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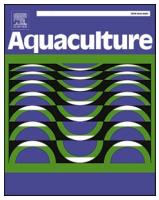

Comparison of intestinal bacterial communities in asymptomatic and diseased Asian seabass (*Lates calcarifer*) with chronic enteritis and mixed bacterial infections

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ABSTRACT

Asian seabass (*Lates calcarifer*) is a major aquaculture food fish species in Singapore. Farming of this species is increasingly threatened by frequent outbreaks of infectious diseases, resulting in mortality exceeding 50–70%. In this study, we investigated the comparative gut bacterial microbiota using 16S rRNA metasequencing between asymptomatic and diseased juvenile fish collected during a disease outbreak soon after stocking. Mild to severe chronic granulomatous enteritis was observed histopathologically in both asymptomatic and diseased fish. Kidneys of diseased fish tested PCR positive for the 'big belly' novel *Vibrio* spp., *Streptococcus iniae* and *Vibrio harveyi*. These bacteria were also readily detected by PCR in water samples corresponding to tanks fish were sampled from. Potentially beneficial microbes that promote gut health such as *Firmicutes*, *Bacteroidota* and *Actinobacteriota* were the dominant phyla in the intestinal microbiota of asymptomatic fish. Moreover, the bacteria with probiotic potential such as *Lactobacillus* only presented in asymptomatic fish, and *Weissella* was unique and prevalent (47.59%) in asymptomatic fish during the recovery phase of the disease outbreak, making them candidate biomarkers for monitoring health status of L. *calcarifer*. Conversely, diseased fish showed reduced diversity of their gut microbiome, with high abundance of members of the phylum *Proteobacteria*. *Vibrio* was the most dominant genus (87.3%) and *Streptococcus iniae* was only detected in diseased fish. These findings provide a baseline study for understanding changes in intestinal microbiota in newly stocked fish with mixed bacterial infection, biomarker assisted health monitoring, and future host-derived probiotics screening in L. *calcarifer*.

1. Introduction

Asian seabass, *Lates calcarifer,* also known as barramundi, is a major food fish species widely cultured in Southeast Asia and Australia, with an annual global production of 90,000 tons in 2017 (Jerry, 2013; Khang et al., 2018; Longbaf Dezfouli et al., 2019). Aquaculture production of L. *calcarifer* is increasingly threatened by frequent outbreaks of multiple infectious diseases resulting in heavy mortalities often exceeding 50–70% (Gibson-Kueh, 2012; Domingos et al., 2021). Important pathogens of L. *calcarifer* include *Vibrio harveyi* (Dong et al., 2017), novel *Vibrio* sp. in 'big belly disease' (Gibson-Kueh et al., 2004; Gibson-Kueh et al., 2021), *Streptococcus iniae* (Jiang et al., 2014; Van Khang et al., 2019), scale drop disease virus (SDDV) (Gibson-Kueh et al., 2012; de Groof et al., 2015; Domingos et al., 2021), *Lates calcarifer* herpes virus (LCHV) (Chang et al., 2018; Meemetta et al., 2020), and nervous necrosis virus (NNV) (Hick et al., 2011; Jaramillo et al., 2017).

In relation to bacterial-mediated diseases in L. *calcarifer*, *V. harveyi*, an opportunistic pathogen, is recognized as one of the major causes of vibriosis in L. *calcarifer* (Ransangan et al., 2012; Dong et al., 2017; Chin et al., 2020a; Deng et al., 2020). This acute disease transmits rapidly among L. *calcarifer* farmed in sea cages, resulting in significant constraints for productive farming of the species (Crosbie and Nowak, 2004; Ransangan et al., 2012). 'Big belly' disease is a severe chronic, granulomatous bacterial enteritis caused by a novel *Vibrio* species (spp.), with affected fish becoming emaciated and having a swollen abdomen (Gibson-Kueh et al., 2004; Gibson-Kueh et al., 2021). The mortality rate

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caused by *Vibrio* spp. throughout the production cycle of L. *calcarifer* can range from 40 to 100% (Gibson-Kueh et al., 2004; Gibson-Kueh, 2012; Dong et al., 2017). *S. iniae*, a gram-positive bacterium, causes systemic infections and is also one of the most serious bacterial pathogenic agents to Asian seabass (Hassani et al., 2021). The production losses due to *S. iniae* are reported between 8% and 15% per year, and this figure can go up to 70% in severe outbreaks (Bromage et al., 1999; Creeper and Buller, 2006; Van Khang et al., 2019).

The fish gastrointestinal tract of fish is a complex ecosystem, composed of highly diverse microbiota which are recognized as an important barrier against invading pathogens (Magnadottir, 2010; Desai et al., 2016; Kong et al., 2017; Legrand et al., 2017). This microbial community is largely composed of bacteria and is termed the bacterial microbiome. A healthy gut microbiome plays important roles in protecting against the overgrowth of potentially pathogenic taxa and regulation of the immune system of the host (Austin, 2006). The balance of fish gut microbial composition has been reported to be influenced by various internal and/or external factors, such as host developmental stages, stress, nutritional status and culture system environment (Kim et al., 2021; Xinyuan et al., 2022). Gastrointestinal dysbiosis in the microbiome can subsequently increase susceptibility to enteritis/inflammatory bowel disease in fish, thereby affecting host fitness (Llewellyn et al., 2014; Antonissen et al., 2016; Tran et al., 2018; Abernathy-Close et al., 2021). Hence, maintaining stabilization of the intestinal microbiome has significant impact on the immunity and health in cultured fish by preventing bacterial diseases (Ma et al., 2019). Several studies comparing the gut microbiome between healthy and diseased fish have been reported, including hybrid Yunlong grouper (*Epinephelus moara* ♀ × *E. lanceolatus* ♂) with nervous necrosis virus infection (Wang et al., 2022), Malaysian mahseer (*Tor tambroides*) suffering from infectious abdominal dropsy (Lau et al., 2022), largemouth bronze gudgeon (*Coreius guichenoti*) infected by furunculosis (Li et al., 2016), and hybrid tilapia (*Oreochromis aureus* × *Oreochromis niloticus*) with external signs of parasitic disease (Ofek et al., 2022). The findings from these studies provided an understanding about the interaction between intestinal microbiota and the host under disease infection, which will further help develop effective approaches for better health management of these fish species.

To date, the gut microbial community of L. *calcarifer* sufferring from infectious disease remains poorly understood. The only report was a finding highlighting gut microbiome with tenacibaculosis symptoms, and isolation of one strain of sp. *nov. Tenacibaculum singaporense* (Miyake et al., 2020). In the present study, we investigated the gut microbiome of asymptomatic and diseased fish during a bacterial disease outbreak at a commercial L. *calcarifer* farm. Improving the understanding of these diseases may lead to farmers being able to better manage the health of the species and limit impacts of disease on L. *calcarifer* production.

2. Materials and methods

2.1. Samples collection

Approximately 48,000 juvenile L. *calcarifer* (17.4 ± 5.5 g) transported from a commercial local fish hatchery were stocked into a 80-ton flow-through commercial aquaculture system, with incoming sand-filtered sea water. Water quality parameters were monitored throughout the study period, with ammonia <0.5 mg/L, water temperature at 30 ± 0.5 °C, pH 7.6, salinity 27–28 ppt, and dissolved oxygen ca. 7 mg/L. A disease outbreak was observed on Day 1 post-stocking. Peak mortality was observed on Day 7, with mortality decreasing until no further mortality was observed on Day 22. Asymptomatic fish exhibited active swimming with good feeding response, while diseased fish showed lethargic behaviour at the water surface, no feeding response, darkened body coloration, and pale gills. Asymptomatic (clinically healthy, $n = 3$) and diseased fish (n = 3) on Day 7 and 9, and Asymptomatic fish (n = 3) on Day 22 post-stocking were sampled. Asymptomatic fish were designated AD7, AD9, and AD22, corresponding to samples taken on Days 7, 9, or 22. Similarly, diseased fish were designated DD7, DD9, and DD22. Fish were euthanized on farm using AquiS (40 mg/L) immersion baths and kept chilled on ice during transport to the laboratory for same day aseptic sampling in a Class 2 biological safety cabinet (ESCO). The exterior of the fish was disinfected with 70% ethanol, prior to dissecting out the entire gastrointestinal tract, and gut contents collected into sterile 2 mL screw capped tubes. In addition, kidney tissues were collected for PCR diagnostic analyses. All samples were flash frozen in liquid nitrogen and stored at −80 °C until DNA isolation. Similarly, water samples were collected from the central drainage outlet of the culture tank into sterile 1 L bottles, on Day 7, 9 and 22 post stocking (corresponding to the fish sampling points). Water samples (WD7, WD9 and WD22) were promptly ice-bathed, filtered with a 0.2 μm sterile filter (Sterlitech™) in a Class 2 biological safety cabinet (ESCO), and stored at −80 °C until processed for DNA extraction.

Formalin-fixed fish tissues (major organs such as liver, kidney, spleen, intestine and gill etc.) in 10% phosphate-buffered formalin were sent to Murdoch University Veterinary Histology Laboratory for histoprocessing into haematoxylin and eosin (H&E) slides. Slides were analysed on an Olympus BX53 transmission light microscope (Olympus Corporation). Lipid stores in liver (Domingos et al., 2021) and splenic red blood cell (Gibson-Kueh and Uichanco, 2021) were graded using histopathology as "+" for mild, "++" for moderate and "+++" for severe depletion. Similarly, enteritis were graded as mild for focal, moderate for multifocal, and severe, for multifocal to coalescing chronic granulomatous enteritis (Gibson-Kueh et al., 2004; Gibson-Kueh et al., 2021).

