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Digestive tract microbiota composition of white shrimp with symptoms of White Feces Syndrome (WFS) causing high mortality in Pasuruan, Indonesia

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[3]Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga. Jl. Dr. Ir. H. Soekarno, Surabaya 60115, East Java, Indonesia
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Abstract. *Satyantini WH, Rahardjo KKE, Amin M, Mukti AT, Andriyono S, Yasin ISM. 2025. Digestive tract microbiota composition of white shrimp with symptoms of White Feces Syndrome (WFS) causing high mortality in Pasuruan, Indonesia. Biodiversitas 26: 690-697.* Disease outbreaks remain one of the leading causes of failure in white shrimp (*Litopenaeus vannamei* (Boone, 1931)) farming, with White Feces Syndrome (WFS) continuing to pose a significant challenge. In Pasuruan District, particularly in Gerongan and Kalianyar Villages, shrimp farmers have reported alarming mortality rates associated with this disease, often experiencing high shrimp death rates within a single day. This study aimed to determine the primary pathogen responsible for WFS by analyzing differences in the microbial composition between healthy and infected shrimp using Next-Generation Sequencing (NGS). Shrimp samples were collected from two villages in Pasuruan where WFS outbreaks had been reported. In Gerongan Village, sampling was conducted at DOC 22, while in Kalianyar Village, it was carried out at DOC 51. Each village provided two shrimp samples: healthy shrimp and those showing disease symptoms. Healthy shrimp were identified by the absence of visible disease symptoms, such as pale hepatopancreas, empty digestive tracts, and floating white feces. Sampling was conducted manually between 10 AM and 12 PM to minimize stress. The shrimp were immediately placed in sterile cool boxes with ice packs, maintaining a temperature of approximately 4°C to preserve gut microbiota and prevent microbial degradation. The shrimp gut samples were analyzed to identify the microbial composition, revealing that the dominant bacteria in the guts of WFS-infected shrimp were pathogenic species, including *Photobacterium damselae*, *Vibrio vulnificus*, and *Vibrio coralliilyticus*. These pathogens are suspected to be the main causative agents of WFS outbreaks in the studied areas.

Keywords: Aquaculture, *Litopenaeus vannamei*, next generation sequencing, shrimp mortality, white feces syndrome

INTRODUCTION

With the rapid increase in demand for the shrimp farming sector, focusing on health during farming activities is becoming increasingly important. The productivity of shrimp farming is closely related to shrimp health, and the gut microbiota composition is an important factor that influences shrimp health. Recently, diseases that are becoming hot topics of discussion among shrimp farmers include White Feces Syndrome (WFS) and Acute Hepatopancreatic Necrosis Disease (AHPND). These two diseases have similar clinical symptoms, including atrophy of the shrimp hepatopancreas (Sukarno 2016), peeling of epithelial cells of the hepatopancreas or digestive tract (Kumar et al. 2019; Kumar et al. 2020), lethargy, reduced appetite or anorexia, growth delay, an empty digestive tract, and a pale to white hepatopancreas. AHPND infections typically occur earlier in the cultivation period, often presenting with sudden mortality and hepatopancreatic cell necrosis, whereas WFS usually manifests in the later stages of cultivation. In WFS, the presence of white feces on the surface of the pond water is a distinctive symptom, along with the appearance of empty guts and delayed shrimp growth (Kumar et al. 2021).

The development of the disease is caused by environmental factors such as weather and seasonal changes. Warmer months affect water temperature, salinity and oxygen levels. These are known to stress shrimp and result in a shift in gut microbiota making shrimp susceptible to pathogens. WFS outbreaks, for example, are more commonly reported during the hot and humid season when environmental conditions become more variable and unfavorable for shrimp health (Deris et al. 2022).

In August 2021, white shrimp farmers in Pasuruan District, East Java, specifically in Gerongan and Kalianyar Villages, reported mass deaths. Clinical symptoms began to appear at 22 days of rearing age (DOC 22) in intensive ponds in Gerongan Village, whereas such symptoms were observable at 51 days of rearing age (DOC 51) in the ponds of Kalianyar Village. In both cases, the symptoms were similar, such as pale hepatopancreas, empty shrimp guts, the appearance of white shrimp feces on the surface of the pond water, decreased shrimp appetite, delayed growth, and the presence of uneaten feed residue. In addition, it is accompanied by the continuous death of shrimp in ponds that are infected with the disease at approximately 2-3 kg/d. Some typical clinical symptoms appear in these two villages, such as the presence of shrimp feces floating on the surface of the pond water, which appear as strands of white thread; however, this symptom is not observed in shrimp affected by AHPND. Therefore, the shrimps in Gerongan and Kalianyar Villages were suspected to have WFS.

