increase production, reduce costs and environmental impact all at once (Lounas et al., 2020), but have not managed to solve the inherent problem of population-dense stock rearing. Living in dense populations, even in places where the naturally occurring currents are supposedly utilized to sufficiently wash away feces, remnants of food and dead individuals, make chances of infection grow significantly (Muziasari et al., 2017). In addition, some pathogens are density-dependent in terms of passing a threshold marking a shift from high prevalence to becoming epizootic (i.e., the analog of a human epidemic in a nonhuman animal population), making fish cages 'greenhouses' promoting pathogen spread into the surrounding waters (Krkošek, 2017). On top of that, a common practice amongst farms (with obvious operational logic) to relocate farmed animal livestock, provides pathogens with an opportunity to traverse great distances and spread to new geographical locations where they were never found before, affecting local wildlife. This represents another way in which human-introduced invasive species make a global impact (Kibenge, 2019).

Wild fish, cage escapees and pathogen transmission

In many areas of the world, one of the most important transmission routes of pathogens into wild fish is the vastly growing mariculture farms industry. By introducing millions of fish globally and annually from land based hatcheries into the ocean, pathogens are routinely entering the marine environment and passing from the fish in the cages to wild marine fish. Natural inhabitants of the marine aquaculture environment are important members of the ecosystems which form wherever floating cages are placed at sea. The cages offer a steady source of food (for those feeding on unconsumed remnants flowing out of them), possible refuge for small fish species in an otherwise open sea, and a good 'refueling spot' for migratory fish (Barrett et al., 2019). The presence of organisms of lower trophic levels turns

the cages into an attraction for predators, and the cages soon become an ecological hotspot (Piroddi et al., 2011, Barash et al., 2018). Migratory apex predators have even been recorded to make detours from the course of their usual routes when cages are introduced in the area (Papastamatiou et al., 2011). The interactions between fish within the cages and their peers outside them is sometimes mediated by **escapees** – cultured fish that find their way out of the cages during their upbringing, due to faulty cage design or during maintenance and harvest events (Jackson et al., 2015). This forms a triangular relationship, in which pathogens have many vectors of transmission between the three populations (Arechavala-Lopez et al., 2013). Today, over a quarter of the reared fish are non-native species to their environment (Atalah and Sanchez-Jerez, 2019), meaning escapees are not only a major factor introducing previously unmet pathogens to naïve hosts, but are also, obviously, in competition with native wildlife populations over resources.

Symbiosis, microbiomes and pathogens

Fish, like many other organisms, have evolved to live within a certain range of physiochemical conditions, such as temperature, pH, oxygen and salinity. Above and below optimal ranges renders them stressed and immunosuppressed. Extended periods beyond these ranges may lead to mortality (Johnson et al., 1992, Conte, 2004). Unlike mammals which are homeotherms, most fish are poikilotherms (what is commonly termed "cold blooded"), i.e., they have a limited ability to regulate their core body temperature. This entails that both the fish and its microbial inhabitants are physiologically tied to the environment and are more prone to experience stress under temperature fluctuations. The infection triangle, describing the triangular relations between the environment, a host and a pathogen (King et al., 2019), forms the basis of research aimed at understanding the effects of pathogens on marine animals, partially because it offers a way to conduct experiments with clearly defined dependent and independent variables – usually an abiotic environmental stress factor (Elarabany et al., 2017, Andrade et al., 2017, Wang et al., 2018, Zarantoniello et al., 2021, Genin et al., 2020). Though many times limited by this simplification of conditions, experiments as such still teach us a lot about the subjects of study and provide important insights and an improved ability to make predictions, especially in the wake of the climate crisis we are facing. Adding into this triangular array another vertex in the form of a microbiome, increases the system's degrees of complexity. Nevertheless, the knowledge accumulated through the works of scientists conducting experiments of the former kind and the tools developed both in DNA sequencing and in data analyses, enables researchers

nowadays to add more layers to that framework, and lets them get hold of higher resolution data, bringing forth new fields of study which were practically inaccessible previously.

The idea of the holobiont – describing the relationship between host and the microorganisms that are inhabitants of its body – has become a topic of great interest in recent years, as the understanding of the intimate partnership between hosts and their symbiotic microbiota in the well-being maintenance of the host, deepens (Apprill, 2017, Aschenbrenner et al., 2016). Symbiosis is often categorized in definite terms: mutualism, where both species benefit; commensalism, where one organism benefits while the other is unharmed; and parasitism, in which one party benefits at the expense of the other. Obviously, the reality of things is that in nature, relationships are not distinguishable as clear cut cases of each of these terms, bringing forward subdivisions that form groups under the roof of each. Moreover, between mutualism and parasitism lies a dynamic scale, in which the relationship can shift from one form to the other (and back) within the life span of the organisms involved, depending on changes in internal and environmental factors (Cunning et al., 2017). In some cases, this may be referred to as switching between different steady states, points of equilibrium – a tactic of evolutionary adaptation that increases survivability (Araújo et al., 2003). Not only that a host may acquire a symbiont, then release it when conditions require (and vice versa), but it can switch between different symbionts – each being optimal for certain environmental conditions (Sudakaran et al., 2017).

Keeping the balance of the microbiome has been found to be important for maintenance of fishes' health (Llewellyn et al., 2014). Hence, following changes in the microbial communities – usually caused by external pressures and stress factors such as naturally occurring environmental changes (Pérez-Ruzafa et al., 2018), anthropogenic activities (Halpern et al., 2008, Nguyen and Liou 2019), and/or changes in the host's internal physiology (Yildirimer and Brown 2018) – may serve as a bio-indicator enabling assessment of the host's health, even before clinical signs are made visible. Combined with data on shifts in pathogen prevalence, these two aspects become key factors in our aim to understand the clockworks of host-pathogen relations. Furthermore, looking into bacterial community dynamics has brought the notion that (at least some) members of these microbiomes help regulate pathogenic activity, serving as additional defense layers to the host's immune system (Vorburger and Perlman, 2018, Ramsey et al., 2016). It is this complex relationship array that we wish to explore in this study, in hope that it will provide another tool in making predictions over the potential for disease outbreaks in fish communities.

Zoonotic diseases

Zoonotic diseases, i.e., diseases that can be transmitted from animals to humans, have been notorious throughout human history, being responsible for taking the lives of many millions of people worldwide (Jordà et al., 2020). A pre-Covid-19 research estimated that in the U.S. alone, zoonotic diseases are responsible for over a third of the annual fatalities and injuries caused by animals (Conover, 2019). It has become clear that globalization and massive biodiversity loss – in part due to destruction of wildlife habitats – puts people in danger of increased exposure to both known and novel pathogenic agents, bearing a potential to mutate and become the source of zoonotic epidemics (Aguirre, 2017, Allen et al., 2017). Zoonoses of fish origin are well known in the industry, affecting mainly personnel coming in close encounter with fish (both fishermen and cultured fish industry employees) (Gauthier, 2015). Vibriosis, caused by species of the *Vibrio* genus (Soto-Rodriguez et al., 2019) and mycobacteriosis, caused by non-tuberculous mycobacteria (NTM) (Regev et al., 2020), are two examples being studied at the Marine Animals Pathogens Lab of the Morris Khan Marine Research Station situated in Sdot Yam.

Genera of interest:

Vibrio

Vibrio (family Vibrionaceae, class Gammaproteobacteria) are aquatic, Gram-negative rod-shaped, motile bacteria, which are facultative anaerobes. There is a dispute amongst bacteriologists regarding the extent of the phylogenetic umbrella under which *Vibrio* species lie, and the continuous discovery of novel species reshapes that structure constantly (Thompson et al., 2004). Within the *Vibrio* genus are 123 known species to date, many of them are part of the natural flora of marine organisms, with a variety of species renown for carrying pathogenic potential either to marine organisms, humans – or both (Ina-Salwany et al., 2019). Several known marine pathogens include: *V. parahaemolyticus* (Li et al., 2017), *V. harveyi* (Mondal et al., 2016), *V. anguillarum* (Zhao et al., 2016), *V. alginolyticus* (Mustapha et al., 2014), *V. ponticus* (Xie et al., 2007), *V. scophthalmi* (Sohn et al., 2019), *V. mediterranei* (Andree et al., 2020), many of which are zoonotic. Some *Vibrio* species' impact is more severe when they co-infect, perhaps due to interspecific competition or the release of molecules by certain *Vibrio* species, leading to changes in host microbiome that favor the pathogenic potential of the entire *Vibrio* community (Rubio-Portillo et al., 2020). There are also several human pathogens among the *Vibrio* genus, *V. cholera*, being the usual suspect, *V. vulnificus*, *V. parahaemolyticus* and others (Martinez-Urtaza et al., 2018, Thompson et al., 2004). Yet vibrios are not only about pathogenesis, as many of them are commensals, such as

V. diazotrophicus, *V. natriegens* and *V. cininnatiensis*, performing important ecological roles, like nitrogen-fixation and oxidative metabolism (Takemura et al., 2014). Pathogenic vibrios may cause Vibriosis, a disease generally attributed by septicemia, in which hemorrhages can be observed at the base of the fins, eyes and gills, and in internal organs like the liver, spleen, and kidney (Meneses-Márquez et al., 2019). *Vibrio*-related infections cause extensive losses in fish stocks worldwide and are a major concern to aquaculture and fisheries (Austin and Zhang, 2006, Rubio et al., 2019).

Photobacterium

The genus *Photobacterium* (Gammaproteobacteria: Vibrionaceae) was formerly identified as part of the *Vibrio* genus, then later raised to the status of a genus on its own, and has nearly 30 species described thus far (Machado and Gram, 2017). It too is Gram-negative, a facultatively aerobic, motile bacterium, commonly found in association with marine animals (though some are free-living, especially in coastal marine ecosystems). Some of the *Photobacterium* spp. bacteria are responsible for bioluminescence expression in marine organisms (Urbanczyk et al., 2011). A few of the species are pathogenic, with *P. damselae* being the most prominent. It has two subspecies, *Photobacterium damselae* subsp. *damselae* and *Photobacterium damselae* subsp. *piscicida*, which produce wound infections and hemorrhagic septicemia with high mortality rates in many species of fish, crustaceans, mollusks and cetaceans. They are also dangerous zoonotic pathogens, with a potential to cause fatal infections in humans (Rivas et al., 2013, Pham et al., 2020).

Shewanella

The genus *Shewanella* (Gammaproteobacteria: Alteromonadaceae) consists of a diverse group of facultative anaerobic, rod-shaped Gram-negative bacteria, widely distributed in marine and freshwater environments. The trademark of many Shewanellae is their capability to utilize a myriad of final electron acceptors in the absence of oxygen, an ability allowing them to survive in diverse habitats. They have been isolated from a wide range of salt concentrations, temperatures, and barometric pressures and have a variety of ecological roles ranging from being symbionts, epibionts, and opportunistic pathogens, to primary food spoilage organisms. Some *Shewanella* species have been reported to cause diseases in humans, with a large variety of symptoms (Hau and Gralnick, 2007, Jung-Schroers et al., 2017).

Staphylococcus

The *Staphylococcus* genus (Bacilli: Staphylococcaceae) members are cocci-shaped Gram-positive bacteria of low G-C content (Suzuki et al., 2012). There are several well-documented species of *Staphylococcus* that are commensals and/or opportunistic pathogens in humans,

some of which show multidrug resistance. *S. aureus*, for example, specializes in evading the human immune system and is notorious for contaminating medical devices (Pollitt, et al., 2018). Other members of the genus are associated with marine animals, and the pathogenic group members pose a great threat to aquaculture worldwide (Çanak and Timur, 2020).

Streptococcus

The *Streptococcus* genus (Bacilli: Streptococcaceae), has 103 known species and 9 subspecies. These bacteria are Gram-positive, cocci-shaped and can be divided into 4 groups: "pyogenic streptococci", the "*Streptococcus bovis*" group, "viridans streptococci" and "miscellaneous streptococci". Amongst each of these four groups there are several members that are responsible for diseases in marine animals as well as humans, making them bacteria of great importance in both (Janda, 2014, Patel and Gupta, 2018).