2.2. DNA extraction and pathogen screening using PCR

DNA was extracted from kidney tissues of the nine asymptomatic (AD7, AD9 & AD22) and six diseased fish (DD7 & DD9), using a DNeasy Blood & Tissue kit (250) (QIAGEN, Germany). Microbial DNA was extracted from intestinal contents of the 15 fish using a Stool Nucleic Acid Isolation Kit (Norgen Biotek). Finally, a DNeasy PowerWater Kit (50) (QIAGEN, Germany) was used to extract DNA from the 0.2 μm sterile filters (Sterlitech™) used for water samples. A blank negative control (ultra-pure water in sterile 1 L bottles transported to farm and brought back to laboratory) was processed in parallel with the farm water samples. Extracted DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and stored at −20 °C until analysed.

DNA extracts from fish kidney and water samples were tested by PCR for common pathogens reported in L. *calcarifer*: Scale Drop Disease Virus (SDDV) (Sukhontip et al., 2019), and three bacterial pathogens such as *S. iniae* (Torres-Corral and Santos, 2021), *V. harveyi* (Domingos et al., 2021) and "Big belly disease" novel *Vibrio* spp. (Segers and Grisez, 2005). The PCR was conducted using a Taq PCR Core KitTM (QIAGEN), in 12.5 μL reactions containing 9.4 μL nuclease free water, 1.25 μL 10× Taq buffer, 0.25 μL dNTPs (10 μM), 0.25 μL of forward and reverse primers (10 μM), 0.1 μL of Taq DNA polymerase (5 units/μL) and 1 μL of extracted DNA (at least 20 ng/μL), on a T100TM Thermal Cycler (Bio-Rad Laboratories, Inc.) (using cycling conditions as stated in the above publications). PCR products were visualized on a 2% agarose using gel with a 100-bp DNA ladder (BioLabs, New England).

2.3. Library preparation and Illumnia Novaseq sequencing

V3-V4 hypervariable regions of the bacteria 16S rDNA gene were amplified with Forward (5'-CCTACGGRRBGCASCAGKVRVGAAT-3′) and Reverse (5'-GGACTACNVGGGTWTCTAATCC-3′) primers (Ren et al., 2019). Indexed adapters were added to the ends of the 16S rDNA amplicons during the PCR. DNA libraries were cleaned up with Agencourt AMPure XP (Beckman Coulter, USA) and quantified on a Qubit 2.0

Fluorometer, and size validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Clean DNA libraries were loaded on an Illumina Novaseq instrument, according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 250 paired-end (PE) configuration, and image analysis and base calling were conducted by the NovaSeq Control Software (MCS) embedded in the NovaSeq instrument.

2.4. Sequence analysis

The Quantitative Insights into Microbial Ecology (QIIME) package was employed for 16S rRNA analysis. Forward and reverse reads were joined and assigned to samples based on barcode, and truncated by cutting off the barcode and primer sequence. Sequences with length < 200 bp were discarded, and the UCHIME algorithm applied to eliminate the chimera sequences. Effective sequences were assembled into Operational Taxonomic Units (OTUs) with a sequence similarity at 97%, by using the clustering program VSEARCH (1.9.6) against the Silva 119 database. Taxonomic information was obtained using RDP classifier (Version 2.2, https://sourceforge.net/projects/rdp-classifer). Alpha diversity indices (Chao1, Ace, Shannon and Simpson) were calculated using QIIME. Beta diversity was calculated using weighted and unweighted UniFrac, and principal coordinate analysis (PCoA) performed. An unweighted Pair Group Method with Arithmetic mean (UPGMA) tree was constructed based on the beta diversity distance matrix. LDA effect size (LEfSe) and biomarkers were identified to investigate differential bacterial taxa abundances up to the genus level (Segata et al., 2011). Tax4Fun was employed to predict the functional profiles of microbial communities (Aßhauer et al., 2015). Bacterial functional data were mapped and annotated at different KEGG (Kyoto Encyclopedia of Genes and Genomes) levels (Kanehisa et al., 2016).

3. Results

3.1. Histopathology and identification of causative disease agents using PCR

Severe chronic granulomatous enteritis with intralesional, clusters of large coccobacilli, along with severe depletion of lipids in liver and splenic red blood cells, were consistently observed in all diseased fish (Table 1 & Fig. 1A). Asymptomatic fish generally presented with mild to moderate enteritis, and depletion of hepatic lipid stores and splenic red blood cells (Table 1 & Fig. 1A). Interstitial nephritis with coccoid shaped bacteria were often observed in the kidney of diseased fish, but not in asymptomatic fish (Fig. 1A). Gills with diffuse epithelial lifting (oedema) and mild branchitis, were observed in both asymptomatic and diseased fish.

PCR was performed on kidney of all 15 fish and the three water samples. A total of 5 out of 6 diseased fish tested PCR positive for "big belly" novel *Vibrio* spp., while one asymptomatic fish (AD9) also tested positive, although amplification of the target as visualized on the gel was weak in this sample. Diseased fish were often concurrently PCR positive for "big belly' novel *Vibrio* spp., *S. iniae* and *V. harveyi*. All asymptomatic and diseased fish tested PCR negative for SDDV. All water samples tested PCR positive for "big belly' novel *Vibrio* spp., *S. iniae* and *V. harveyi*. (Fig. 1B, i-iv).

Table 1
Summary of histopathology findings in asymptomatic and diseased *Lates calcarifer*.

Tissue	Asymptomatic			Diseased	
	AD7	AD9	AD22	DD7	DD9
Liver (Lipid depletion)	+(2); ++(1)	+++(1)	-(3)	+++(3)	+++(3)
Spleen (RBC depletion)	+(2); ++(1)	++(1)	+(1); ++(1)	NE	++(1); +++(2)
Intestine (Enteritis)	+(1); ++(1)	++(2); +++ (1)	+(2); ++(1)	+++(3)	+++(3)

* Note: '+' mild; '++' moderate; '+++' severe; '-' fish with good lipid stores; 'NE' spleen not examined; '()' Number of fish; RBC = "Red blood cell".

3.2. Reduced microbiota diversity in diseased L. calcarifer intestine

A total of 3,155,965 quality filtered reads from 9 asymptomatic, 6 diseased and 3 water samples (ranging from 81,679 to 284,944 reads per sample) were assigned to 1941 operational taxonomic units (OTU), at 97% sequence identity matched with the Silva132 database. OTUs in water (ranged from 536 to 614) were more diverse than those present in the gut of fish (ranged from 63 to 474), with more OTUs present in asymptomatic fish (average: 223 ± 143) than diseased fish samples (average: 87 ± 40). In addition, the alpha diversity indices of Chao1 (Fig. 2A) and Shannon (Fig. 2B) indicated the higher richness and diversity of gut microbiota in asymptomatic compared to diseased fish, with the lowest value in DD9 and the highest in AD9 samples. Significant difference of both indices were observed for DD7 vs AD9 ($p < 0.05$) and DD9 vs AD9 ($p < 0.01$).

3.3. Differences in bacterial composition among gut content of asymptomatic and diseased fish, and water samples

To further compare the differences in the structure of the bacterial community among the samples, a hierarchical clustering analysis of three-dimensional scatter plot was generated using principal coordinate analysis (PCoA) based on the OTU composition. The results showed the plots of all asymptomatic samples were dispersed widely, while the diseased sample plots tend to be clustered in closer proximity. The asymptomatic fish plots were distinctively separated from the diseased fish in the first principal component (33% explained variance) (Fig. 2C). The three water samples clustered together, but distinctively separated from that of both diseased and asymptomatic fish intestinal samples. The hierarchical cluster analysis of all the fish and water samples aligned with the PCoA results (Fig. 2D).

3.4. Diseased and asymptomatic L. calcarifer maintains different intestinal microbiome profiles at the phylum level

A total 1869 among all of the assigned OTUs were annotated with the rate of 96.29%. The taxonomic mapping of the fish intestinal microbiota identified 24 classifiable phyla. Half of these phyla were common between the asymptomatic and diseased fish, but their abundance varied greatly among the five groups. The top ten most abundant phyla are shown in Fig. 3A. The three most dominant phyla were *Proteobacteria, Firmicutes* and *Fusobacteria* in diseased (DD7, DD9) and asymptomatic fish samples (AD7, AD22), contributing 95.7% to 99.6% of total bacterial diversity in all the four fish groups (Fig. 3A). AD9 was rich in *Proteobacteria, Bacteroidota, Actinobacteriota* and *Firmicutes*, together reaching a relative abundance of 85.8%. In addition, a high abundance (10.6%) of an unidentified phylum (k_Bacteria_Unclassified) was detected in AD9, which significantly differed from the other fish samples. *Proteobacteria* was the most dominant phylum in all five fish sample groups, with nearly 97.3% and 99.2% mean relative abundance in DD7 and DD9, respectively, while a significant decrease was observed in asymptomatic fish from 82.9% in AD7, 74.97% in AD9 and 38.09% in AD22. In contrast, the relative abundance of *Firmicutes*, *Fusobacteria Bacteroidota* and *Actinobacteriota* was generally high in asymptomatic fish. *Firmicutes* had increased abundance across all asymptomatic fish sampled over time (1.5% in AD7; 3.3% in AD9), and accounted for nearly half (47.8%) of the microbial community in AD22. The most predominant phyla in water samples was also *Proteobacteria* (66.8% to 75.9%), followed by *Bacteroidota* (13.2% to 30.1%), *Fusobacteriota* (1.3% to 8.7%) and *Firmicutes* (0.4% to 1.8%) (Fig. 3A).