In the last four years, mass deaths have also been reported in several shrimp ponds spread across several Indonesian shrimp farming centers, such as Banyuwangi and Madura (Supono et al. 2019) Aceh Besar District (Arisa et al. 2021, Tambak Pinang Gading-South Lampung (Islamey et al. 2019), and in several countries, such as Malaysia, Vietnam, Thailand, India, and Ecuador (Sajiri et al. 2021), presenting the same clinical symptoms. To date, the main causes of WFS are still inconclusive and include parasites such as *Enterocytozoon hepatopenaei* (Sajiri et al. 2021), *Vibrio parahaemolyticus* (Zhang et al. 2021), *Vibrio anguillarum*, *Vibrio fluvialis*, *Vibrio alginolyticus*, and *Vibrio mimicus* (Jayadi et al. 2016; Supono et al. 2019).

The ambiguity regarding the cause of the disease (the causative agent) of WFS hinders identifying methods to prevent and treat infected shrimps. Unfortunately, studies on aquaculture microbiomes in Indonesia are limited. Therefore, research on the composition and dominance of bacteria in the guts of healthy shrimp or those affected by WFS, along with accompanying clinical symptoms that are considered to helpful to confirm WFS in shrimp ponds in Gerongan and Kalianyar villages.

This study aimed to use the 16S rRNA amplicon sequencing method (V3-V4 region) to compare the composition, microbial diversity, and abundance in the guts of shrimp with clinical WFS symptoms and healthy shrimp. The chosen metagenomic approach will provide a new perspective from both composition and potential functions to explore the main disease-causing agents in two different shrimp health conditions.

MATERIALS AND METHODS

Study area

Shrimp sampling was performed on shrimp farms in Pasuruan District, East Java, Indonesia in Gerongan Village, Kraton Sub-district (DOC 22) (7°35'46.94"S, 112°51'18.87"E) and Kalianyar Village, Bangil Sub-district (DOC 51) (7°33'49.24"S, 112°48'38.26"E), as indicated by mass shrimp death (Figure 1). The selected villages had been experiencing continuous mortality rates ranging from 2-3 kg per day, which helped to determine the timing and location for sampling.

Procedures

This study employed a structured survey design with a purposive sampling approach to ensure that the sample selection aligned with the objectives (Purnamasari et al. 2017). The main parameters analyzed were bacteria in the shrimp gut from four prepared samples.

Shrimp sampling

Shrimp sample were collected from two villages in Pasuruan District, East Java, Indonesia, where WFS outbreaks had been reported. In Gerongan Village, samples were taken at 22 days of rearing age (DOC 22), and in Kalianyar Village, at 51 days of rearing age (DOC 51). Each village provided two samples: one healthy shrimp and one exhibiting WFS symptoms. Healthy shrimp were identified by the absence of visible disease symptoms, such as pale hepatopancreas, empty digestive tracts, and floating white feces. Shrimp showing these clinical signs were considered infected. Shrimp were harvested manually during late morning hours, between 10:00 AM and 12:00 PM, to minimize stress. After collection, they were immediately transported to the laboratory in a sterile cool box with an ice pack to preserve gut microbiota, maintaining a temperature of approximately 4°C to prevent microbial degradation.