Choosing subject fish species

When researching fish pathogens, considerable thought should be put into choosing the fish species, which in turn, depends very much on the research questions and objectives. There are several possible reasons to pick a certain species, depending on what we want to study. We could: (i) compare native to invasive species; (ii) cultured to wild fish; (iii) benthic to pelagic species; (iv) seasonal/multi-yearly changes; or (v) compare geographical influences on a certain species. The original plan of this study was aimed at addressing some of these questions (ii, iv and v), as it was set to follow up on previous studies performed at the lab (on kidneys and livers) (Regev et al., 2020, Meron et al., 2020), but due to COVID-19 lockdowns, a change in plans was made (twice), and the focus of the study was shifted to gills. Yet, since the lab has not worked with gill samples prior to this study, no such samples were available in the lab's -80°C repository, which created the need to obtain new tissue samples from freshly collected fish. This entailed limiting the study to 89 samples of three common wild fish species – all of ecologic and economic regional importance – provided by the Haifa branch of Israel Oceanographic and Limnological Research (IOLR), from its June 2020 expedition (see **Materials and Methods** for details). The fact that these three fish species are commonly found in the region and come up in large numbers by IOLR trawler expeditions, opens the possibility to perform temporal studies in the future.

Species selected for this study

(Atlantic chub mackerel. Photo by © Pedro Niny Duarte © ImagDOP. fishbase.org)

Scomber colias

The Atlantic chub mackerel (*Scomber colias* Gmelin, 1789) is an abundant middle-sized pelagic fish distributed in warm and temperate waters of the Atlantic Ocean and in the Mediterranean Sea across the shelf and upper slope (Martins et al., 2013). It can reach 65cm in length and weigh as much as 2.9 kg (Navarro et al., 2012). It feeds on zooplankton, cephalopods, crustaceans and small pelagic fish. It is considered a relatively low market priced fish (usually brought up by trawlers as bycatch), but still an important part of local diets in many coastal regions around the Mediterranean and has become one of the major fish species being fished worldwide, being 24 of the top 25 mostly fished (in weight), with over 500K tonnes being taken out of seas globally in 2016 (FAO 2018).

(Lessepsian lizardfish. Photo by: Oz Rittner)

Saurida lessepsianus

The Lessepsian lizardfish (*Saurida lessepsianus* Russell et al., 2015) originates, as its name suggests, from the Red sea. It is an invasive species in the Mediterranean, which made its way like many other Lessepsian fish, through the Suez Canal. It is found mainly on sandy or muddy substrates reaching depths of 100m, but observed more commonly inshore, in depths of 20-30m. Its diet consists mostly of other fish. Mature fish range in length from 20-30cm, though a specimen collected from an IOLR expedition in 2019 measured 35.3cm, and can weigh up to 300gr (same specimen).

(Bluespotted seabream. Photo by © Shibl Dammous. fishbase.org)

Pagrus caeruleostictus

The Bluespotted seabream (*Pagrus caeruleostictus* Valenciennes, 1830) is a common resident of the Eastern Atlantic and the Mediterranean, and is considered a native benthopelagic fish, living in depths ranging from surface water to 200m (though usually found at 30-50m). Mature fish average at ~50cm in length (yet can even reach 90cm), they feed mostly on bivalves, crustaceans and small fish, and are regarded as being of commercial significance in local fish markets (Bauchot and Hureau, 1990, Ismail et al., 2018).

Why gills?

As indicated above, kidneys were initially targeted (with liver as a secondary organ, for inter-organ comparison), mainly because an earlier study found interesting results (Meron et al., 2020), and this study aimed at first to be a follow up on it. In addition, being internal organs which do not come in direct contact with the external environment, the presence of pathogenic bacteria in kidneys and livers may be an indication of an immune system fault, the sign of a disease or the remains of one (Gorrisen and Flik, 2016). However, difficulties in obtaining amplicons of sufficient quality, in part due to the use of a sub-optimal forward primer and/or an unsuitable DNA extraction kit, and combined with a tight schedule, made it compelling to move on to plan b (and then c). Gills are an important gateway into the fish body. They are a mucosal organ involved in gas exchange, osmoregulation, acid-base regulation and excretion of nitrogenous waste, making them vital to maintaining systemic homeostasis in the face of changing internal (e.g., acidosis) and environmental (e.g., salinity) conditions (Evans et al., 2005). Gills express immune responses on both independent and systemic levels, and are not necessarily affected by pathogens entering the body. It should be noted that being in direct interaction with the surrounding waters, entails that the microbial communities within gills would reflect to some extent those of the water (Kuang et al., 2020) – including the presence of pathogens – raising an intriguing possibility which requires deeper exploration, to prove the viability of using gills as a proxy for pathogen detection in the water. Moreover, it has been shown that mucosal vaccines are simpler and more cost effective than traditional delivery systems (Koppang et al., 2015).

Gills microbiome – the known and unknown

The worldwide research body on fish gill microbiome is still relatively poor and in its infancy (Merrifield and Rodiles, 2015). Previous 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, Yuan et al., 2021), and occasionally on other organs, such as kidneys and liver (Sevellec et al., 2014, Meron et al., 2020). Gills are an organ receiving more attention of late and being studied many times alongside skin, as they enable sampling live fish (Mohammed and Arias, 2015), yet, there are still only a handful of studies aimed at farmed (Rosado et al., 2019, Minich et al., 2020a, Brown et al., 2019) or wild fish (Hess et al., 2015, Minich et al., 2020b). The three fish species studied here have not yet been reported on, to the farthest of our knowledge (though some kin species have been studied) (Minich et al., 2020b). A study on several reef fish species found that gill and intestinal microbiomes from the same individual were more similar to one another than to gill and intestinal microbiomes

from different individuals, and managed to deduce the presence of a core microbiome, amidst the intra and inter-species variances (Pratte et al., 2018). The use of 16S rRNA amplicon sequencing is considered common practice in these studies (Brown et al., 2019, Mohammed and Arias, 2015, Krotman et al., 2020, Meron et al., 2020, Sevellec et al., 2014, Minich et al., 2020a, Minich et al., 2020b, Pratte et al., 2018).

Validating NGS results by conventional PCR

The process of establishing the NGS method as a routine monitoring tool for detecting specific pathogens included performing validation experiments (Meron et al, 2020). In this study we validated the detection of NGS for three important fish pathogens: *Photobacterium damselae*, *Vibrio harveyi* and *Streptococcus iniae* by our in-house laboratory, sets of primers and PCR systems, as also described in earlier studies performed at the lab (Berzak et al., 2019 Regev et al., 2020). We found during these validation experiments that a vast majority of positive NGS results for specific pathogens were also positive by conventional PCR, using long fragments of multiple genes specific to each of the pathogens. In this current project, due to the large amount (41) of NGS detected pathogens, it became impractical to validate all candidate pathogens by conventional PCR systems. Some of the more important ones are now undergoing further validation and characterization in the lab.

Hypotheses

I: Fish gills host species-specific microbiota.

II: Fish gills accommodate species-specific arrays of pathogens.

III: Putative pathogens can be detected in fish gills.

Objectives

There are three main objectives set for this study: (i) to create protocols for screening fish gills microbiota; (ii) to assess the prevalence of pathogens in fish gills; and (iii) to find correlations between pathogenic species residing together.

Significance

Living off the marine environment demands understanding how its major aspects function and interact. Marine pathogens play an important role in this environment, since they affect members of all trophic levels in marine ecosystems (Harvell et al., 1999, Harvell et al., 2002). Pathogens, naturally, are also a significant factor affecting the economics of mariculture (Rosa et al., 2012, Stentiford et al., 2017), and since some pathogens are zoonotic, they pose threats to public health. Furthermore, as the climatic crisis is expected to deepen and its effects to become more pronounced in the upcoming decades (Lejeusne et al., 2009, Li et al.,

2020), and as anthropogenic pollution of the marine environment becomes more severe (Walters et al., 2011), studying pathogens may also teach us of the extent of our impact on the seas and how that affects us in return (Smith et al., 2019).

This research is part of the Mediterranean monitoring program performed by researchers at Haifa University's Morris Kahn Marine Research Station (MKMRS). The aim of this study is to help establish a scientific baseline of marine pathogens in the Eastern Mediterranean, using fish gills as a proxy to study the pathogen presence in ambient sea water. Since gills provide a niche which can be thought of as an "enrichment culture" for pathogens, studying them can help gain knowledge of potential pathogen prevalence – including in the water. The fish species that were sampled and analyzed for *Streptococcus*, *Staphylococcus*, *Shewanella*, *Photobacterium* and *Vibrio*, are fish with both ecologic and economic regional importance.

Materials and Methods

Fish collection

All fish samples used were collected during the May-June 2020 IOLR trawler surveys (occurring twice a year at depths of 20-80m, at constant locations south west of Ashdod, and eight kilometers away from cultured fish cages – a distance which some migrating fish can traverse within hours (Kristensen et al., 2019); IOLR usually runs a second round during early winter). The fish were placed on ice on board the trawler, and kept on ice until being picked up from the IOLR station at Shikmona, Haifa, by members of the Marine Animals Pathogens Lab, who then brought them to the research station at Sdot Yam. The fish brought in included *Saurida lessepsianus* (N=24), *Pagrus caeruleostictus* (N=25) and *Scomber colias* (N=40), a total of **89** individual fish. All three species persist in relatively large numbers through biannual IOLR trawler expeditions, which makes them good candidates for long term monitoring. Some of the samples were immediately dissected while others were frozen at a temperature of -20°C until necropsy was performed.

Tissue sampling

Fish specimens were gradually thawed in small batches, weighed and had their length recorded, and were then dissected aseptically according to the fish necropsy protocol (Yanong, 2003). Gills tissue samples were removed and placed in predesignated test tubes, then frozen at a temperature of -80°C until undergoing DNA extraction.

DNA extraction

Extraction of DNA was done using the GeneMATRIX Soil DNA Purification Kit (EURx), following the manufacturer's DNA purification protocol instructions for tissue lysates, with one additional step – for improving yield – a two-hour incubation at 55°C right after placing the sample tissue in the beads tube with the lysis buffer. DNA quality was examined by NanoDrop spectrophotometry analysis as well as 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. PCR reactions were performed on a SimpliAmp Thermal Cycler (Applied Biosystems, Waltham, MA). Each reaction consisted of a total of 50µl in volume and included: 25µl of GoTaq Green Master mix (Promega, Fitchburg, WI), 2µl of mixed forward and reverse primers (in a concentration of 1nM), 2µl of bovine serum albumin (BSA), 18µl of ultra-purified water (UPW) and 3µl of sample DNA (DNA concentrations showed relative low variance, on average ~80ng/µl, thus enabled keeping the quantity uniform). The primers contained 5' linker sequences compatible with Access Array

primers for Illumina sequencers (Fluidigm, South San Francisco, CA) (Caporaso et al., 2012). The primers used for amplification were (linker sequences in **bold**, sequences shown from primers' 5' to 3'): CS1_518F: **ACACTGACGACATGGTTCTACA**CCAGCAGCCGCGGTAATACG (Nakasaki et al., 2008) and CS2_806Rc: **TACGGTAGCAGAGACTTGGTCT**GGACTACNVGGGTWTCT (Walters et al., 2015).

The PCR conditions were set 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, Illinois) for library construction and sequencing. For library preparation, each sample received a separate primer set with a unique 10-base barcode (Fluidigm, South San Francisco, CA; Item #100–4876) and received an Illumina sequencing adapter. Then, libraries were pooled and augmented with a 20% phiX DNA spike-in control. Libraries were then sequenced using an Illumina MiniSeq instrument (2x150 bp). Sequence data was retrieved as FASTQ formatted files (two files per sample).

Sequence data processing

Sequence data was analyzed using the Dada2 pipeline (Callahan et al., 2015) using R package 'dada2' (version 1.14.1). Fastq formatted reads were trimmed and filtered for low quality using the command 'filterAndTrim' with parameters maxEE=2, maxN=0, trimleft=20 and the trunclen=150. Error rate estimation was carried out using the 'learnerror' command with default parameters, but with the randomize parameter set to TRUE, in order to sample nucleotides and reads for model building randomly across all samples. Following, the dada2 algorithm was implemented for error correction and a count table containing the amplicon sequence variants and counts per sample was produced. Merging of forward and reverse reads was done using the 'mergePairs' command with a minimum overlap of 8 bases. Then, suspected chimeras were detected and removed using the command 'removeBimeraDenovo', with default parameters. For each ASV, taxonomy (up to the species level) was inferred by alignment to the Silva non redundant small subunit ribosomal RNA database (version 138), using commands 'assignTaxonomy' and 'addSpecies' with default parameters, while setting the minimum bootstrap confidence value to 80%.