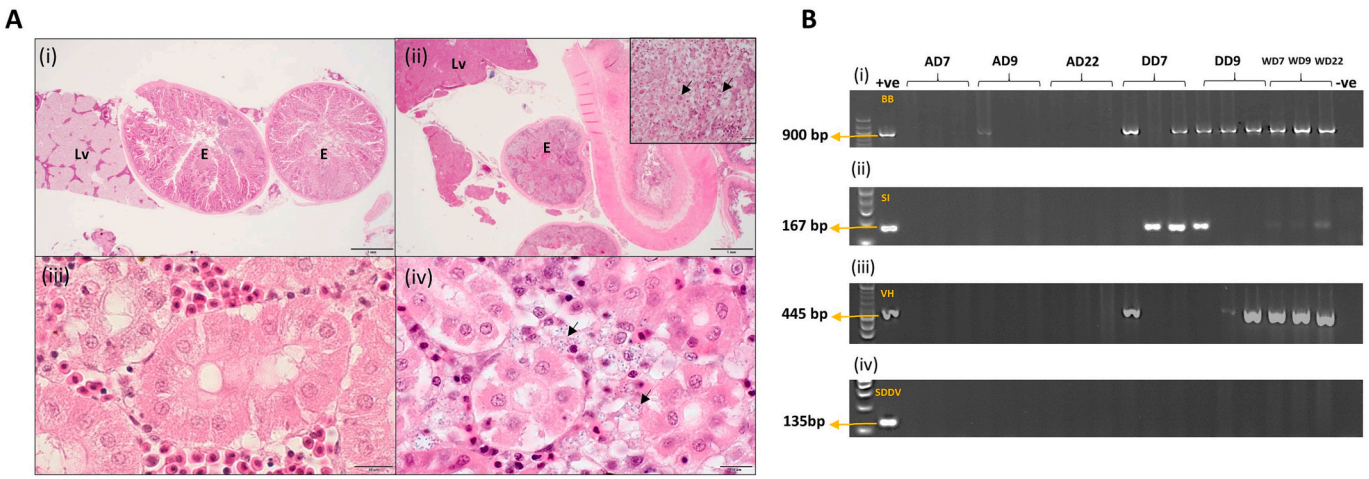

Fig. 1. A) Histopathology on asymptomatic and diseased L. *calcarifer*. Chronic granulomatous enteritis (E) were observed in both asymptomatic (i) as well as diseased fish (ii). Asymptomatic fish had good lipid stores in liver (Lv) (i), as compared to darker staining liver (Lv) due to lipid loss in diseased fish (ii). Diseased fish had more severe and extensive enteritis, often with intralesional, clusters of large coccobacilli (ii-inset, arrows). Kidney in asymptomatic fish (iii). Kidney of diseased fish (iv) with interstitial nephritis and coccoid shaped bacteria (arrows). **B)** PCR on fish and water samples collected on Days 7, 9 and 22 post-stocking, (i) Nearly all diseased fish (5/6) and all water samples tested PCR positive for big belly (BB) disease novel *Vibrio* spp. (ii) Half of the diseased fish (3/6) and all water samples tested PCR positive for *S. iniae* (SI). (iii) Some diseased fish (3/6) and all water samples tested positive for *V. harveyi* (VH). (iv) All fish and water samples tested negative for scale drop disease virus (SDDV). +ve positive control; −ve negative control. The expected size of PCR product for each pathogen is indicated.

3.5. Intestinal microbiota at genus level correlates with disease-state in L. calcarifer

Differences in intestinal microbiome composition and relative abundance at a genus level were observed between the asymptomatic and diseased fish groups (Fig. 3B). The top five highest abundant genus in DD7 were *Vibrio* (77.3%), *Burkholderia-Caballeronia-Paraburkholderia* (BCP) (6.2%), *Pseudomonas* (4.9%), *Photobacterium* (2.5%) and *Cetobacterium* (1.8%), while *Vibrio* (97.2%), *BCP* (0.8%), *Ralstonia* (0.6%), *Streptococcus* (0.4%) and *Comamonas* (0.2%) were rich in DD9 (Fig. 3B). The members of the genera that contributed significantly to the microbial diversity of the three asymptomatic groups were *BCP* (24.7%), *Ralstonia* (13.9%), *Photobacterium* (13.6%), *Cetobacterium* (11.2%) and *Pseudomonas* (9.9%) in AD7; *Ralstonia* (29.1%), *Brucella* (10.7%), *Pelomonas* (9.3%), *BCP* (4.2%) and *Pseudomonas* (3.6%) in AD9; *Weissella* (47.6%), *Photobacterium* (12.3%), *Cetobacterium* (9.8%), *Ralstonia* (9.3%), *Comamonas* (2.9%) and *Brucella* (2.7%) in AD22 (Fig. 3B).

Vibrio was the most abundant genus and prevalent in diseased fish with a relative abundance of 77.3% in DD7 and 97.2% in DD9, while it was rarely present in the asymptomatic fish (0.6% in AD7, 2.4% in AD9, 0.3% in AD22) (Fig. 3B & 4A). *S. iniae* was detected at the species level with greater relative abundance in diseased fish as compared to the asymptomatic fish groups (Fig. 4A). In contrast, the relative abundance of *Ralstonia*, *Brucella, pelomonas* and *allorhizobium_neorhizobium_pararhizobium_rhizobium* (ANPR) were significantly high in the asymptomatic fish compared to the diseased fish ($p < 0.05$) (Fig. 4A & 4B). Notably, *Weissella* was unique in the asymptomatic group AD22 with a rather high relative abundance (47.59%) (Fig. 3B & Fig. 4A). In addition, *Lactobacillus* was also only presented in asymptomatic fish (Fig. 4A).

The analysis of the microbiome of water revealed that *Pseudoalteromonas* (34.8 to 45.5%) was the most dominant genus across all of the three sample collection time points, followed by *Nautella* (7.6 to 12.2%), *Lishizhenia* (6.1 to 7.7%), *Vibrio* (3.7 to 8.5%), *NS10_marine_group* (0.06 to 14.5%), *Cetobacterium* (1.0 to 8.6%) and *Photobacterium* (1.9 to 6.1%) (Fig. 3B). Although there is a strong difference in microbiota composition at the genus level between water and fish, a decline in the relative abundance of *Vibrio* in water was observed overtime (8.51% to 3.66%) with a drop in mortality rate (Fig. 3B), which was similarly observed in the gut over time.

3.6. LEfSe analyses

LEfSe analyses were performed to characterize the microbial communities exhibiting significant differences in abundance between the asymptomatic and diseased groups. A total of 21 bacterial taxa with significant differential relative abundances were identified with LDA score > 3.0. Sixteen of these bacterial taxa were higher in the asymptomatic fish and five were higher in the diseased fish samples ($p < 0.05$) (Fig. 4B & 4C). In particular, the analysis revealed that the genus *Vibrio*, family *Vibrionaceae*, class *Gammaproteobacteria* and phylum *Proteobacteria* were more dominant in diseased fish; whereas in the asymptomatic fish, a higher representation of the phyla *Firmicutes, Bacteroidota* and *Actinobacteriota*, the families of *Burkholderiaceae*, *Rhizobiaceae* and *Comamonadaceae*, as well as genera of *Ralstonia*, *Brucella, Pelomonas* and ANPR were identified.

3.7. Immune-related pathways with significant differences between diseased and asymptomatic L. calcarifer

The potential physiological associations of the gut microbiota in diseased and asymptomatic groups were compared. A total of 72 functional pathways related to human disease (KEGG level 1) and the immune system (KEGG level 2) were identified. Of these, 18 pathways showed significant differences between the two groups (Fig. 5) including *Vibrio cholerae* infection, NOD-like receptor signaling pathway, Pertussis, Salmonella infection, and Th17 cell differentiation. In addition, two pathways of Biofilm formation - *Vibrio cholerae* (infection) and bacterial secretion system were the most abundant (Fig. 5).

4. Discussion

This study is based on field samples (sourced from a local fish hatchery), collected during disease outbreak soon after stocking in a commercial fish farm. Mild to severe, chronic granulomatous enteritis was observed histopathologically in both asymptomatic and diseased juvenile L. *calcarifer*. All diseased L. *calcarifer* in this study tested PCR positive for the 'big belly' novel *Vibrio* spp., *V. harveyi, S. iniae*, or concurrent infections. We also observed *Streptococcus iniae* unique presented and high abundance of *Vibrio* (87.3%) was in diseased L. *calcarifer* albeit with reduced diversity of gut microbiome based on 16S rRNA