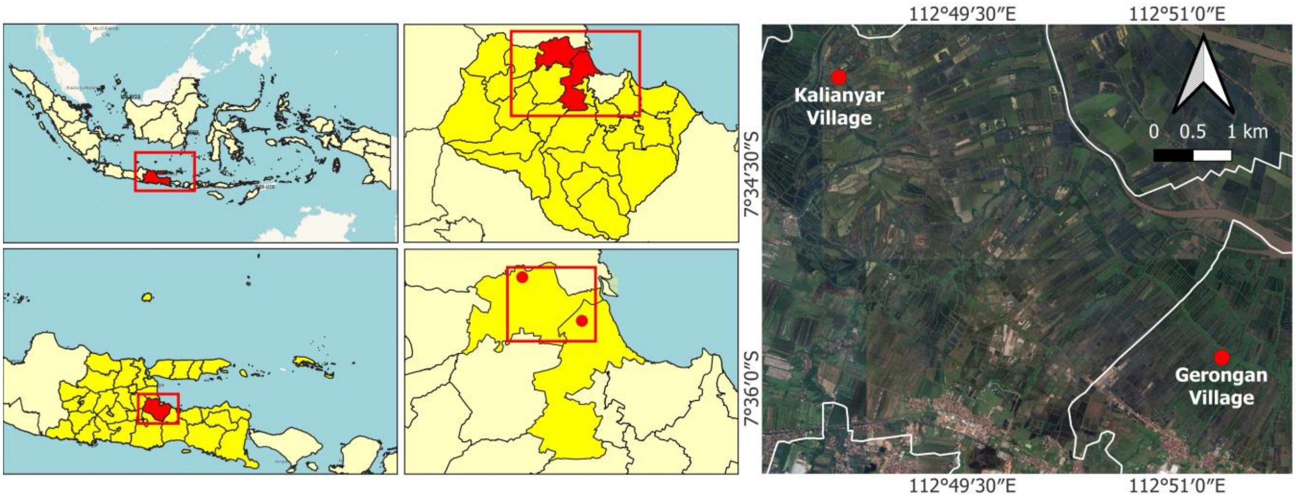

Figure 1. Location of shrimp farms in Pasuruan District, East Java, Indonesia, indicating the villages affected by mass shrimp deaths: Gerongan Village, Kraton Sub-district (DOC 22) and Kalianyar Village, Bangil Sub-district (DOC 51)

Analysis of microbial composition

The bacterial composition of the shrimp gut was assessed using Next Generation Sequencing (NGS), which enables high-throughput sequencing of the bacterial genome and provides insight into genetic diversity and evolutionary relationships among bacterial communities. Simultaneous sequencing of bacterial genomes allows the identification of pathogenic species and their phylogenetic relationships to support species reclassification based on genomic data (Deurenberg et al. 2017).

In the laboratory, Next-Generation Sequencing (NGS) was employed to analyze the microbial composition of the shrimp's digestive tracts. The shrimp guts were dissected under sterile conditions, and DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Germany) along with essential reagents such as absolute ethanol, ATL Buffer, Proteinase K, AW1 Buffer, AW2 Buffer, and AE Buffer. A Benchmark MC-12 microcentrifuge was used to facilitate the extraction. DNA concentration was measured using a NanoDrop Spectrophotometer. The 16S rRNA gene (V3-V4 region) was amplified for sequencing using primers 341F and 805R, as described by Deurenberg et al. (2017). Amplified products were purified, and sequencing was performed on an Illumina MiSeq platform. Sequences were analyzed for microbial diversity and abundance using QIIME2, comparing bacterial species between healthy and WFS-infected shrimp.

RESULTS AND DISCUSSION

Overview of mass mortality in white shrimp

The mass death of white shrimp has caused significant losses to shrimp farmers. Disease began to be observed at different maintenance age ranges of 22 and 51 DOC in Gerongan and Kalianyar, respectively. In general, shrimp affected with WFS are very difficult to save; therefore, all existing shrimp must be harvested or discarded. The differences in the visual appearance of the guts and hepatopancreas of healthy shrimp and those affected by WFS (Figure 2).

Role of gut microbiota in disease susceptibility

The gut is easily exposed to disease-causing agents, such as bacteria and viruses (Okon et al. 2023). It is very possible for bacteria, fungi, viruses, and parasites to inhabit the gut. The natural microbiota of the gut varies significantly (Hou et al. 2022). Microbiota plays an important role in maintaining the host's balance and resistance to pathogens; however, microbiota in the gut can also cause disease in the host. Several studies have revealed that many diseases caused by bacteria are associated with shifts and imbalances in gut microbiota (Li et al. 2016). Shrimp diseases have been shown to substantially alter the composition of gut microbiota (Cornejo-Granados et al. 2017).

Bacterial composition in healthy and diseased shrimp

This study focused on community diversity and the number of bacteria associated with mass mortality in white shrimp in two areas at comparable times. To visualize the profile and composition of bacteria at the genus level in a hierarchical manner in the gut of healthy shrimp and those affected by WFS, a pie chart was created to represent the abundance of bacteria (Figures 4 and 5).

Figures 3 and 4 showcase the dominance of the genera *Vibrio* (29.05%), *Chloroplast* (9.32%), and *Algoriphagus* (9.20%) in the gut samples of shrimps with WFS. In contrast, three genera dominated the gut of healthy shrimp: *Chloroplast* (19.07%), *Vibrio* (9.53%), and *Algoriphagus* (8.06%). The abundance of the *Vibrio* genus in the group of diseased shrimp gut samples was greater (29.05%) than that in the healthy shrimp gut samples. Based on the test results, there were differences in the abundance of bacteria in shrimp guts under the two different health conditions. This difference in numbers could be caused by shrimp carrying out strong selection against external microbial colonization, resulting in differences in microbial communities among the shrimp gut microbiota, rearing media water, and microbiota in the sediment (Chen et al. 2017; Zheng et al. 2017; Zhao et al. 2018).