Data analysis

For data analysis and generation of figures shown in **Results**, the online tool MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml) (Chong et al., 2020, Dhariwal et al., 2017), was used. The option Marker Data Profiling (MDP) was picked and three CSV files were uploaded – ASV counts, taxonomy and metadata files. For taxonomy labels the SILVA taxonomy option was chosen. Data filtering settings defined as follows: (i) minimum counts: 4; (ii) low count filter – prevalence in samples (%): 10; (iii) low variance filter – percentage to remove (%): 5, and filter based on inter-quantile range. This process removed 1093 low abundance/variance features and had 283 others kept. In order to produce alpha-diversity graphs, data normalization settings were adjusted to have: (i) data rarefied to the minimum library size; (ii) no data scaling; and (iii) no data transformation. Furthermore, the alpha-diversity profiling settings were placed at: (i) data input – filtered; (ii) experimental factor kept as 'species'; (iii) taxonomic level kept on 'feature-level'; (iv) diversity measure – three options were used – observed, Shannon and Simpson; and (v) the statistical method chosen was Mann-Whitney/Kruskal-Wallis;

Then, for all other graphs and analyses, the data normalization settings required not rarefying data, while transforming the data using relative log expression (RLE).

To produce taxa abundance stacked-bar graphs, the graph type chosen was percentage-abundance, while merging small taxa with counts < 8,000 and grouping samples by fish species. In order to produce beta-diversity graphs, the NMDS ordination method was chosen, with Jaccard index used for the distance method calculation. The statistical method chosen was Permutational MANOVA (PERMANOVA) and other options kept as default. For the correlation analysis, the algorithm applied was Spearman's rank correlation, the *P*-value threshold set to 0.05 and the correlation threshold placed at 0.5. All other values used default settings.

Phylogenetic trees

Sequences identified as belonging to genera-of-interest were copied from the main data file (each genus separately) and converted to FASTA format, and then 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), with the following parameters: (i) gene: ssu; (ii) unaligned remaining bases – attached to the last aligned base; (iii) search and classify (checked) with minimum identity with query sequence set to 0.98, and number of neighbours per query sequence at 2; (iv) compute tree (checked) with its workflow set to

'denovo including neighbours', 'FastTree' as the program to use, and 'gamma' as the rate model for likelihoods; (v) the output format: FASTA and file zip-compressed; and (vi) taxonomies selected for classification: SILVA and RDP (only). All other parameters kept as default. Output TREE format files extracted for visualization with the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

For matters of simplicity, in figures and text of the **Results** hereafter, *Pagrus caeruleostictus* is simply referred to as BSSB, which stands for Bluespotted seabream, LLF for Lessepsian lizardfish (*Saurida lessepsianus*) and ACM is an abbreviation of Atlantic chub mackerel (*Scomber colias*).

Fish gills' microbiome – the genus level

To get some understanding of the attributes and compositions of microbial communities within the gills of the different fish species, and in order to find patterns which may lead to valuable insights, we ran several types of analyses (all presented on the taxonomic level of Genus, for reasons discussed later on): a community structure analysis (Simpson index: **Figure 1**; Observed alpha-diversity and Shannon index: **Supplementary Figure 1**, **Supplementary Material**), followed by a comparison of compositions (**Figure 2**), an interaction network (**Figure 3**), and a relative abundance bar graph (**Figure 4**). Each of these figures sheds light on a different aspect of the data, and adds another layer to a map growing in resolution.

In search of patterns in species-related microbiota, a community structure analysis was performed, producing box plots for the Observed alpha diversity and Shannon index (**Supplementary Figure 1**, **Supplementary Material**) and another for the Simpson index (**Figure 1**). All tests reflected a similar trend (and thus only the Simpson index box plot is presented here) – ACM has on average a high and much richer gill microbiome than the other two fish species (Simpson index average: 0.9). LLF (0.75) and BSSB (0.81) have quite similar (medium-high) rich microbiomes, with LLF expressing a larger variance between samples. A further analysis was performed to find whether outliers (especially at the bottom-end of bacteria species richness) show an unusual increase in pathogenic agent presence, however no such relation could be detected.

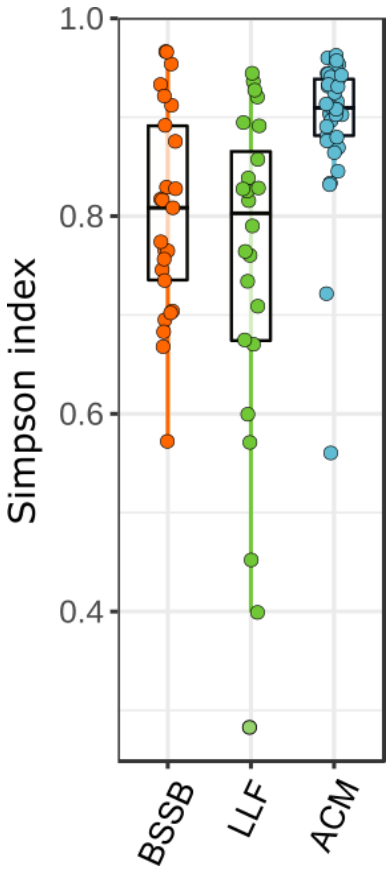

Figure 1 | Community structure of fish gills samples. Data clustered by fish species. Kruskal-Wallis statistic: 24.928, *P*<.001.

A comparison of compositions was then made to view how much these microbiomes are species-related. Each of the 89 samples is represented by a different combination of shape and color, for ease of identification. Metadata on length and weight of each fish sample was not incorporated into the calculation. The results (**Figure 2**) show a rather apparent clustering of microbiomes among species, with ACM possessing a composition of bacteria which is least similar to the others, and BSSB sharing most of its microbiome with either (or both) of the two other fish species. The ellipses representing the area which is characteristic of each of the fish species do not contain all samples belonging to their respective species. These seemingly outliers express similarity closer to compositions typical of other fish species, and show the variance occurring.

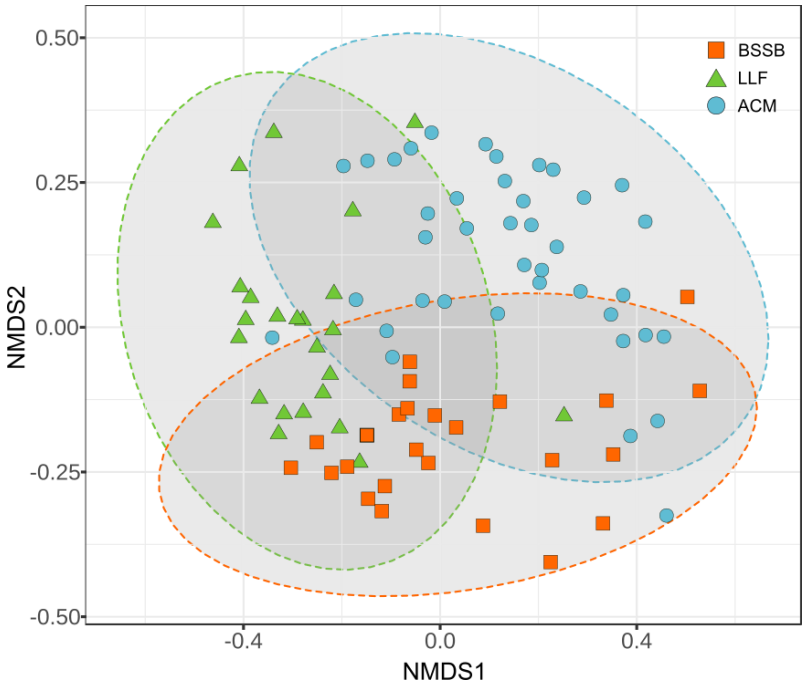

Figure 2 | Comparison of compositions of the gills' microbiome between the sampled fish species. PERMANOVA: F: 7.7605, R^2: 0.1529, P<0.001, NMDS stress = 0.1740.

An interaction network (**Figure 3**) – expressing the strength of ties between bacteria genera to each other and the extent to which they tend to be hosted by the different fish species – may serve as a tool to focus a search of genotypic aspects characteristic of certain fish species' gills. Spearman's rank was used for the calculation and the threshold settings placed at: correlation>0.5; P<0.05. The network highlights three main cohorts: the largest, henceforward named the '*Psychrobacter* cohort', is the most diverse and contains the least number of severe pathogenic agents. It is mostly associated with ACM; The '*Photobacterium* cohort', expressing correlation especially to BSSB samples, presents ties between the Gammaproteobacteria class members *Vibrio*, *Aliivibrio*, *Photobacterium* (all part of the Vibrionaceae family) and *Shewanella*, together with *Cetobacterium* (class Fusobacteriia) – all attributed by being Gram-negative bacteria; and the *'Staphylococcus-Streptococcus* cohort', most strongly associated with LLF, and exhibiting correlations between the Bacilli class members *Staphylococcus*, *Streptococcus* and *Gemella*, with *Actinomyces*, *Cutibacterium*,

Micrococcus and *Rothia* (Actinobacteria) – all of which are Gram-positive bacteria – and also with the Gram-negative *Cloacibacteria* (Bacteroidia) and *Enhydrobacter* (Gammaproteobacteria). Out of the three cohorts, we chose to focus on the *Photobacterium* cohort (and more specifically on *Photobacterium*, *Vibrio* and *Shewanella*) and on the *Streptococcus-Staphylococcus* cohort (but especially on those two genera). Henceforward, these five genera are collectively referred to as 'genera of interest'.

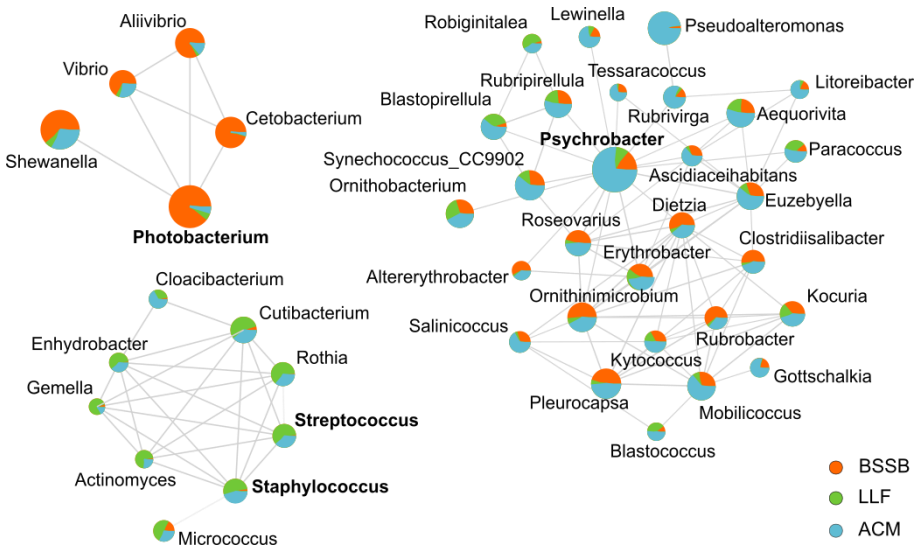

Figure 3 | An interaction network between bacteria genera, in reference to fish species. Size of circles represents abundance and the coloring associates bacteria with host in regards to mean abundance. Names of 4 species were emphasized by bold, in order to identify them as those after which each cohort was named (i.e., *Photobacterium* cohort, *Psychrobacter* cohort and *Staphylococcus-Streptococcus* cohort).

The relative abundance on the bacteria-genus level per fish species (**Figure 4**), reveals that the ACM gills' microbiome consists of *Psychrobacter* (28.3%), with 27.7% of the reads classified as 'Not-assigned', *Shewanella* (6.3%), *Pseudoalteromonas* (5.3%), *Photobacterium* (2.4%), *Synechococcus* (2.1%), and 27.9% 'Others' (low counts);

BSSB, owes 30.2% of its gills microbiome to *Photobacterium*, 18.4% Not-assigned, *Shewanella* (11.3%), *X2013Ark19i* (10.1%), *Cetobacterium* and *Psychrobacter* (5.2% each), *Aliivibrio* (3.2%), *Vibrio*

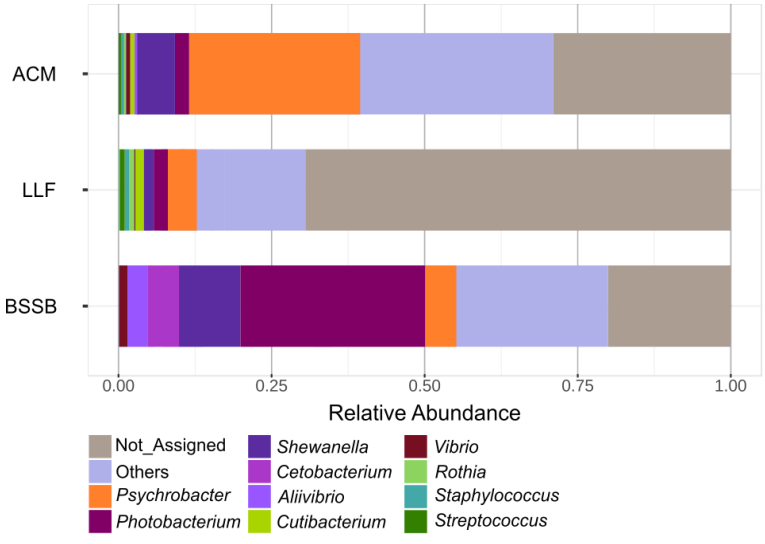

Figure 4 | Relative abundance of the fish species' major bacterial populations within the gills' community. The graph presents groups by relative size – from right to left – where the largest (more abundant) group is on the right and the least is on the left. The grey colored portions of the bars represent bacteria unidentified to the genus taxonomic level. Low count bacteria genera were aggregated into the 'Others' group. The three fish species express big differences in prominent genera, including presence of potential pathogens.