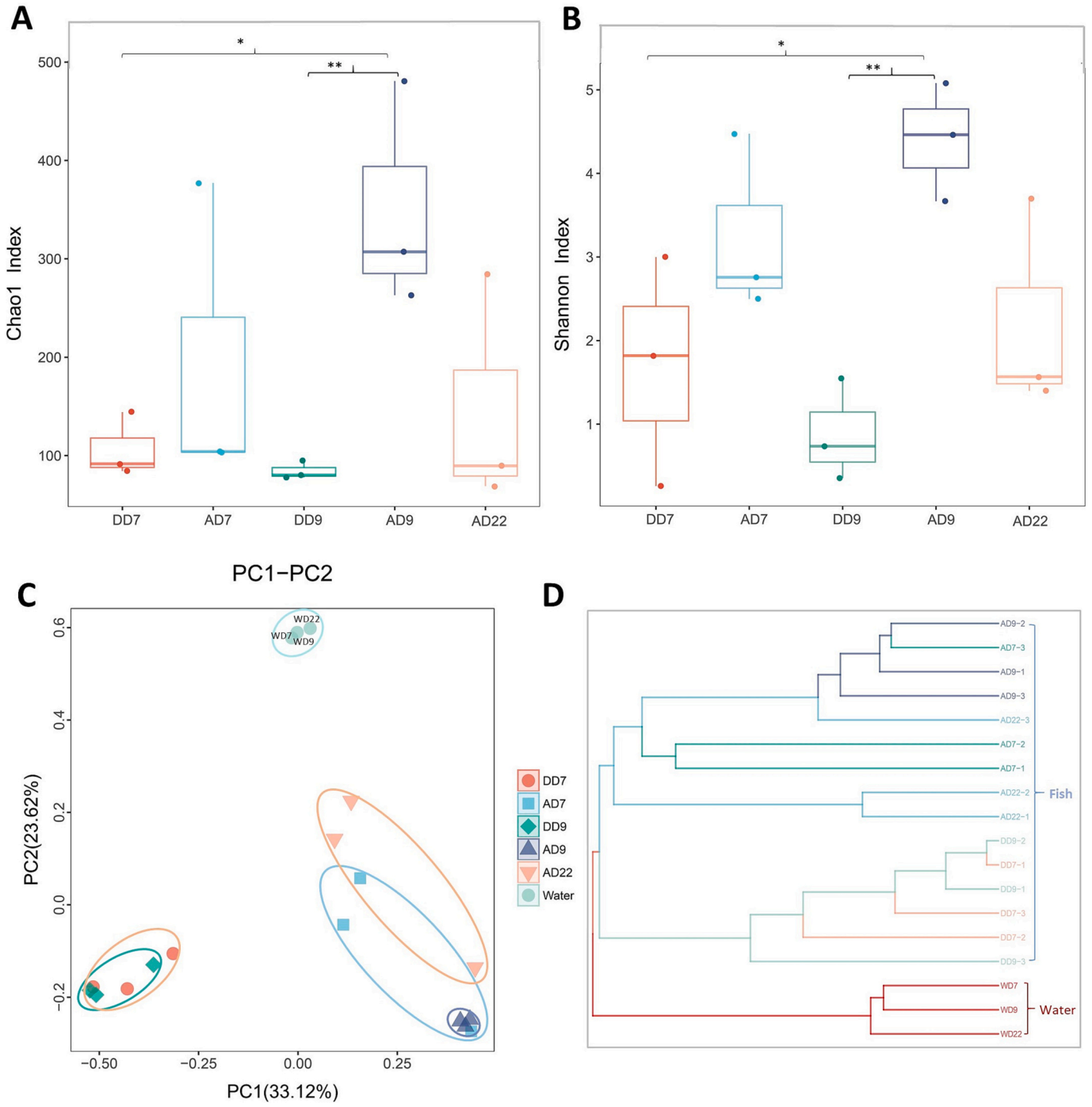

Fig. 2. Summary of the alpha and beta diversities of asymptomatic and diseased L. *calcarifer* gut microbiomes from 16S rRNA metasequencing. **A)** alpha diversity index of Chao1. **B)** alpha diversity index of Shannon index. Statistically significant pairwise differences among the groups is highlighted with asterisk (*, $p < 0.05$; **, $p < 0.01$). **C)** Principal coordinates analysis (PCoA) of bacterial community in fish and water. **D)** Beta diversity analysis of UPGMA cluster tree of fish and water.

sequencing. *Vibrio* and *S. iniae* are common environmental bacteria and likely opportunistic infections in fish with significant gut damage in advanced chronic bacterial enteritis. Farmed fish, especially newly stocked fish can be more susceptible to disease due to many forms of stress such as transportation (Barton, 2000; Manuel et al., 2014), handling (Wedemeyer, 1972; Falahatkar et al., 2009; Aguirre-Guzman et al., 2016), stocking process and environmental changes in their new habitat. Prolonged starvation or acute stress in newly stocked fish can weaken the fish's immune system and alter the composition of gut microbial community, making it more vulnerable to opportunistic pathogens and decreasing its ability to maintain a healthy intestinal microbial community (Olsen et al., 2005; Xia et al., 2014; Desai et al., 2016; Huang et al., 2020; Brocca et al., 2022). Our study suggests that the stress factors may compromise the immunity and physiology of L. *calcarifer*, trigger significant dysbiosis in gut microbiota by predisposing to opportunistic pathogens such as *Vibrio* and *S. iniae*, and lead to enteritis in newly stocked fish. Our results demonstrated the sensitivity of the newly stocked juvenile L. *calcarifer* microbiome to stressors, with potential associated health impacts on the host.

The most dominant bacterial phylum in the gut microbiota of diseased L. *calcarifer* was *Proteobacteria* (98.3%) in the present study, whereas its relative abundance significantly decreased in asymptomatic

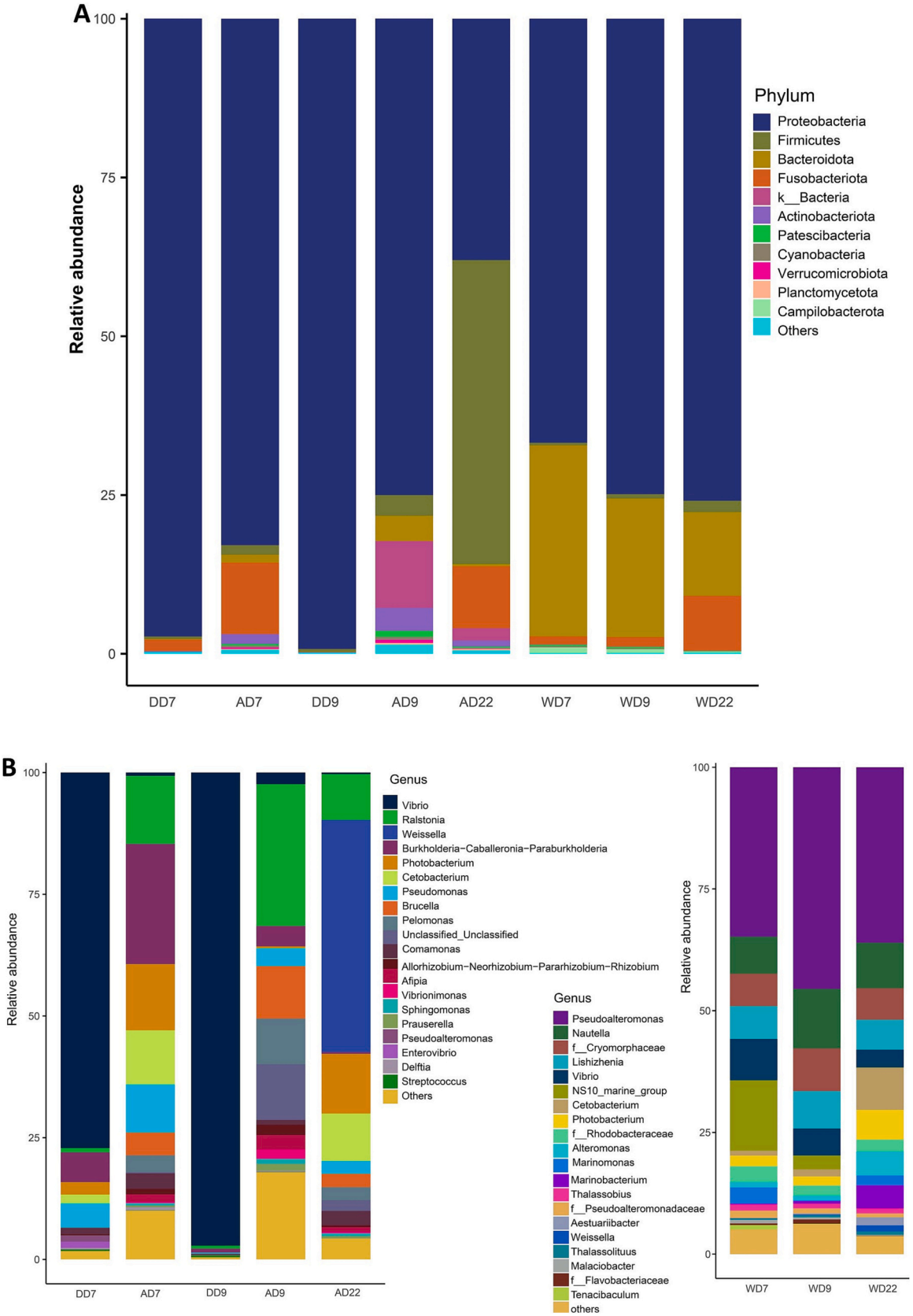

Fig. 3. Comparison of the relative abundances of bacterial OTUs among asymptomatic and diseased L. *calcarifer*, and water samples. **A)** Phyla. **B)** Genus.