(1.4%) and 15.2% Others;

In LLF 65.8% is Not-assigned at the genus level, 5.3% *Psychrobacter*, 4.8% *MD3_55*, *Photobacterium* (2.6%), *X2013Ark19i* (2.4%), *Shewanella* (1.8%), and several species including *Staphylococcus* and *Streptococcus* make up ~1% of the reads (each), with an additional 8% to Others.

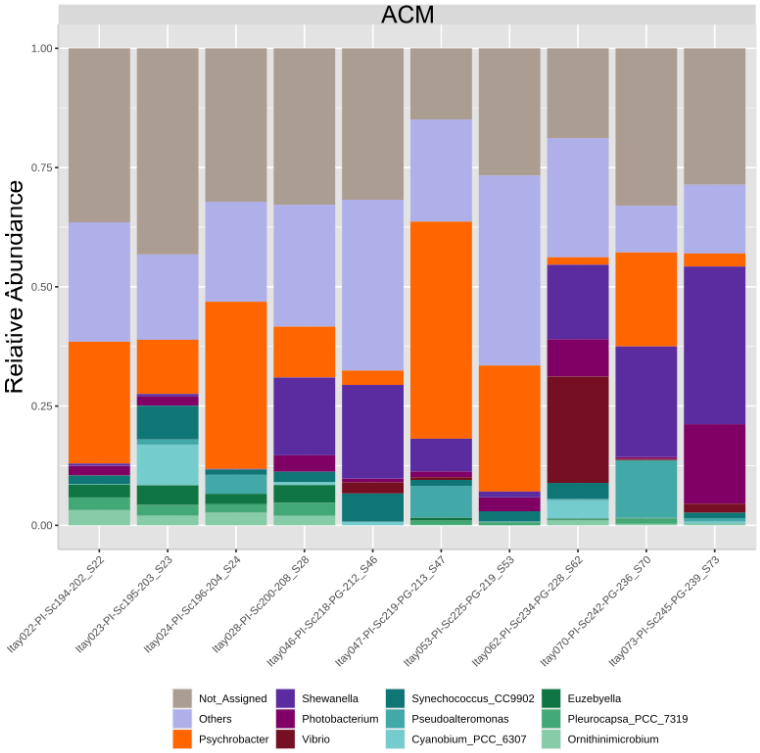

Figure 5 | Atlantic Chub Mackerel (ACM) relative abundance – 10 random samples.

A few relative abundance graphs were produced which take 10 random samples of each fish species and compare their individual relative abundances to each other. They serve the purpose of a 'control group', to evaluate how much the representation in **Figure 4** is indeed true to random individuals within the population. They are shown below (**Figures 5-7**).

The relative abundance of 10 randomly chosen ACM samples is presented in **Figure 5**. The patterns observed here are relatively similar to those shown in **Figure 4**.

The relative abundance of 10 randomly chosen LLF samples is presented in **Figure 6**. The patterns observed here are dominated by the Not-Assigned (NA) ASVs, which is also present in **Figure 4** (though not as pronounced). What is also

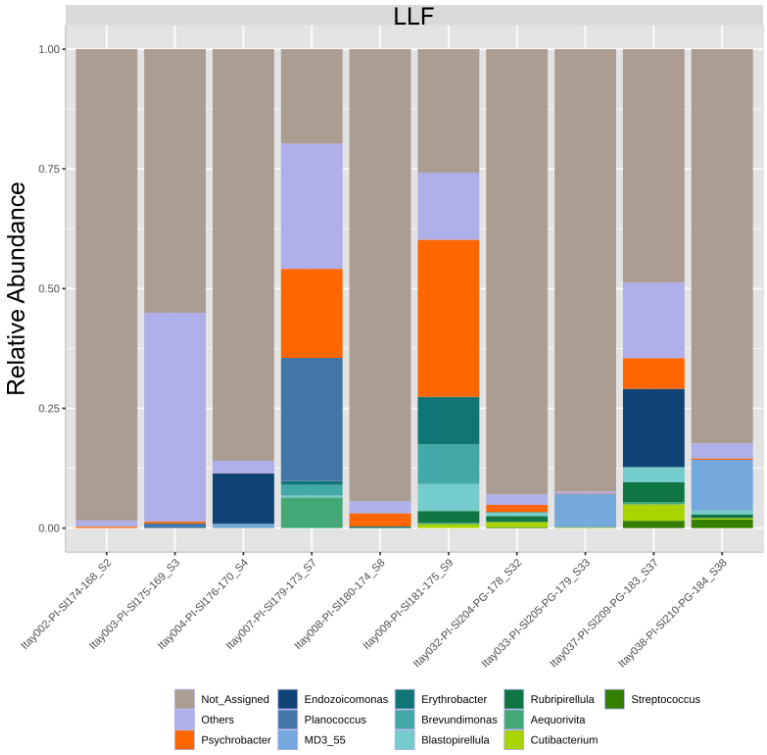

Figure 6 | Lessepsian Lizardfish (LLF) relative abundance – 10 random samples.

evident is the large variance within the sample group. That may be related to the analysis presented in **Figure 1**, where LLF samples express a large variance in species richness.

The relative abundance of 10 randomly chosen BSSB samples is presented in **Figure 7**. The patterns observed here are relatively similar to those shown in **Figure 4**. What is evident in this presentation of BSSB samples alone is the rearrangement of genera, so that *Photobacterium* is presented as very dominant.

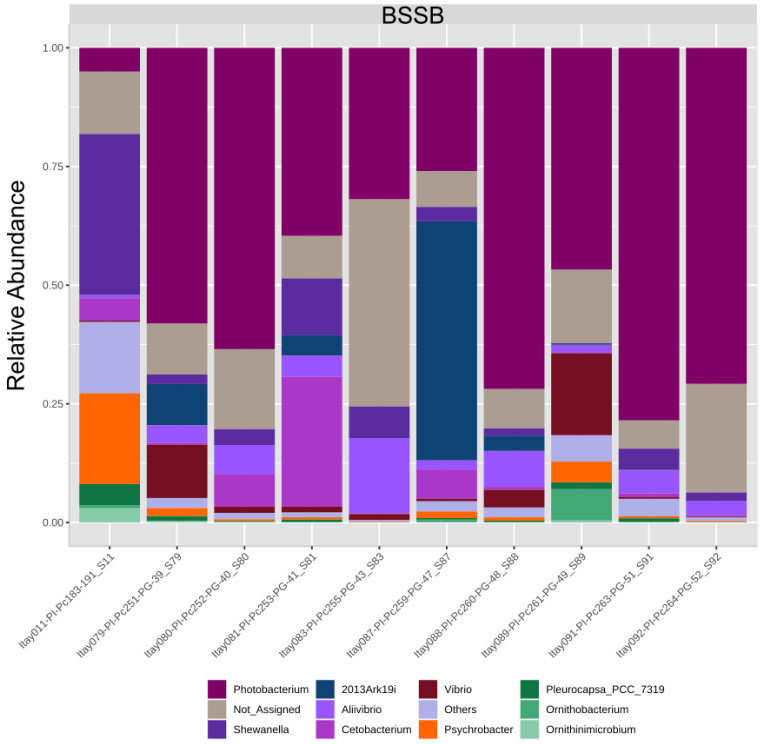

Figure 7 | Bluespotted Seabream (BSSB) relative abundance – 10 random samples.

From genus to species: the search of pathogens

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, 5 as eukaryotes and the rest were unidentified. None of the ASVs appeared in 100% of the samples, nor in 100% of the samples of a certain fish species. Of those bacterial ASVs, 189 were initially identified to the taxonomic level of species, with 177 unique values (i.e., species) and 12 duplicates. All 177 species were looked up 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 several genera of interest (and identified at least to the Genus level) were run through BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This raised the total number of species to 181. After combining these two sources of information, it was found that amongst these selected species, 41 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', through 'Pathobiont' to 'Opportunistic' and 'Yes', and eventually 'Obligatory'. Species were labeled **Unknown** whenever the literature provided no evidence of pathogenicity at all. **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

Table 1 | Top 20 most prevalent potentially pathogenic bacterial species found in the gills of fish sampled. The data presented combines ASVs identified as being associated with a pathogenic species to a high level of certainty. The percentage accounts for the number of samples out of the total (per species) that this potential pathogen was found in. Marine Pathogenicity marks where the literature indicated pathogenicity to marine animals, whereas Clinical Relevance marks pathogenicity to humans. Bacteria species are listed from the most prevalent at the top, to the least.

Bacteria	ACM	LLF	BSSB	Marine Pathogenicity	Marine Pathology	Clinical Relevance	Human Pathology
Shewanella baltica	95.0%	45.8%	92.0%	Unknown		Yes	Food spoilage (Zhu et al., 2015)
Photobacterium damselae	72.5%	37.5%	84.0%	Yes	Hemorrhages, ulcerative lesions, septicemia (Pham et al., 2020; Rivas et al., 2013; Terceti et al., 2016)	Opportunistic	Necrotizing fasciitis (Matanza & Osorio, 2020)
Kytococcus sedentarius	47.5%	12.5%	36.0%	Unknown		Pathobiont	Hemorrhagic pneumonia (Levenga et al., 2004)
Cutibacterium acnes	22.5%	58.3%	-	Unknown		Pathobiont	Acne vulgaris (Castillo et al., 2019)
Rothia dentocariosa	17.5%	29.2%	-	Unknown		Pathobiont	Endophthalmitis (MacKinnon et al., 2001)
Vagococcus carniphilus	10.0%	-	32.0%	Yes	Liver, myocardium, spleen, kidney necrosis (Chang et al. 2018)	Unknown	
Lawsonella clevelandensis	12.5%	16.7%	-	Unknown		Yes	Breast abscess (Menezes et al., 2018)
Streptococcus sanguinis	17.5%	4.2%	-	Unknown		Pathobiont	Endocarditis (Zhu et al., 2018)
Tenacibaculum maritimum	17.5%	-	-	Yes	Gill abrasion (Powell et al., 2005)	Unknown	
Acinetobacter lwoffii	12.5%	4.2%	-	Yes	Tissue lesions (Cao et al., 2018)	Opportunistic	Acute gastroenteritis (Regalado et al., 2009)
Cardiobacterium hominis	7.5%	8.3%	-	Unknown		Rarely	Endocarditis (Malani et al., 2006)
Flavobacterium johnsoniae	15.0%	-	-	Yes	Skin erosion (Li et al., 2015)	Unknown	
Granulicatella elegans	2.5%	12.5%	-	Unknown		Yes	Bacteremia, endocarditis (Alberti et al., 2016)
Staphylococcus epidermidis	2.5%	12.5%	-	Opportunistic	Fin, gills haemorrhages (Kubilay & Uluköy, 2004)	Pathobiont	Bloodstream infections (Otto, 2009)
Corynebacterium lipophiloflavum	10.0%	4.2%	-	Unknown		Yes	Bacterial vaginosis (Funke et al., 1997)
Rothia mucilaginosa	10.0%	4.2%	-	Unknown		Pathobiont	Pneumonia (Maraki & Papadakis, 2015)
Vibrio nereis	5.0%	-	8.0%	Yes	Vibriosis (Mondal et al., 2016)	Unknown	
Vibrio scophthalmi	5.0%	-	8.0%	Opportunistic	Liver, intestine hemorrhage (Qiao et al., 2012; Zhang et al., 2020)	Unknown	
Actinomyces odontolyticus	2.5%	8.3%	-	Unknown		Rarely	Lung abscess (Takiguchi et al., 2003)
Lautropia mirabilis	2.5%	8.3%	-	Unknown		Pathobiont	Periodontitis (Colombo et al., 2012)

symbiont becoming virulent, while the latter refers to a commensal symbiont of pathogenic capabilities. **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, as turns out, is a rare behavior found in bacteria and in any case, no bacterial species which are obligatory pathogens were found in this study, thus it was unused. The list is presented in **Table 1** and **Supplementary Table 1** (**Supplementary Material**). Of the 41 pathogenic agents listed, 36 had varying human clinical relevance, 9 of which were also pathogenic to marine animals. They were divided between taxonomic classes as such: 15 Gammaproteobacteria; 10 Actinobacteria; 8 Bacilli; 1 species belongs to Campylobacteria; 1 to Alphaproteobacteria; and 1 to Fusobacteriia. There were a total of 14 marine animal pathogens, 9 of them from the class Gammaproteobacteria; 3 are Bacilli; and 2 Bacteroidia. 13 of the fish pathogens appeared in samples belonging to ACM, 6 in LLF and 4 in BSSB.

To provide a visually simple view of **Table 1**, the top 20 most prevalent pathogens are also presented in **Figure 8**. It should be noted that prevalence does not equal abundance, i.e., each appearance of a sequence read in any given sample was treated equally to sequence reads in other samples, regardless of the total amount of reads. Hence, a single read was regarded the same as 10,000 reads in a given fish sample. Nevertheless, some statistical correlation does exist between prevalence and abundance, making this simplification valid and useful. From this graph, we see *S. baltica* is prevalent in the gills of these fish

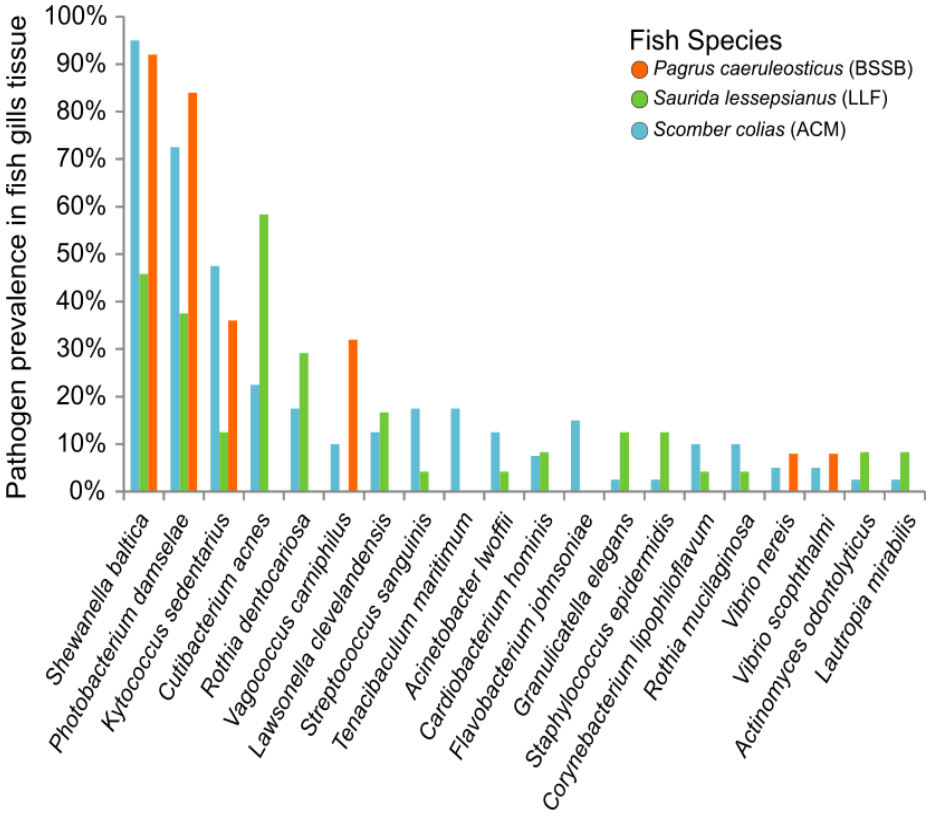

Figure 8 | Prevalence of the 20 most commonly found pathogens in the gills of the fish sampled. The percentage (y axis) refers to amount of samples per species found to contain these specific potential pathogens. Pathogens arranged (on the x axis) from the most (overall) prevalent (left) to the least (right).

species (95% of ACM, 46% of LLF and 92% of BSSB samples contain sequences identified as *S. baltica*), and that *P. damselae* is also highly prevalent (73%, 38% and 84% of the ACM, LLF and BSSB samples, respectively). On the other hand, pathogenic *Streptococcus*, *Staphylococcus* and *Vibrio* are much less prevalent.

Phylogenetic trees of genera of interest show similarity of ASVs to sequences

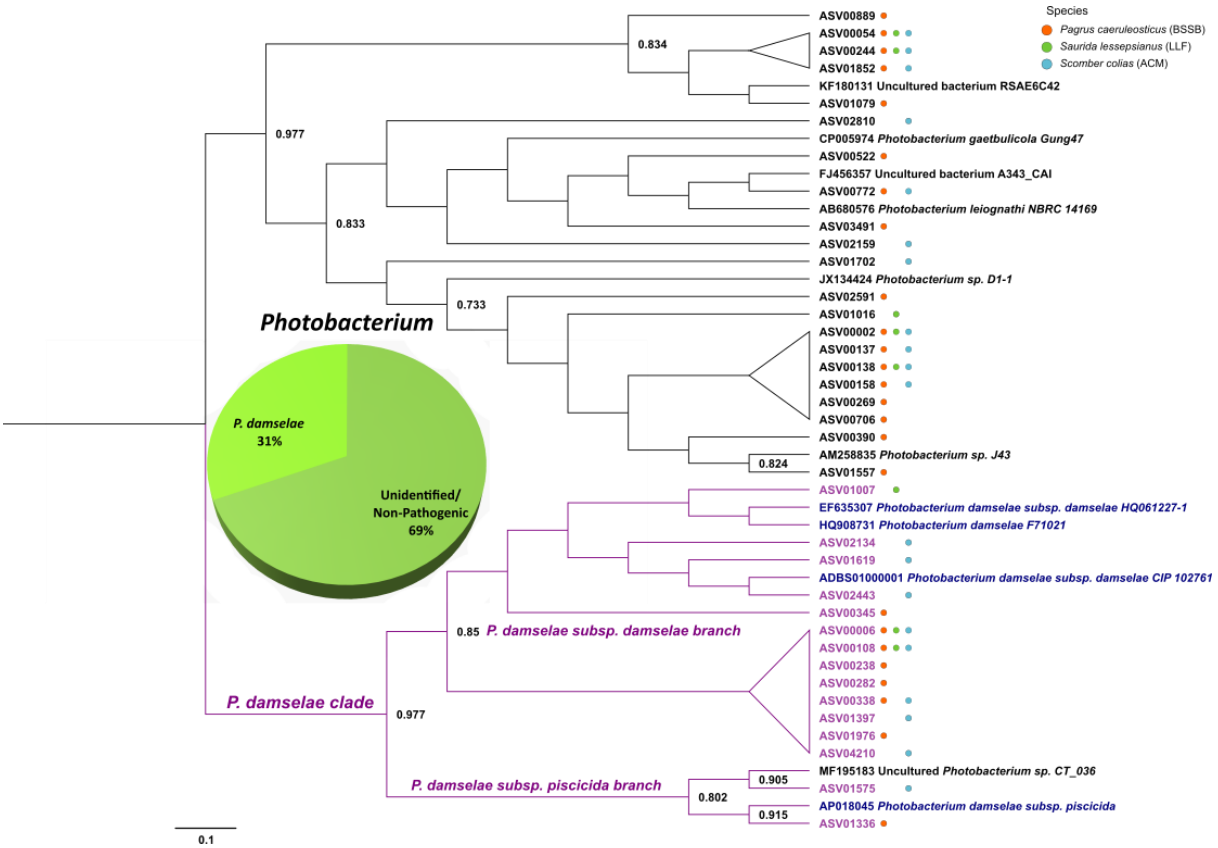

Figure 9 | A phylogenetic tree for *Photobacterium*-related ASVs. A cutoff of 0.7 (70% bootstrap support) was made for nodes, thus any lower value is not presented. Triangular shaped tips represent sequences found to be practically identical. Colored dots (to the right of ASV numbers) represent fish species in which the ASVs appeared. Reference sequences include their GenBank accession numbers. Blue-colored sequence names are of pathogenic species. Purple-colored sequence names mark ASVs identified as bearing a similarity to pathogenic species-related sequences. The pie chart figures express percentage out of total number of reads. Smaller ASV numbers indicate they were more common (in terms of total reads) than ASVs with large numbers. The scale bar represents 0.1 nucleotide substitution per site.

uploaded to GenBank and help identify which ASVs are likely to be of pathogenic potential. The *Photobacterium damselae* clade (**Figure 9**, marked in purple), appears divided between ASVs associated with *P. damselae subsp. damselae* and *P. damselae subsp. piscicida*, in which *P. damselae subsp. damselae* is predominant – both in number of ASVs and the total reads. Yet, due to the limitations of 16S, further inspection is required to validate: (i) that it is indeed *P. damselae* and (ii), which subspecies it is. These are actions in progress at the lab, which results are to be expected soon. In total, *P. damselae* makes up >30% of the species' ASV reads.

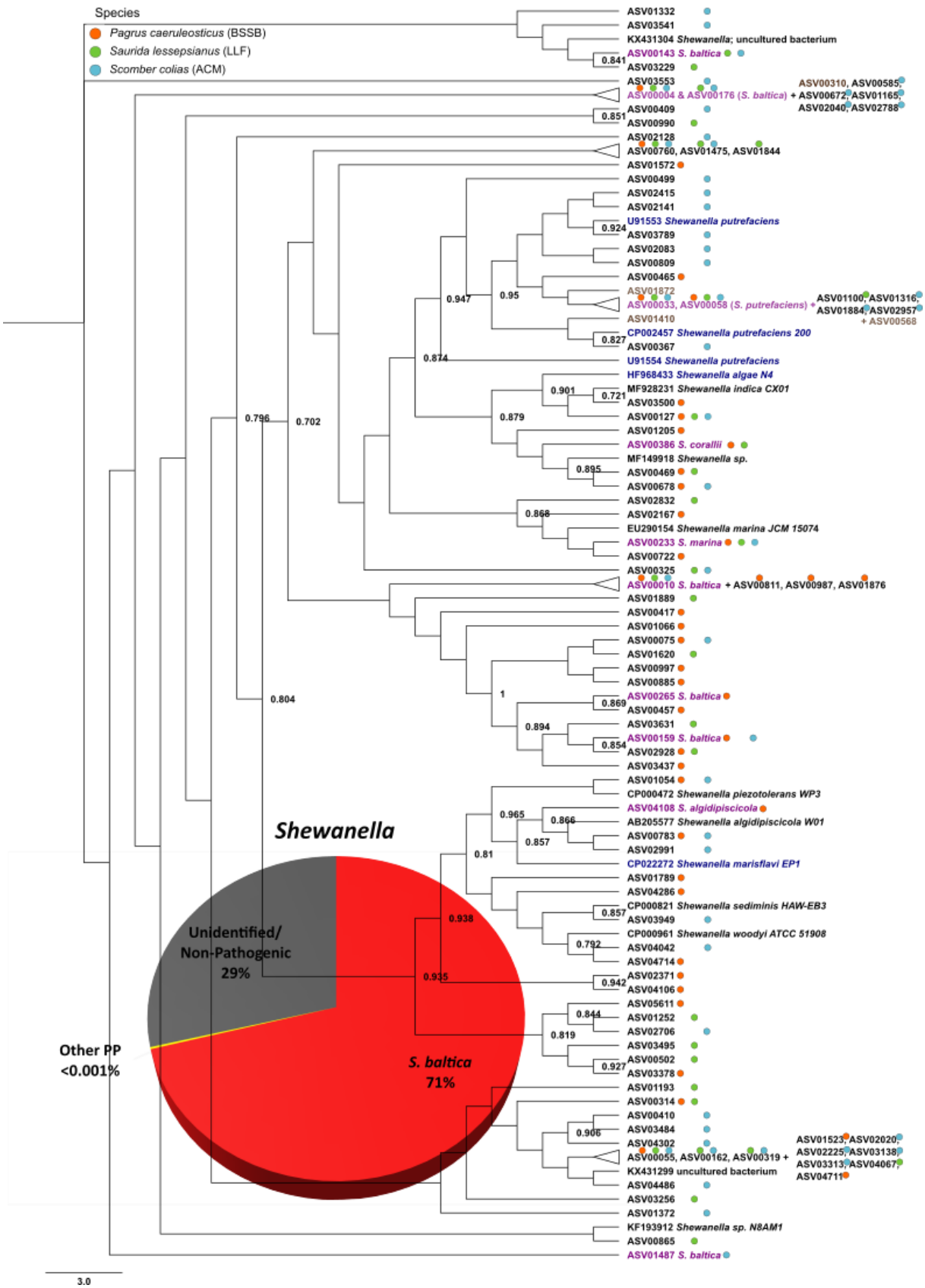

Figure 10 | A phylogenetic tree for Shewanella-related ASVs. Due to amount of ASVs associated with Shewanella spp., many of which are practically identical (e.g., ASV00055, ASV00162, ASV00319 and the group to their right), the presentation of the dots marking the fish species in which they were found were placed either above or to the top-right of the ASV number. Beige-colored ASVs were found in negative control (NC) samples only. The scale bar represents 0.3 nucleotide substitution per site. All other remarks provided under **Figure 9** apply here as well.