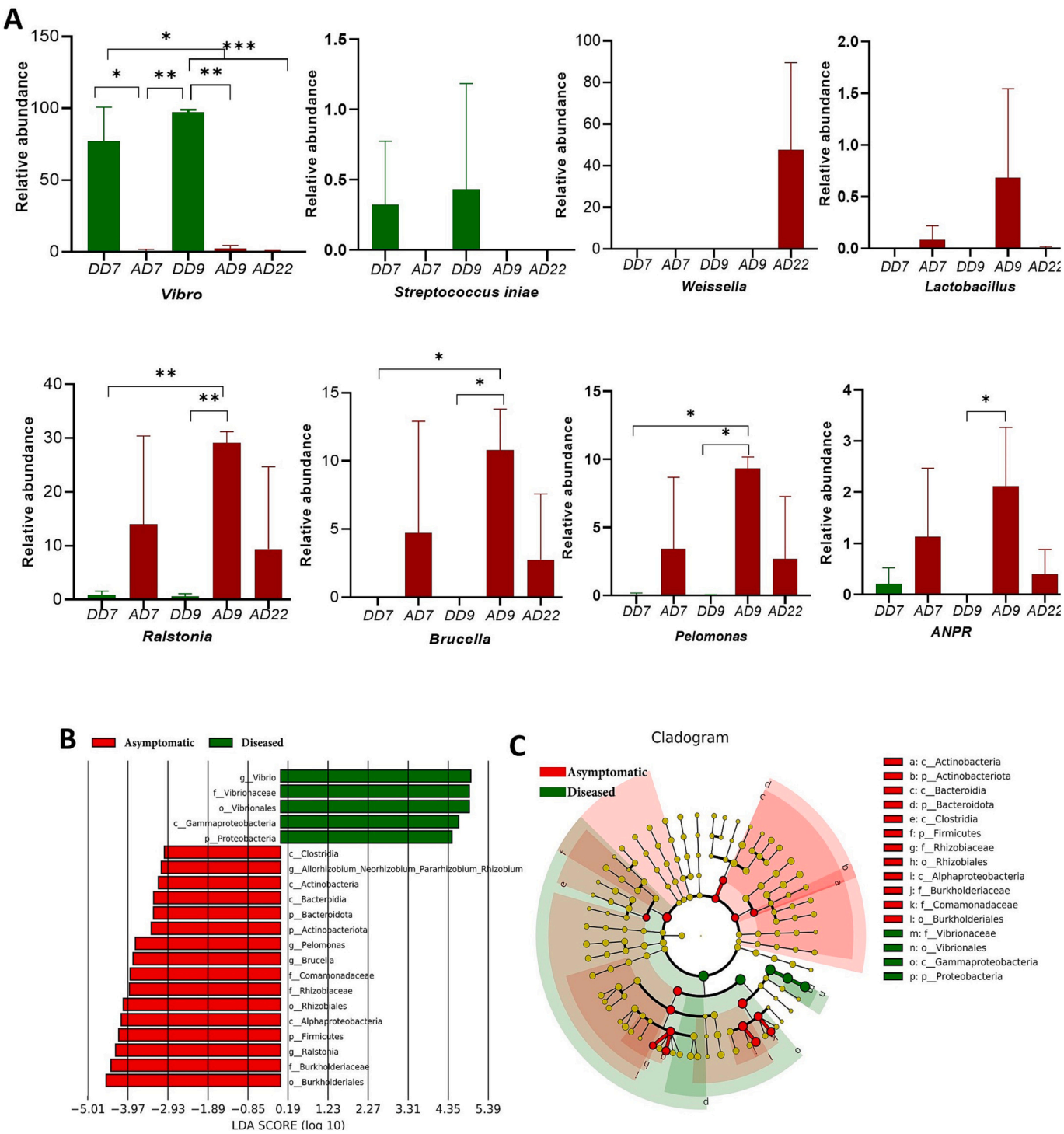

Fig. 4. A) Comparison of the most abundant observed bacteria genera in gut microbiota of asymptomatic and diseased fish. Bars with '*' indicated significant difference between fish sample groups. **B)** Histogram of Linear discriminant analysis (LDA) scores (log10) to identify differentially abundant bacterial taxa between diseased and asymptomatic groups. **C)** Circular Cladogram showed taxonomic distribution of bacterial abundance between asymptomatic and diseased groups.

fish, particularly by the end of the disease outbreak on Day 22 post-stocking (38.1%). Similarly, diseased Asian seabass experimentally and naturally infected with tenacibaculosis exhibited gut microbiota dominated by *Proteobacteria* (83.1–87.0%) (Miyake et al., 2020). In addition, domination by *Proteobacteria* has also been observed in diseased fish in other species, such as grass carp (Tran et al., 2018) and groupers (Ma et al., 2019). Increased prevalence of *Proteobacteria* has been proposed as a potential diagnostic signature of dysbiosis, enteritis and risk of disease in mammals (Shin et al., 2015; Litvak et al., 2017).

LEfSe analysis identified *Firmicutes*, *Actinobacteriota* and *Bacteroidota* as dominant phyla in the intestinal microbiota of asymptomatic fish (this study). These phyla of bacteria are potentially beneficial microbes that promote gut health. The *Firmicutes* with the highest LDA score were present in significantly lower abundance in diseased L. *calcarifer* (0.4%) than in asymptomatic fish (47.8%), especially at the recovery phase on Day 22 post stocking. *Firmicutes* has been recorded previously as primarily beneficial phylum comprising beneficial genera *Bacillus, Lactobacillus, Lactococcus, Enterococcus* and *Clostridium* in healthy fish of

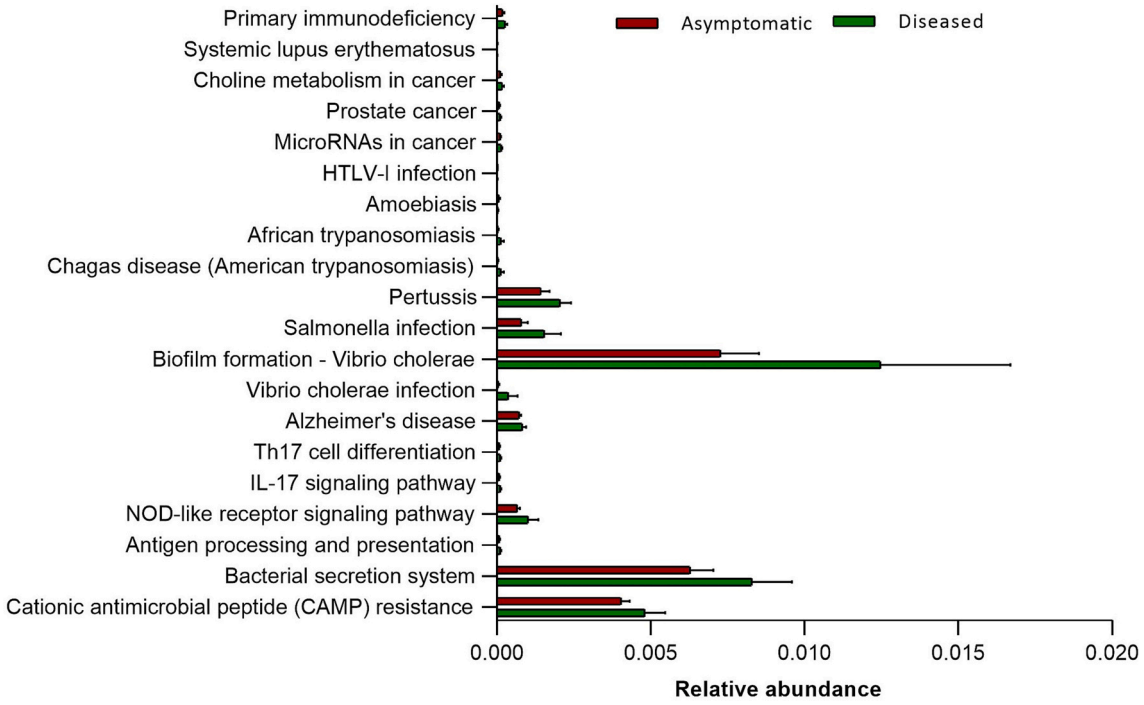

Fig. 5. The KEGG Orthologies by Tax4Fun classification at level 3 of OTUs identified by 16S rRNA sequencing in the gut of diseased and asymptomatic L. *calcarifer*.

several other species (Yang et al., 2022). For example, *Bacillus* spp. are naturally occurring in the gut of cultured fish like grass carp (Wu et al., 2012b) and tilapia (Sookchaiyaporn et al., 2020), and thought to fight gut infections (Silva et al., 2020). In addition to *Firmicutes,* phylum *Actinobacteria* can biosynthesize secondary metabolites such as antibiotics used against invasive pathogens (Penn et al., 2009); *Bacteroidota* is a known core taxa of the adult human microbiome, with roles in producing beneficial metabolites that enhance immunity (Falony et al., 2016). Overall, the shift in gut microbiota in asymptomatic fish of this study to elevated abundance of *Firmicutes*, *Bacteroidota* and *Actinobacteriota* suggests an important role of these bacteria in maintaining a healthy microbiota community in the intestine of L. *calcarifer*. Furthermore, it appears that the increase in *Proteobacteria* in intestines of diseased fish was at the expense of these three beneficial phyla, especially *Firmicutes*, which may contribute to the gut dysbiosis in L. *calcarifer*.

Vibrio was detected as the most dominant gut microbial genus in diseased fish on Day 7 (77.3%) and Day 9 (97.2%) compared to asymptomatic fish (average 1.1%) in this study. *Vibrio* spp. are opportunistic bacteria within the phylum *Proteobacteria* (de Souza Valente and Wan, 2021), which can exist naturally as part of the microbiota of marine fish without causing disease (Wu et al., 2012a; Li et al., 2017; Chin et al., 2020b). However, they may turn pathogenic when fish immunity and physiology is compromised due to husbandry stress (Thune et al., 1993; Creeper and Buller, 2006; Krupesha Sharma et al., 2012). Especially when in high numbers, *Vibrio* spp. can cause substantial damage to the intestinal lining of the fish gut by produce exotoxins causing gastroenteritis and ultimately mortality (Lee et al., 2002; Liu et al., 2004; Macpherson et al., 2012; Zhang et al., 2014). *Vibrio* spp. such as *V. harveyi* (Ransangan et al., 2012; Dong et al., 2017), "Big belly" bacteria (a novel *Vibrio* species) (Gibson-Kueh et al., 2021), *V. alginolyticus* (Krupesha Sharma et al., 2012) and *V. anguillarum* (Rajesh Kumar et al., 2007; Kumaran et al., 2010) have been linked to multiple disease outbreaks in L. *calcarifer* farms, resulting in severe economic losses. In addition, Miyake et al. (2020) reported that experimentally infected and naturally diseased L. *calcarifer* with tenacibaculosis had increased *Vibrio* levels (57.3 ± 22.7% and 31.4 ± 28.7% respectively). In the present study, the microbiota composition in diseased fish showed *Vibrio* were the most prevalent genus (average 87.3%) which was in alignment with the PCR-based diagnosis where diseased fish tested PCR positive for two *Vibrio* spp. of *V. harveyi* and "big belly" novel *Vibrio* spp. Hence, we speculate that these two *Vibrio* spp. were potentially pathogenic species responsible for the disease-state of juvenile L. *calcarifer* in this study.

S. iniae in the present study was observed as the predominant species within the genus *Streptococcus* with an average relative abundance in the microbiome of 0.38% in diseased fish. The species was absent in asymptotic fish. This finding agreed with the PCR-based diagnosis where *S. iniae* was not detected in any asymptomatic fish. Streptococcosis, caused by *S. iniae*, is one of the most important bacterial disease in L. *calcarifer* and causes enormous economic losses (Kayansamruaj et al., 2017). *S. iniae* can persist in the intestine for extended periods in L. *calcarifer* subjected to an oral challenge, and hence serve as an important reservoir of infection (Bromage and Owens, 2002).

It is noteworthy that the genus *Weissella* was unique in the intestine of asymptomatic L. *calcarifer* in significantly high abundance on Day 22 post-stocking (47.59%) in our study. In addition, *Lactobacillus* was only detected in asymptomatic fish (0.26%). *Weissella* and *Lactobacillus* are lactic acid bacteria within the phylum *Firmicutes,* and reported as beneficial gut bacteria that colonize the intestinal regions of fish to stimulate host immune response against disease, or inhibit pathogenic bacterial species (He et al., 2017; Ringø et al., 2018). Several *Weissella* spp., such as *W. cibaria*, *W. confusa, W. paramesenteroides* and *W. hellenica,* have been suggested to act as probiotics due to their antimicrobial activity against fish pathogens (Cai et al., 1998; Abriouel et al., 2015; Kahyani et al., 2021). In the present study, the unique presence of *Weissella* and *Lactobacillus* in asymptomatic fish suggests its potential as a biomarker for monitoring health in L. *calcarifer*, and is worthy of future study.

Similar water microbiome profiles in our study were observed across all three sampling time points, with *Pseudoalteromonas*, *Nautella, Lishizhenia, Vibrio, NS10_marine_group* and *Cetobacterium* etc. being core genera present in all water samples. The most abundant genus was *Pseudoalteromonas* (34.8% to 45.5%), which have been known to produce various antibacterial and antiviral compounds (Neu et al., 2014).

The composition and relative abundance of bacteria in water was observed significantly different from the intestine in L. *calcarifer* (at genus level). In addition, the beta diversity analysis as plotted on PCoA showed that the three water samples clustered together, distinctively separated from both fish groups, especially the diseased fish. These findings may suggest a weak correlation between intestinal microbiota composition in L. *calcarifer* and the surrounding water microbiome. However, there was a decline in the relative abundance of *Vibrio* spp. (8.51% to 3.66%) in water towards the end of the disease outbreak, which correlated with its changing trend in the fish intestine. Future research should further explore the interaction among various stressors (water column, handling, transportation etc.), intestinal microbiome and L. *calcarifer* under disease infection by increasing the number of samples for both fish and water to eliminate individual differences, adding accuracy and reliability to the study.

5. Conclusion

This is the first field study that investigated the changes in the gut microbiome in diseased and asymptomatic newly stocked juvenile L. *calcarifer* with chronic enteritis and mixed bacterial infections. This work showed that the fish gut microbiome changes according to their health status (diseased and asymptomatic). *Proteobacteria* at phylum level, *Vibrio* and *S. iniae* at genus and species level, respectively, were observed increasing in abundance in diseased L. *calcarifer*. Understanding this relationship between the host, gut microbiome and pathology would allow farmers of L. *calcarifer* to better manage disease soon after stocking. Moreover, the unique presence of beneficial bacteria such as *Weissella* and *Lactobacillus* within phylum *Firmicutes* in asymptomatic fish, make them potential candidate biomarkers for monitoring health status of L. *calcarifer*. In addition, the abundance and composition of the microbiome in the water column were significantly different from the intestinal microbiome of L. *calcarifer,* and the relationship is worthy of future study. These findings provide a baseline study for understanding changes in intestinal microbiome in newly stocked stressed fish with mixed bacterial infection, biomarker assisted health monitoring, and future host-derived probiotics screening in L. *calcarifer*.

Author contributions

XS and XZC conceived and designed the experiments. XZC conducted the field sampling and PCR analysis. XZC and SK were responsible for performing data curation of histopathology analysis. XS performed PCR and 16S rRNA sequencing data analysis. XZC, SK and XS wrote the first draft of the manuscript. DJ reviewed and edited the MS. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Ethics approval and consent to participate

The study was conducted at James Cook University in Singapore, under Institute Animal Care and Use Committee (IACUC) approved project IACUC-2019-A08.

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Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Raw sequence data were submitted to the National Center for Biotechnology Information Sequence Read Archive under the BioProject accession number PRJNA890882.

References

Abernathy-Close, L., Barron, M.R., George, J.M., Dieterle, M.G., Vendrov, K.C., Bergin, I. L., Young, V.B., 2021. Intestinal inflammation and altered gut microbiota associated with inflammatory bowel disease render mice susceptible to clostridioides difficile colonization and infection. mBio 12 (3). https://doi.org/10.1128/mBio.02733-20 e0273320-e0273320.

Abriouel, H., Lerma, L.L., Casado Muñoz, M.D.C., Montoro, B.P., Kabisch, J., Pichner, R., Cho, G.-S., Neve, H., Fusco, V., Franz, C.M.A.P., Gálvez, A., Benomar, N., 2015. The controversial nature of the Weissella genus: technological and functional aspects versus whole genome analysis-based pathogenic potential for their application in food and health. Front. Microbiol. 6, 1197. https://doi.org/10.3389/fmicb.2015.01197.

Aguirre-Guzman, G., Carvajal-de-la-Fuente, V., Neri-Coronado, M., Loredo-Osti, J., Rábago-Castro, J.L., 2016. Hematological and clinical chemistry changes induced by acute stress during handling and capture of catfish (Ictalurus punctatus). Revista MVZ Córdoba 21 (2), 5345–5354. https://doi.org/10.21897/rmvz.601.

Antonissen, G., Eeckhaut, V., Van Driessche, K., Onrust, L., Haesebrouck, F., Ducatelle, R., Moore, R.J., Van Immerseel, F., 2016. Microbial shifts associated with necrotic enteritis. Avian Pathol. 45 (3), 308–312. https://doi.org/10.1080/03079457.2016.1152625.

Aßhauer, K.P., Wemheuer, B., Daniel, R., Meinicke, P., 2015. Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. Bioinformatics 31 (17), 2882–2884. https://doi.org/10.1093/bioinformatics/btv287.

Austin, B., 2006. The bacterial microflora of fish, revised. Sci. World 6, 931–945. https://doi.org/10.1100/tsw.2006.181.

Barton, B.A., 2000. Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. N. Am. J. Aquac. 62 (1), 12–18. https://doi.org/10.1577/1548-8454(2000)062<0012:SFDITC>2.0.CO;2.

Brocca, G., Zamparo, S., Pretto, T., Calore, A., Marsella, A., Xiccato, R.L., Cornaggia, M., Cortinovis, L., Bano, L., Toffan, A., Quaglio, F., Verin, R., 2022. Severe gastroenteropathy associated with Clostridium perfringens isolation in starving juvenile sturgeons. J. Fish Dis. 45 (3), 471–477. https://doi.org/10.1111/jfd.13579.

Bromage, Owens, L., 2002. Infection of barramundi Lates calcarifer with Streptococcus iniae: effects of different routes of exposure. Dis. Aquat. Org. 52 (3), 199–205. https://doi.org/10.3354/dao052199.

Bromage, Thomas, A., Owens, L., 1999. Streptococcus iniae, a bacterial infection in barramundi Lates calcarifer. Dis. Aquat. Org. 36 (3), 177–181. https://doi.org/10.3354/dao036177.

Cai, Y., Benno, Y., Nakase, T., Oh, T.-K., 1998. Specific probiotic characterization of Weissella hellenica DS-12 isolated from flounder intestine. J. Gen. Appl. Microbiol. 44 (5), 311–316. https://doi.org/10.2323/jgam.44.311.

Chang, S.F., Ng, K.S., Grisez, L., de Groof, A., Vogels, W., van der Hoek, L., et al., 2018. Novel fish pathogenic virus. In: International Publication Number WO 2018/029301 A1. World Intellectual Property Organization International Bureau, Geneva.

Chin, Y.K., Al-saari, N., Zulperi, Z., Mohd-Aris, A., Salleh, A., Silvaraj, S., Mohamad, A., Lee, J., Zamri-Saad, M., Ina-Salwany, M.Y., 2020a. Efficacy of bath vaccination with a live attenuated Vibrio harveyi against vibriosis in Asian seabass fingerling, *Lates calcarifer*. Aquac. Res. 51 (1), 389–399. https://doi.org/10.1111/are.14386.

Chin, Y.K., Ina-Salwany, M.Y., Zamri-Saad, M., Amal, M.N.A., Mohamad, A., Lee, J.Y., Annas, S., Al-Saari, N., 2020b. Effects of skin abrasion in immersion challenge with Vibrio harveyi in Asian seabass Lates calcarifer fingerlings. Dis. Aquat. Org. 137 (3), 167–173. https://doi.org/10.3354/dao03435.

Creeper, J.H., Buller, N.B., 2006. An outbreak of Streptococcus iniae in barramundi (Lates calcarifera) in freshwater cage culture. Aust. Vet. J. 84 (11), 408–411. https://doi.org/10.1111/j.1751-0813.2006.00058.x.

Crosbie, Nowak, B., 2004. Immune responses of barramundi, Lates calcarifer (Bloch), after administration of an experimental Vibrio harveyi bacterin by intraperitoneal injection, anal intubation and immersion. J. Fish Dis. 27 (11), 623–632.

de Groof, A., Guelen, L., Deijs, M., van der Wal, Y., Miyata, M., Ng, K.S., van Grinsven, L., Simmelink, B., Biermann, Y., Grisez, L., van Lent, J., de Ronde, A., Chang, S.F., Schrier, C., van der Hoek, L., 2015. A novel virus causes scale drop disease in Lates calcarifer. PLoS Pathog. 11 (8), e1005074 https://doi.org/10.1371/journal.ppat.1005074.

de Souza Valente, C., Wan, A.H.L., 2021. Vibrio and major commercially important vibriosis diseases in decapod crustaceans. J. Invertebr. Pathol. 181, 107527. https://doi.org/10.1016/j.jip.2020.107527.

Deng, Y., Zhang, Y., Chen, H., Xu, L., Wang, Q., Feng, J., 2020. Gut-liver immune response and gut microbiota profiling reveal the pathogenic mechanisms of Vibrio harveyi in pearl gentian grouper (Epinephelus lanceolatus ♂ × E. fuscoguttatus ♀). Front. Immunol. 11, 607754. https://doi.org/10.3389/fimmu.2020.607754.

Desai, M.S., Seekatz, A.M., Koropatkin, N.M., Kamada, N., Hickey, C.A., Wolter, M., Pudlo, N.A., Kitamoto, S., Terrapon, N., Muller, A., Young, V.B., Henrissat, B., Wilmes, P., Stappenbeck, T.S., Núñez, G., Martens, E.C., 2016. A dietary Fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. Cell 167 (5), 1339–1353.e1321. https://doi.org/10.1016/j.cell.2016.10.043.