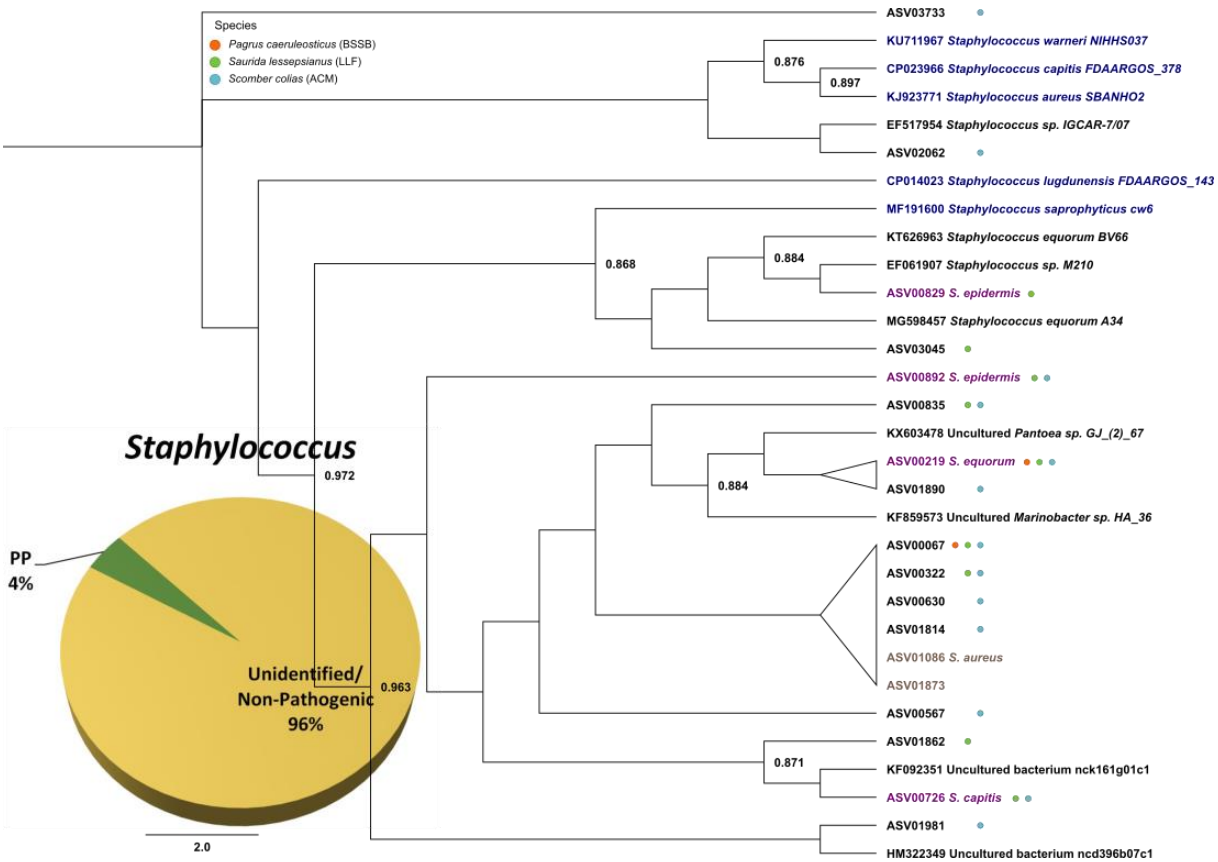

Figure 11 | A phylogenetic tree for *Staphyloccocus*-related ASVs.

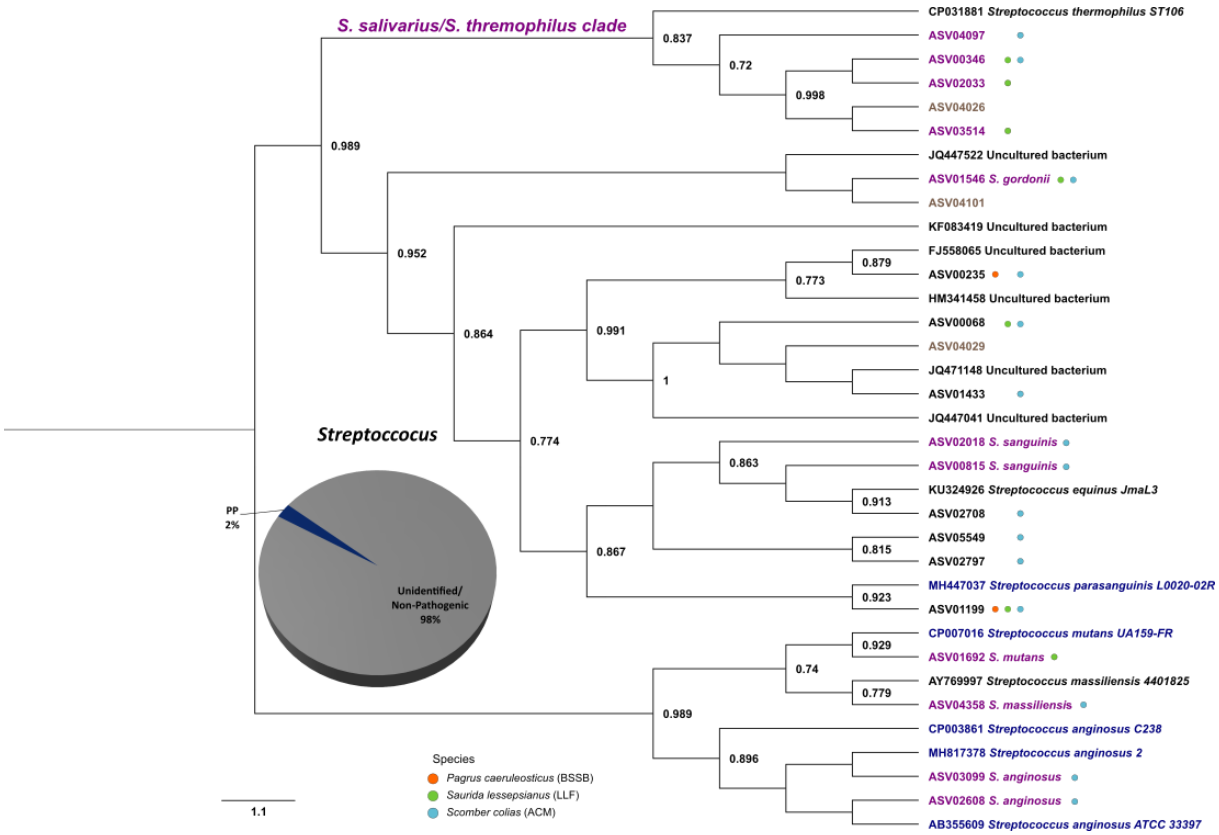

Figure 12 | A phylogenetic tree for *Streptoccocus*-related ASVs

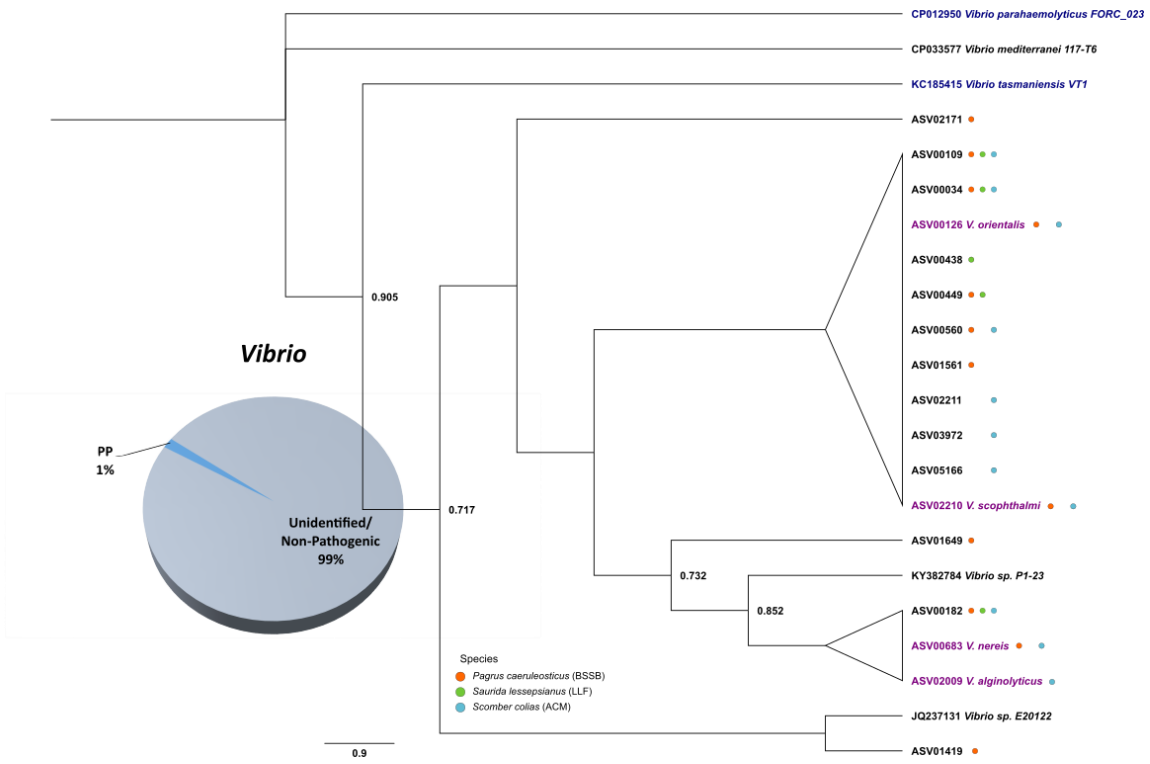

Figure 13 | A phylogenetic tree for *Vibrio*-related ASVs

The *Shewanella* phylogenetic tree (**Figure 10**) is comprised of many ASVs, as this genus is the most prevalent of all genera appearing in the results. What is more important is the amount of *S. baltica* associated ASVs, collectively responsible for >70% of all *Shewanella* reads. The possible reason behind this is discussed further below.

The trees of *Staphylococcus*, *Streptococcus* and *Vibrio* (**Figures 11 - 13**, respectively) are all quite similar in terms of the pathogenic/non-pathogenic ratios they exhibit: between 1-4%. This is summarized in **Figure 14** (**Discussion**).

Before explaining the results of what was found, it is worth noting what was missing: the genus *Mycobacterium* was absent altogether and *Vibrio harveyi*, a notorious fish pathogen common in the region, was too undetected. That fact is both intriguing and perhaps a proxy testament of the health of the fish communities sampled, but is also worth registering for follow up researches, to learn whether it has something to do with the microbial inhabitants present in the gills of these specific fish species.