Domingos, J.A., Shen, X., Terence, C., Senapin, S., Dong, H.T., Tan, M.R., Gibson-Kueh, S., Jerry, D.R., 2021. Scale drop disease virus (SDDV) and Lates calcarifer herpes virus (LCHV) coinfection downregulate immune-relevant pathways and cause splenic and kidney necrosis in barramundi under commercial farming conditions. Front. Genet. 12, 666897. https://doi.org/10.3389/fgene.2021.666897.

Dong, Taengphu, S., Sangsuriya, P., Charoensapsri, W., Phiwsaiya, K., Sornwatana, T., Khunrae, P., Rattanarojpong, T., Senapin, S., 2017. Recovery of Vibrio harveyi from scale drop and muscle necrosis disease in farmed barramundi, Lates calcarifer in Vietnam. Aquaculture 473, 89–96. https://doi.org/10.1016/j.aquaculture.2017.02.005.

Falahatkar, B., Poursaeid, S., Shakoorian, M., Barton, B., 2009. Responses to handling and confinement stressors in juvenile great sturgeon Huso huso. J. Fish Biol. 75 (4), 784–796. https://doi.org/10.1111/j.1095-8649.2009.02334.x.

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., Kurilshikov, A., Bonder, M.J., Valles-Colomer, M., Vandeputte, D., 2016. Population-level analysis of gut microbiome variation. Science 352 (6285), 560–564.

Gibson-Kueh, S., 2012. Diseases of Asian Seabass (or Barramundi), *Lates calcarifer* Bloch. Murdoch University.

Gibson-Kueh, S., Uichanco, J.A., 2021. The pathology associated with putative algal toxicosis in red snapper, Lutjanus species (Bloch 1790). J. Fish Dis. 44, 857–861.

Gibson-Kueh, S., Crumlish, M., Ferguson, H.W., 2004. A novel 'skinny pot-belly' disease in Asian seabass fry, Lates calcarifer (Bloch). J. Fish Dis. 27 (12), 731–735. https://doi.org/10.1111/j.1365-2761.2004.00594.x.

Gibson-Kueh, S., Chee, D., Chen, J., Wang, Y.H., Tay, S., Leong, L.N., Ng, M.L., Jones, J. B., Nicholls, P.K., Ferguson, H.W., 2012. The pathology of 'scale drop syndrome' in Asian seabass, Lates calcarifer Bloch, a first description. J. Fish Dis. 35 (1), 19–27. https://doi.org/10.1111/j.1365-2761.2011.01319.x.

Gibson-Kueh, S., Terence, C., Chew, X.Z., Uichanco, J.A., Shen, X., 2021. PCR, in-situ hybridization, and phylogenetic analysis suggest that 'big belly' disease in barramundi, Lates calcarifer (Bloch), is caused by a novel Vibrio species. J. Fish Dis. 44 (12), 1985–1992. https://doi.org/10.1111/jfd.13512.

Hassani, F., Peyghan, R., Alishahi, M., Ghorbanpour, M., Ahangarzadeh, M., 2021. Molecular and biochemical investigation of the role of streptococcus iniae in mortality of Lates calcarifer cages culturing in the Persian Gulf. Experiment. Anim. Biol. 9 (3), 19–27.

He, S., Ran, C., Qin, C., Li, S., Zhang, H., De Vos, W.M., Ringø, E., Zhou, Z., 2017. Anti-infective effect of adhesive probiotic Lactobacillus in fish is correlated with their spatial distribution in the intestinal tissue. Sci. Rep. 7 (1), 1–12.

Hick, P., Schipp, G., Bosmans, J., Humphrey, J., Whittington, R., 2011. Recurrent outbreaks of viral nervous necrosis in intensively cultured barramundi (Lates calcarifer) due to horizontal transmission of betanodavirus and recommendations for disease control. Aquaculture 319 (1), 41–52. https://doi.org/10.1016/j.aquaculture.2011.06.036.

Huang, X., Zhong, L., Fan, W., Feng, Y., Xiong, G., Liu, S., Wang, K., Geng, Y., Ouyang, P., Chen, D., Yang, S., Yin, L., Ji, L., 2020. Enteritis in hybrid sturgeon (Acipenser schrenckii♂ × Acipenser baeri♀) caused by intestinal microbiota disorder. Aquacult. Rep. 18, 100456 https://doi.org/10.1016/j.aqrep.2020.100456.

Jaramillo, Hick, P., Whittington, R.J., 2017. Age dependency of nervous necrosis virus infection in barramundi Lates calcarifer (Bloch). J. Fish Dis. 40 (8), 1089–1101. https://doi.org/10.1111/jfd.12584.

Jerry, D.R. (Ed.), 2013. Biology and culture of Asian seabass, *Lates calcarifer*. CRC Press, Boca Raton, FL, USA, pp. 1–326.

Jiang, J., Miyata, M., Chan, C., Ngoh, S.Y., Liew, W.C., Saju, J.M., Ng, K.S., Wong, F.S., Lee, Y.S., Chang, S.F., 2014. Differential transcriptomic response in the spleen and head kidney following vaccination and infection of Asian seabass with Streptococcus iniae. PLoS One 9 (7), e99128.

Kahyani, F., Pirali-Kheirabadi, E., Shafiei, S., Shenavar Masouleh, A., 2021. Effect of dietary supplementation of potential probiotic Weissella confusa on innate immunity, immune-related genes expression, intestinal microbiota and growth performance of rainbow trout (Oncorhynchus mykiss). Aquac. Nutr. 27 (5), 1411–1420.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44 (1), D457–D462. https://doi.org/10.1093/nar/gkv1070.

Kayansamruaj, P., Dong, H.T., Nguyen, V.V., Le, H.D., Pirarat, N., Rodkhum, C., 2017. Susceptibility of freshwater rearing Asian seabass (Lates calcarifer) to pathogenic Streptococcus iniae. Aquac. Res. 48 (2), 711–718.

Khang, P.V., Phuong, T.H., Dat, N.K., Knibb, W., Nguyen, N.H., 2018. An 8-year breeding program for Asian seabass Lates calcarifer: genetic evaluation, experiences, and challenges. Front. Genet. 9, 191.

Kim, P.S., Shin, N.-R., Lee, J.-B., Kim, M.-S., Whon, T.W., Hyun, D.-W., Yun, J.-H., Jung, M.-J., Kim, J.Y., Bae, J.-W., 2021. Host habitat is the major determinant of the gut microbiome of fish. Microbiome 9 (1), 1–16.

Kong, W., Huang, C., Tang, Y., Zhang, D., Wu, Z., Chen, X., 2017. Effect of Bacillus subtilis on Aeromonas hydrophila-induced intestinal mucosal barrier function damage and inflammation in grass carp (Ctenopharyngodon idella). Sci. Rep. 7 (1) https://doi.org/10.1038/s41598-017-01336-9, 1588–1511.

Krupesha Sharma, S.R., Rathore, G., Verma, D.K., Sadhu, N., Philipose, K.K., 2012. Vibrio alginolyticus infection in Asian seabass (Lates calcarifer, Bloch) reared in open sea floating cages in India. Aquac. Res. 44 (1), 86–92. https://doi.org/10.1111/j.1365-2109.2011.03013.x.

Kumaran, S., Deivasigamani, B., Alagappan, K., Sakthivel, M., 2010. Infection and immunization trials of Asian seabass (Lates calcarifer) against fish pathogen Vibrio anguillarum. J. Environ. Biol. 31 (4), 539–541.

Lau, M.M.L., Kho, C.J.Y., Lim, L.W.K., Sia, S.C., Chung, H.H., Lihan, S., Apun, K., 2022. Microbiome analysis of gut bacterial communities of healthy and diseased Malaysian mahseer (Tor tambroides). Malays. J. Microbiol. 18 (2), 170–191.

Lee, K.K., Liu, P.C., Chuang, W.H., 2002. Pathogenesis of gastroenteritis caused by Vibrio carchariae in cultured marine fish. Marine Biotechnol. (New York, N.Y.) vol. 4 (3), 267–277. https://doi.org/10.1007/s10126-002-0018-9.

Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A.J., Qin, J.G., Oxley, A.P.A., 2017. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. Front. Microbiol. 8, 2664. https://doi.org/10.3389/fmicb.2017.02664.

Li, T., Long, M., Ji, C., Shen, Z., Gatesoupe, F.-J., Zhang, X., Zhang, Q., Zhang, L., Zhao, Y., Liu, X., Li, A., 2016. Alterations of the gut microbiome of largemouth bronze gudgeon (Coreius guichenoti) suffering from furunculosis. Sci. Rep. 6 (1), 30606. https://doi.org/10.1038/srep30606.

Li, T., Li, H., Gatesoupe, F.-J., She, R., Lin, Q., Yan, X., Li, J., Li, X., 2017. Bacterial signatures of "red-operculum" disease in the gut of crucian carp (Carassius auratus). Microb. Ecol. 74 (3), 510–521. https://doi.org/10.1007/s00248-017-0967-1.

Litvak, Y., Byndloss, M.X., Tsolis, R.M., Bäumler, A.J., 2017. Dysbiotic Proteobacteria expansion: a microbial signature of epithelial dysfunction. Curr. Opin. Microbiol. 39, 1–6. https://doi.org/10.1016/j.mib.2017.07.003.

Liu, P.C., Lin, J.Y., Chuang, W.H., Lee, K.K., 2004. Isolation and characterization of pathogenic Vibrio harveyi (V. carchariae) from the farmed marine cobia fish Rachycentron canadum L. with gastroenteritis syndrome. World J. Microbiol. Biotechnol. 20 (5), 495–499. https://doi.org/10.1023/B:WIBI.0000040402.44340.0e.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5, 207. https://doi.org/10.3389/fmicb.2014.00207.