Discussion

The results presented above indicate that monitoring pathogen prevalence and the spread of diseases by studying bacterial community populations in gills of wild fish, may hold an interesting potential, provided that it is applied on data which enables intra-species comparison, such as temporal, spatial, treatment differences, etc. It is also suggested that this method should be further tested to validate if it enables deducing pathogen prevalence in ambient waters, based on a holistic view of a given ecosystem: looking at the water and the fish swimming in it as a local spatio-temporal holobiont. If it can be proven so, it is an approach that can be applied over a wide range of ecosystems – from fish farms to fish breeding grounds and other sensitive ecological niches – and can become part of long term monitoring programs. This study is one of a handful of similar studies held worldwide surveying gill microbiomes in search of pathogens, and to the furthest of our knowledge, it is the first to be carried out in the region of the Eastern Mediterranean. In all of these studies, the presence of pathogens (if at all identified) was inferred from taxonomic levels higher than species (Rosado et al., 2019, Pratte et al., 2018), sometimes supported by data on shifts in microbiome composition between treatment and control groups (Brown et al., 2019, Hess et al., 2015, Mohammed and Arias, 2015, Minich et al., 2020a) or spatio-temporal differences (Minich et al., 2020b). In this current study, we based our findings (regarding pathogens) only on ASVs we could identify at the species level, to a high level of certainty.

It is interesting to note that *S. colias*, which is host to the richest microbial community of all three, also shows the largest total number of bacterial species with pathogenic potential: it had 35 out of the 41 pathogenic bacteria species (and 13 of 14 fish pathogens), while *S. lessepsianus* had 26 (6/14) and *P. caeruleostictus* had just 6 (5/14 fish pathogens). Perhaps the reason for *S. colias* to have the richest bacterial communities (and potential pathogens) has to do with its pelagic-migratory nature, which means it passes through a diversity of environments, in which it can accumulate a variety of bacteria in its gills. Yet, the sheer number of pathogenic species can be deceiving, since it does not take into account the total number of reads nor does it relate to the relative abundance, and treats *Photobacterium damselae* (ASV00006 – amongst others – with >90,000 reads across samples from all three fish species) equally to *Corynebacterium durum* (ASV04283; 15 reads in just a single *S. colias* sample). The data demonstrates that *P. caeruleostictus* has a high relative abundance of *Photobacterium*, and that within the *Photobacterium* genus ~30% of the reads belong to pathogenic species. This brings forward the question whether *Photobacterium damselae* – both *Photobacterium damselae subsp. damselae* and *Photobacterium damselae subsp.*

piscicida (sub-speciation could not be performed in this study) – is an obligatory pathogen or not and furthermore, is it opportunistic or a pathobiont. Given that all fish seemed to be healthy when inspected during dissection, the analysis suggests it is not obligatory. This coincides with Terceti et al. (2017), which found a pathogenicity-regulating gene, but lacks reference in many other papers on this species (which were referred to in the **Introduction**). To further assess whether it is opportunistic or a pathobiont, there is a need to look at its ecological role in order to identify functionalities such as fending off other pathogens or providing some other beneficial 'service' to the host. The high correlation observed between Gammaproteobacteria of the *Photobacterium* cohort (and especially the Vibrionaceae family members, *Vibrio*, *Aliivibrio* and *Photobacterium*) hints that the ecological niche existing in the form of (most specifically) *P. caeruleostictus* gills, provides these genera with preferable conditions. A similar assumption can be made regarding the *Streptococcus*-*Staphylococcus* cohort and the gills of *S. lessepsianus*, while bearing in mind two big differences between both cases: one being the fact that within the *Streptococcus*/*Staphylococcus* genus-related sequences, the pathogenic ones make up only a small percentage of the total reads; and the other, that these two genera are far less dominant in terms of relative abundance in *S. lessepsianus* than *Photobacterium* is within *P. caeruleostictus* samples.

One difficulty inherent to the data analysis process is the degree of identification of sequences down to the species taxonomic level. The rate of unidentified ASVs (marked Not Assigned, or NA) grew, naturally, for each taxonomic level (Phylum: ~3%; Class: 6%; Order: 15%; Family: 25%; Genus: 55%; and Species: nearly 97%). What this means is that while it was possible to present data regarding the gills' microbiome at higher taxonomic levels, in order to be able to provide trustworthy analysis on potential pathogens, it is necessary to make use of the ~3% identified species and add ASVs identified to the genus level and belonging to genera of interest, to see if they can be identified. It was this further identification – done by running sequences of genera of interest through SILVA and BLAST – that revealed the true identity of those specific ASVs we focused on, and provided a better understanding of the pathogen/benevolent ratios per genus (**Figure 14**). It is noteworthy that the great majority of *Shewanella*-associated pathogenic ASVs were identified as *S. baltica*. This bacterium is known to prefer cold waters, is not known to be pathogenic to marine animals, but rather a major cause of food spoilage, thus afflicting grave economic losses on the global supply chain of seafood and affecting humans consuming spoiled food (Zhang et al., 2020).

It is clear, however, that the data was not fully exhausted and contains considerably more information, which may have been overlooked, and that further investigation may detect more potentially pathogenic species. It also raises the possibility that better identification would require lengthening the sequences obtained from the gene by using a different set of primers, as suggested by Morales and Holben (2009) and Martínez-porchas et al. (2016), especially in light of the difficulty to tell apart *Vibrionaceae* family members via their 16S genes (Machado and Gram, 2015), or to complement the identification using Multilocus Sequence Analysis (MLSA), targeting protein-encoding genes such as *toxR* and *rpoD* (Pascual et al., 2010). To make the story slightly more complex, it was suggested 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 virulent factors. In other words, many cases in which *P. damselae* is highly prevalent in fish but does not cause disease, may not be due to the lack of a suitable and immunosuppressed host, but that there is a need for a few variants of this pathogen to 'join forces' to create effective infection (Osorio et al., 2018). This idea is strengthened by another study, conducted on *P. damselae* subsp. *piscicida*, which found that some strains are virulent, while others are avirulent, and that a protein exotoxin, AIP56, secreted by virulent *P. damselae subsp. piscicida* in large quantities (but not by avirulent strains), is a key factor responsible for apoptogenic activity targeting fish macrophages and neutrophils (do Vale et al., 2005). In summary, there is a tradeoff between the sensitivity level of the gene sequences amplified and the ability to determine the bacterial species, where 16S partial gene amplicons enable us to identify a wide variety of bacteria, but they lack specificity. So in order to make accurate identifications of certain pathogens, another complimenting step is required: to amplify amplicons of pathogen-specific genes (perhaps functional ones, as suggested above).

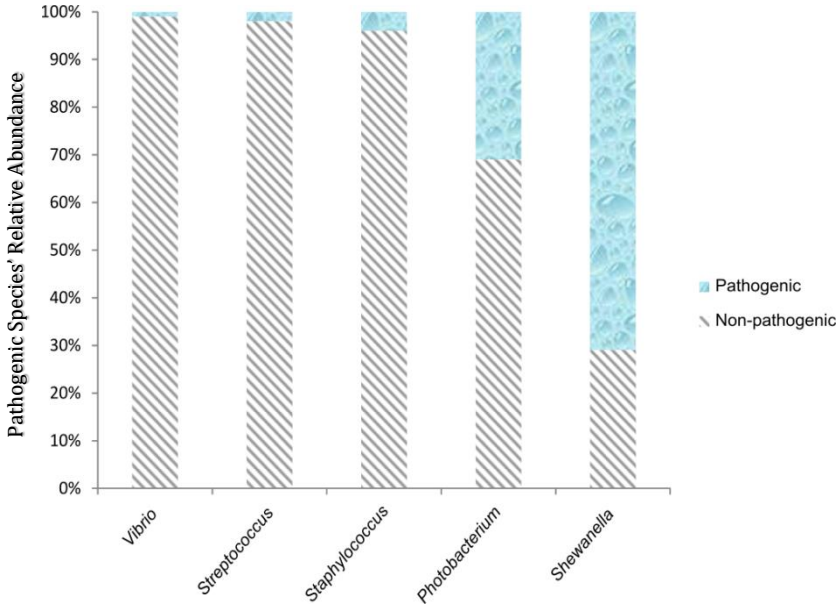

Figure 14 | A comparison of the Pathogenic/Non-pathogenic ratios per genera of interest. Values represent relative abundance of potential pathogenic species identified out of all ASVs associated with a certain genus.

Stabilizing a cooperative microbial community reduces pathogenicity

Besides host-pathogen relations, there is yet another factor affecting virulence expression – both in terms of creating the conditions favoring virulence, and also in the ability to control virulence expression in the first place – and that is: bacterial social interactions. 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). These interactions are found to be a stabilizing element widespread in *Vibrio* species, creating a pro-cooperation community, which shows increasing growth yield (in part by producing "Public Goods", costly products that increase individual fitness, but whose resulting benefits can be shared between all members of a population, including non-producers), 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 (Lyn and De Leenheer, 2019). One important example coinciding with this theory is the pathogenic habits of *Vibrio harveyi*. 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 (Montánchez et al., 2019).

Defining pathogens

Knowing that some pathogens have the ability to survive for substantial time periods in the open water, explains how fish cage originated secretions can travel many kilometers with the currents and still affect wildlife in large regions downstream (Shapiro et al., 2013, Viau et al., 2011). Of course, without nutrients to feed on, and as these secretions dissipate over time and space, such pathogens become more and more limited in their ability to propagate, making the detection of them in large numbers – less likely. That corresponds with the claim that pathogens are prevalent in hotspots, be it an immunosuppressed animal, a naïve host, or near the outflow from fish farms and other anthropogenic pollution-hit areas (Lyons et al., 2010, Lobelle and Cunliffe, 2011). Following that line of argument, one may therefore claim that **Pathogenicity**, being defined as "the quality of producing or the ability to produce pathologic changes or disease" (Miller et al., 2003), 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 return may have an effect on the pathogenicity of a given pathogen.

That, in part, may help explain why asserting whether some less known bacteria are pathogenic and to which degree, may prove to be tricky: some pathogens are not necessarily the cause of a certain disease, but are rather secondary agents of disease, which can either help progress it, or just gain benefit off the change in conditions of a host's infected group of cells, being a good substrate for them to proliferate on (Brink et al., 2019). Many times bacteria are labeled 'pathogenic' based on association with disease, not necessarily on unequivocal evidence. Furthermore, many a times a certain species are misidentified, leading to false associations with pathogenesis (Broly et al., 2020). It would also be plausible that the same pathogen would have different relations with different hosts, posing no or little threat to some, while wreaking havoc on another. Given this elusive property of many (possibly) disease-causing bacteria, it would be better to label the great majority of them as 'Potentially Pathogenic', and further study their relationships with the host and the organ-specific microbiota they are found in.

Challenges in pathogen detection

When seeking for the **best ways** to detect marine pathogens, we are actually asking several different questions: (i) which pathogens are we focused on? (ii) where do we expect to find them? and (iii) which tools and techniques should be applied in order to track them?

The first question assumes that (i) there is a myriad of pathogens out there; (ii) our search almost always starts with a host of interest – not pathogen of interest – usually due to economic interests and/or logistic constraints, making the host and its environment the focus of the study; and (iii) we know that some pathogens developed into becoming symbionts of opportunistic pathogenesis capabilities, using pathogenicity as a survival tool, which may hint on the reason why co-infection is common – an arrival of a non-symbiotic pathogen on the scene (due to injury) may cause the host to become immunosuppressed and create conditions favorable of pathogenesis for the symbiotic pathogen. For all the reasons abovementioned, it is clear that we can't cover all marine pathogens – even when confining ourselves only to those found in the geographic region in which we are searching.

The second question assumes that pathogens are not to be expected everywhere alike, but rather mostly in places where conditions induce them, such as: ill and dead organisms; surrounding and downstream of pollution sources; especially in warmer/acidic/saline waters; in certain animal organs – rather than others, etc., and therefore, requires the researcher to carefully choose the subjects and conditions of study. Now, it should be noted that in the wild, unhealthy social fish reduce locomotion and tend to stray away from their peers, as a counter-pathogen spread measure (Kirsten et al., 2018), which makes them an easy target for predators, and may explain their absence from wild fish samples.

The answer to the third question is based on those given to the previous two and sometimes requires some trial and error to fine-tune (as occurred in this study – targeting different organs to focus on, finding the proper DNA extraction kit, the right pair of primers, optimizing the PCR settings, etc.).