Longbaf Dezfouli, M., Ghaedtaheri, A., Keyvanshokooh, S., Salati, A.P., Mousavi, S.M., Pasha-Zanoosi, H., 2019. Combined or individual effects of dietary magnesium and selenium nanoparticles on growth performance, immunity, blood biochemistry and antioxidant status of Asian seabass (Lates calcarifer) reared in freshwater. Aquac. Nutr. 25 (6), 1422–1430. https://doi.org/10.1111/anu.12962.

Ma, C., Chen, C., Jia, L., He, X., Zhang, B., 2019. Comparison of the intestinal microbiota composition and function in healthy and diseased Yunlong grouper. AMB Express 9 (1), 1–11. https://doi.org/10.1186/s13568-019-0913-3.

Macpherson, H.L., Bergh, Ø., Birkbeck, T.H., 2012. An aerolysin-like enterotoxin from Vibrio splendidus may be involved in intestinal tract damage and mortalities in turbot, Scophthalmus maximus (L.), and cod, Gadus morhua L., larvae. J. Fish Dis. 35 (2), 153–167. https://doi.org/10.1111/j.1365-2761.2011.01331.x.

Magnadottir, B., 2010. Immunological control of fish diseases. Marine Biotechnol. (New York, N.Y.) vol. 12 (4), 361–379. https://doi.org/10.1007/s10126-010-9279-x.

Manuel, R., Boerrigter, J., Roques, J., Heul, J.V.D., Bos, R.V.D., Flik, G., Vis, H.V.D., 2014. Stress in african catfish (clarias gariepinus) following overland transportation. Fish Physiol. Biochem. 40 (1), 33–44. https://doi.org/10.1007/s10695-013-9821-7.

Meemetta, W., Domingos, J.A., Dong, H.T., Senapin, S., 2020. Development of a SYBR green quantitative PCR assay for detection of Lates calcarifer herpesvirus (LCHV) in farmed barramundi. J. Virol. Methods 285, 113920. https://doi.org/10.1016/j.jviromet.2020.113920.

Miyake, S., Soh, M., Azman, M.N., Ngoh, S.Y., Orbán, L., Seedorf, H., 2020. Insights into the microbiome of farmed Asian sea bass (Lates calcarifer) with symptoms of tenacibaculosis and description of Tenacibaculum singaporense sp. nov. Antonie Van Leeuwenhoek 113 (6), 737–752. https://doi.org/10.1007/s10482-020-01391-9.

Neu, A.K., Månsson, M., Gram, L., Prol-García, M.J., 2014. Toxicity of bioactive and probiotic marine bacteria and their secondary metabolites in Artemia sp. and Caenorhabditis elegans as eukaryotic model organisms. Appl. Environ. Microbiol. 80 (1), 146–153.

Ofek, T., Lalzar, M., Izhaki, I., Halpern, M., 2022. Intestine and spleen microbiota composition in healthy and diseased tilapia. Anim. Microbiom. 4 (1), 50. https://doi.org/10.1186/s42523-022-00201-z.

Olsen, R.E., Sundell, K., Mayhew, T.M., Myklebust, R., Ringø, E., 2005. Acute stress alters intestinal function of rainbow trout, Oncorhynchus mykiss (Walbaum). Aquaculture 250 (1), 480–495. https://doi.org/10.1016/j.aquaculture.2005.03.014.

Penn, K., Jenkins, C., Nett, M., Udwary, D.W., Gontang, E.A., McGlinchey, R.P., Foster, B., Lapidus, A., Podell, S., Allen, E.E., 2009. Genomic islands link secondary metabolism to functional adaptation in marine Actinobacteria. The ISME J. 3 (10), 1193–1203.

Rajesh Kumar, S., Parameswaran, V., Ahmed, V.P.I., Musthaq, S.S., Hameed, A.S.S., 2007. Protective efficiency of DNA vaccination in Asian seabass (Lates calcarifer) against Vibrio anguillarum. Fish and Shellfish Immunol. 23 (2), 316–326. https://doi.org/10.1016/j.fsi.2006.11.005.

Ransangan, J., Lal, T.M., Al-Harbi, A.H., 2012. Characterization and experimental infection of Vibrio harveyi isolated from diseased Asian seabass (Lates calcarifer). Malaysian J. Microbiol. 8 (2), 104–115. https://doi.org/10.21161/mjm.03512.

Ren, Z., Qu, X., Peng, W., Yu, Y., Zhang, M., 2019. Nutrients drive the structures of bacterial communities in sediments and surface waters in the river-Lake system of Poyang Lake. Water 11 (5), 930.

Ringø, E., Hoseinifar, S.H., Ghosh, K., Doan, H.V., Beck, B.R., Song, S.K., 2018. Lactic acid bacteria in finfish—an update. Front. Microbiol. 9, 1818.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12 (6), R60. https://doi.org/10.1186/gb-2011-12-6-r60.

Segers, R.P.A.M., Grisez, L., 2005. Novel Bacterium and Vaccine. Australian Patent No. AU2005203202. Retrieved from. http://patentimages.storage.googleapis.com/14/da/7f/3cfa9d11c66137/AU2005203202C1.pdf.

Shin, N.-R., Whon, T.W., Bae, J.-W., 2015. Proteobacteria : microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. (Regular ed.) 33 (9), 496–503. https://doi.org/10.1016/j.tibtech.2015.06.011.

Silva, B.R.D.S., Derami, M.S., Paixão, D.A., Persinoti, G.F., Dias da Silveira, W., Maluta, R.P., 2020. Comparison between the intestinal microbiome of healthy fish

and fish experimentally infected with Streptococcus agalactiae. Aquac. Res. 51 (8), 3412–3420. https://doi.org/10.1111/are.14676.

Sookchaiyaporn, N., Srisapoome, P., Unajak, S., Areechon, N., 2020. Efficacy of Bacillus spp. isolated from Nile tilapia Oreochromis niloticus Linn. On its growth and immunity, and control of pathogenic bacteria. Fish. Sci. 86 (2), 353–365. https://doi.org/10.1007/s12562-019-01394-0.

Sukhontip, S., Chuenchit, B., Siripong, T., Ha Thanh, D., Saengchan, S., 2019. A highly sensitive and specific SYBR green quantitative polymerase chain reaction (qPCR) method for rapid detection of scale drop disease virus in Asian sea bass, Lates calcarifer. In: Cold Spring Harbor. Cold Spring Harbor Laboratory Press.

Thune, R.L., Stanley, L.A., Cooper, R.K., 1993. Pathogenesis of gram-negative bacterial infections in warmwater fish. Annu. Rev. Fish Dis. 3 (C), 37–68. https://doi.org/10.1016/0959-8030(93)90028-A.

Torres-Corral, Santos, Y., 2021. Development of a real-time PCR assay for detection and quantification of Streptococcus iniae using the lactate permease gene. J. Fish Dis. 44 (1), 53–61.

Tran, N.T., Zhang, J., Xiong, F., Wang, G.-T., Li, W.-X., Wu, S.-G., 2018. Altered gut microbiota associated with intestinal disease in grass carp (Ctenopharyngodon idellus). World J. Microbiol. Biotechnol. 34 (6), 71–79. https://doi.org/10.1007/s11274-018-2447-2.

Van Khang, P., Van Nha, V., Nguyen, N.H., 2019. Resistance to Streptococcus iniae and its genetic associations with traits of economic importance in Asian seabass (Lates calcarifer). J. Fish Dis. 42 (12), 1657–1666. https://doi.org/10.1111/jfd.13092.

Wang, L., Tian, Y., Li, Z., Li, Z., Chen, S., Li, L., Li, W., Wang, Q., Lin, H., Li, B., 2022. Comparison of the gut microbiota composition between asymptomatic and diseased Epinephelus moara ♀ × Epinephelus lanceolatus ♂ with nervous necrosis virus infection. Aquac. Res. 53 (2), 633–641. https://doi.org/10.1111/are.15607.

Wedemeyer, 1972. Some physiological consequences of handling stress in the juvenile coho salmon (Oncorhynchus kisutch) and steelhead trout (Salmo gairdneri). J. Fish. Board Canada 29 (12), 1780–1783.

Wu, S., Wang, G., Angert, E.R., Wang, W., Li, W., Zou, H., 2012a. Composition, diversity, and origin of the bacterial community in grass carp intestine. PLoS One 7 (2), e30440.

Wu, Z.X., Feng, X., Xie, L.L., Peng, X.Y., Yuan, J., Chen, X.X., 2012b. Effect of probiotic Bacillus subtilis Ch9 for grass carp, Ctenopharyngodon idella (Valenciennes, 1844), on growth performance, digestive enzyme activities and intestinal microflora. J. Appl. Ichthyol. 28 (5), 721–727. https://doi.org/10.1111/j.1439-0426.2012.01968.x.

Xia, J.H., Lin, G., Fu, G.H., Wan, Z.Y., Lee, M., Wang, L., Liu, X.J., Yue, G.H., 2014. The intestinal microbiome of fish under starvation. BMC Genomics 15 (1), 266. https://doi.org/10.1186/1471-2164-15-266.

Xinyuan, C., Qinrong, Z., Qunde, Z., Yongyong, Z., Hua, C., Guoqi, L., Lifeng, Z., 2022. Research Progress of the gut microbiome in hybrid fish. Microorganisms (Basel) 10 (5), 891. https://doi.org/10.3390/microorganisms10050891.

Yang, H.-L., Liu, Z.-Y., Jian, J.-T., Liu, Z.-X., Ye, J.-D., Sun, Y.-Z., 2022. Host-derived probiotics shape the intestinal microbial composition, but not putative function in juvenile Japanese seabass (Lateolabrax japonicus). Front. Mar. Sci. 9 https://doi.org/10.3389/fmars.2022.878633.

Zhang, X., Li, Y.-W., Mo, Z.-Q., Luo, X.-C., Sun, H.-Y., Liu, P., Li, A.-X., Zhou, S.-M., Dan, X.-M., 2014. Outbreak of a novel disease associated with Vibrio mimicus infection in fresh water cultured yellow catfish, Pelteobagrus fulvidraco. Aquaculture 432, 119–124. https://doi.org/10.1016/j.aquaculture.2014.04.039.