A hint as of the answer to the second question involves some evidence combined with common sense: at large, bacteria of all phyla have each certain 'preferable' physio-biochemical conditions in which they thrive, and a range of conditions that they can tolerate and survive. Within the Eastern Mediterranean Sea's (EMS) water column itself, exists a stratification of layers with different physiochemical properties, affecting the microbial communities present (Techtmann et al., 2015). It is known that 'Plastispheres', a term describing microbial communities forming on top of microplastic debris floating at sea, exhibit consistent genetic differences compared with surrounding waters (Kirstein et al., 2016). 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 therefore should be obvious that fish gills offer a niche with a different (and unique) set of conditions, and thus certain microbial communities will form there, that are different than those in the water the fishes swim in. However, when one attempts to explain pathogenesis based solely on the relations between the four vertices (environment, host, microbiome and pathogen), it should be kept in mind that the gills' microbiomes do not tell the whole story, for several reasons: (i) there may be considerably large variations in the gills' microbiome throughout the life-time of an individual fish (easily explained by the custom of many fish to change habitats during their life cycle, as they grow from larval and juvenile stages to adulthood) (Nagelkerken and van der Velde, 2002, Wilson et al., 2010, Mercier et al., 2012); and (ii) the gills may be home also to ciliate parasites, which frequently facilitate the establishment of secondary microbial species (Jahangiri et al., 2020), as well as many

other pathogenic agents – from viruses to fungi (Bui et al., 2019), and to macro and micro-parasites such as helminths and myxosporea (Nguyen et al., 2021, Liyanage et al., 2003, Molnár 2002). That is to say, fish gills are a dynamic niche for a great variety of interacting organisms, and thus, it would be wrong to be drawing unequivocal conclusions based solely on the results presented here. Instead, they should be treated like a stepping stone, one of several needed to enable making valid predictions regarding fishes' health in their natural or cultured environment.

Conclusions and afterthoughts

Fish gills harbor species-specific microbiomes, exhibiting strong correlations between certain taxonomic groups. Some overlap exists between the three species sampled, perhaps expressing some form of core microbiome. The 'genera of interest' (*Vibrio*, *Photobacterium*, *Shewanella*, *Staphylococcus* and *Streptococcus*) are important players within these fish species gills' microbiomes (especially *Photobacterium* and *Shewanella*), and at least in the case of *Pagrus caeruleostictus* (Bluespotted Seabream), a substantial percent of its gills' microbiome is populated by generalist pathogenic species, which are notorious marine pathogens. Given that all fish sampled appeared healthy and based on the notion that pathogenicity is 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.

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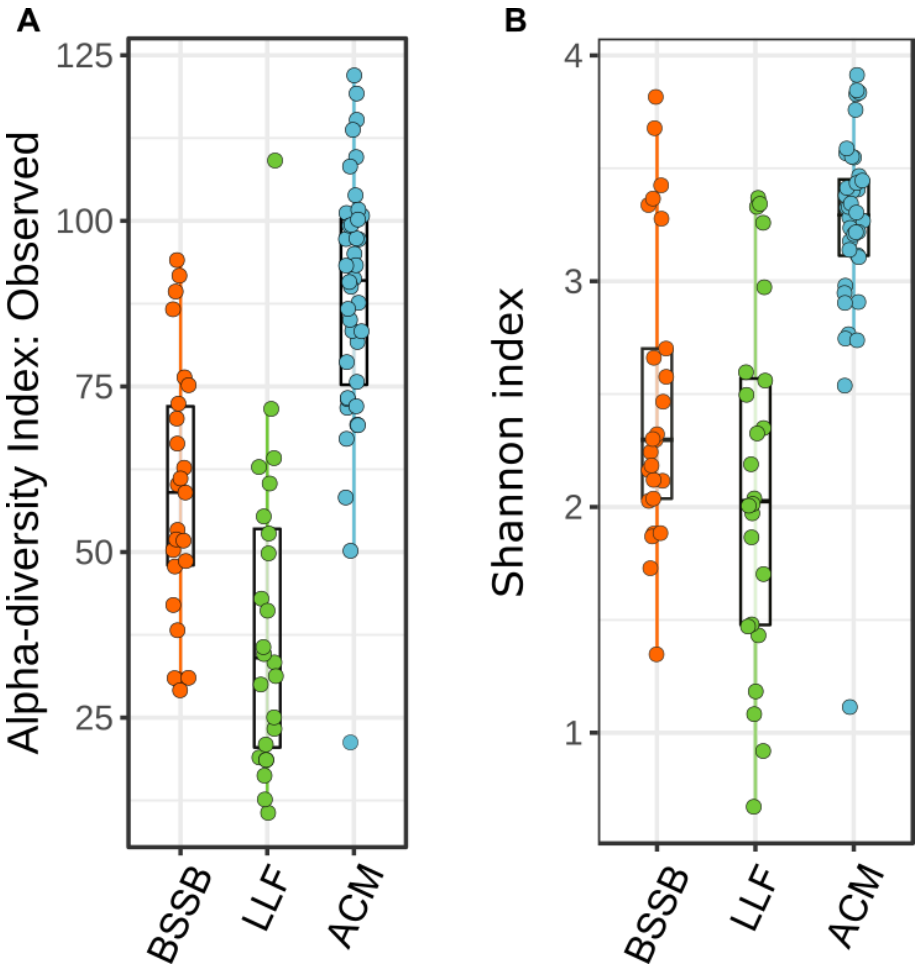
Supplementary Table 1 | The remaining potential pathogenic bacterial species found in fish gills samples. The species are listed by ranking of their prevalence, from most prevalent to least prevalent. Percentages refer to the amount of samples 'infected' from total samples of that species.

Bacteria	*ACM*	*LLF*	*BSSB*	Marine Pathogenicity	Marine Pathology	Clinical Relevance	Human Pathology
Kingella oralis	5.0%	4.2%	-	Unknown		Pathobiont	Periodontitis (Chen, 1996)
Abiotrophia defectiva	2.5%	4.2%	-	Unknown		Yes	Endocarditis (Kiernan et al., 2008)
Lactobacillus iners	2.5%	4.2%	-	Unknown		Pathobiont	Vaginal dysbiosis (Petrova et al., 2017)
Shewanella putrefaciens	2.5%	4.2%	-	Pathobiont	Shewanellosis (Esteve et al., 2017; Paździor, 2016)	Rarely	Otitis, hepatobiliary infection (Vignier et al. 2013)
Sphingomonas koreensis	2.5%	4.2%	-	Unknown		Rarely	Meningitis (Marbjerg et al., 2015)
Staphylococcus capitis	2.5%	4.2%	-	Yes	Skin necrosis (Marcel et al., 2013)	Yes	Prosthetic joint infections (Tevell et al., 2017)
Acinetobacter radioresistens	5.0%	-	-	Unknown		Yes	Bacteremia and pneumonia (Wang et al., 2019)
Janibacter hoylei	5.0%	-	-	Unknown		Rarely	Bacteremia (Lim et al. 2017)
Streptococcus anginosus	5.0%	-	-	Unknown		Yes	Brain, liver abscesses (Brassil et al., 2020)
Alkanindiges hongkongensis	-	4.2%	-	Unknown		Rarely	Parotid abscess (Woo et al., 2005)
Campylobacter gracilis	-	4.2%	-	Unknown		Yes	Periradicular lesions (Siqueira Jr & Rôças, 2003)
Cutibacterium granulosum	-	4.2%	-	Unknown		Pathobiont	Periprosthetic joint infection (Kusejko et al., 2021)
Fusobacterium nucleatum	-	4.2%	-	Unknown		Pathobiont	Brennan & Garrett, 2019
Stenotrophomonas maltophilia	-	4.2%	-	Opportunistic	Visceral hemorrhage, fin/tail rot (Abraham et al., 2016)	Opportunistic	Lung cell lysis in CF patients (Brooke et al., 2017)
Streptococcus mutans	-	4.2%	-	Unknown		Opportunistic	Dental caries (Krzyściak et al., 2014)
Acinetobacter schindleri	2.5%	-	-	Yes	Red eye disease (Reddy & Mastan 2013)	Rarely	Bacteremia (Montaña et al., 2018)
Aeromonas hydrophila	2.5%	-	-	Yes	Gastroenteritis, septicemia, and necrotizing fasciitis (Rasmussen-Ivey et al., 2016)	Yes	Diarrhea (Khajanchi et al., 2010)
Aggregatibacter segnis	2.5%	-	-	Unknown		Pathobiont	Associated with oral cancer (Zhang et al., 2020)
Corynebacterium durum	2.5%	-	-	Unknown		Yes	Blood cultures, gingiva, abscesses (Bernard, 2005)
Psychrobacter arenosus	2.5%	-	-	Unknown		Rarely	Bacteremia (Caspar et al., 2013)
Vibrio alginolyticus	2.5%	-	-	Yes	White spot disease (Selvin & Lipton, 2003; Xie et al., 2005)	Yes	Wound infection (Reilly et al., 2011)



Supplementary Figure 15 | Alpha-diversity analyses comparison between fish samples using Observed (A) and Shannon index (B). Samples clustered by fish species.
Observed: Kruskal-Wallis statistic: 44.181, *P*<.001.
Shannon: Kruskal-Wallis statistic: 29.703, *P*<.001.

Raw data (i.e. Counts, Metadata and Taxonomy – used for analyses as CSV files, as described in **Materials and Methods**) are accessible in Excel format via this link:
https://drive.google.com/file/d/11XzzAT737W2vA1TNwzbP5E2QwfDzDxP_/view?usp=sharing

ניטור פתוגנים של דגים במזרח הים התיכון על ידי ריצוף רקמות זימים באמצעות Next Generation Sequencing

פלג איתי

תקציר

דיווחים מרחבי העולם מצביעים על כך שבשנים האחרונות, התפרצויות של מחלות דגי ים נעשות שכיחות ועוצמתיות יותר. חרף העובדה שפגיעתן הכלכלית משמעותית הן בשטחי דיג והן בחוות דגים, והן מהוות איום גם על בריאות הציבור, מחקר על שכיחותם של גורמי התחלואה שאחראים להן לוקה בחסר באזורים רבים, כולל באגן המזרחי של הים התיכון. במחקר זה, 89 פרטים בריאים למראה משלושה מיני בר נפוצים, נאספו מאזור ים פתוח המרוחק כ-50 קילומטרים דרומית-מערבית לחופי העיר אשדוד, ישראל, ובסמיכות יחסית לכלובי דגים. זימי הדגים עובדו לשם הפקת דנ"א, שלאחר מכן רוצף בעזרת מכשור NGS. המידע שנאסף נותח מתוך מטרה לאתר הבדלים בשכיחות פתוגנים והרכב המיקרוביום. הניתוח העלה כי פתוגנים רבים נוכחים בזימים – כולל כאלו העשויים לעבור לבני אדם – אם כי בכמויות זניחות. אף על פי כן, מתוך המגוון הרחב של סוגי חיידקים התורמים כולם להרכב המיקרוביום של זימי הדגים, חמישה נבחרו לבחינה מדוקדקת יותר: *Photobacterium, Shewanella, Staphylococcus, Streptococcus* ו-*Vibrio* – כולם סוגים נפוצים והכוללים מינים פתוגניים מוכרים, בעלי השפעה גדולה על דגי בר וחוות כאחד ברחבי העולם. מתוך חמשת הסוגים הנבחרים הללו, *Photobacterium* ו-*Shewanella* נמצאו כשכיחים ומצויים בשפע הרב ביותר. הדבר נכון במיוחד במקרה של הפאריד ה (*Pagrus caeruleostictus*), מין שבו 30.2% מהמיקרוביום של הזימים נמצא כשייך למינים של *Photobacterium*, ו-11.3% לכאלו של *Shewanella*, ואלו בתורם נשלטים בידי *Photobacterium damselae* ו- *Shewanella baltica* (בהתאמה). סוגים-נבחרים אחרים נמצאו בכמויות שאינן עולות על 1.4% מהמיקרוביום (Vibrio ב-*P. caeruleostictus*), לגביהם נמצאה נוכחות של מינים פתוגניים בכמויות של 1-4%.

הנתונים מצביעים על כך שדגים מהווים בית גידול עבור מיני חיידקים בעלי זיקה ספציפית למין הדג והמציגים קורלציה חזקה בין קבוצות טקסונומיות מסוימות – אם כי חפיפה מסוימת קיימת בין שלושת מיני הדגים הללו – רמז לקיומה של ליבת מיקרוביום משותפת. בהינתן שכל הדגים שנדגמו נראו בריאים, הוצע כי פתוגנים הינם במקרים נדירים בלבד אובליגטוריים, ושפתוגניות מושפעת מלחצים סביבתיים נגד אלימות (וירולנטיות), שמקורם באינטרקציות הפנימיות בתוך חברות חיידקים, בהן קיימת העדפה ברורה לאסטרטגיות של שיתוף פעולה על פני בגידה – מה שמאפשר למיני פתוגנים לפרוח גם מבלי להפוך אלימים.

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