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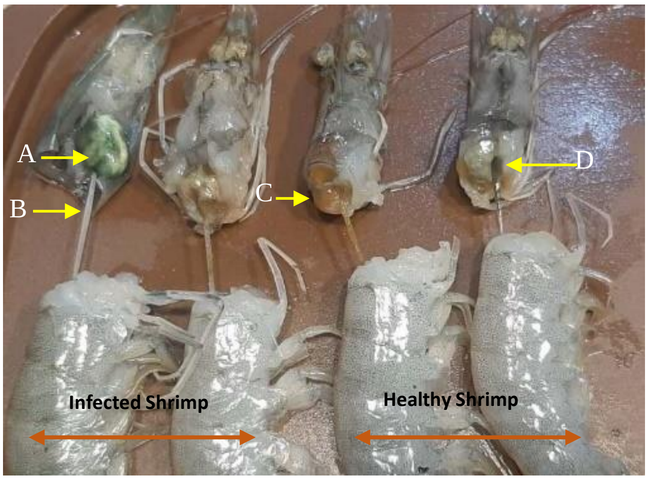

Figure 2. Appearance of gut and hepatopancreas of healthy shrimp and those indicated for WFS after necropsy in infected shrimp: A. Pale hepatopancreas; B. Empty shrimp gut (white color). In healthy shrimp, C. The hepatopancreas is brown; D. The gut appears full and brown

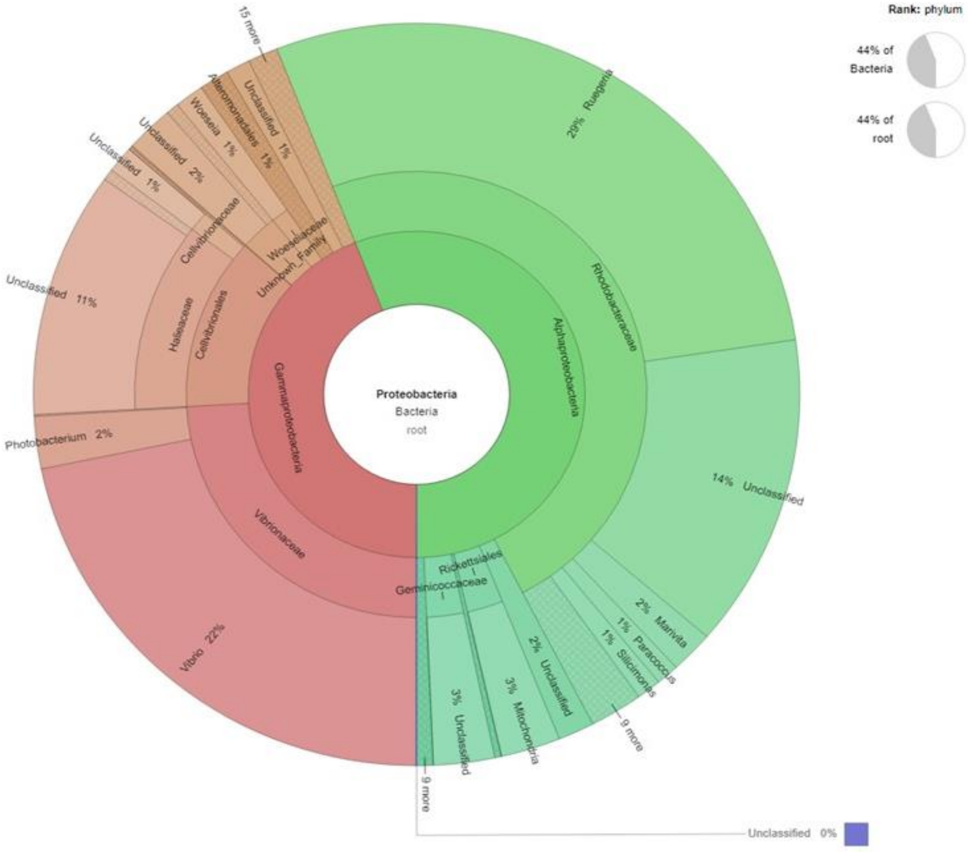

Figure 3. Bacterial composition at the genus level in healthy shrimp guts

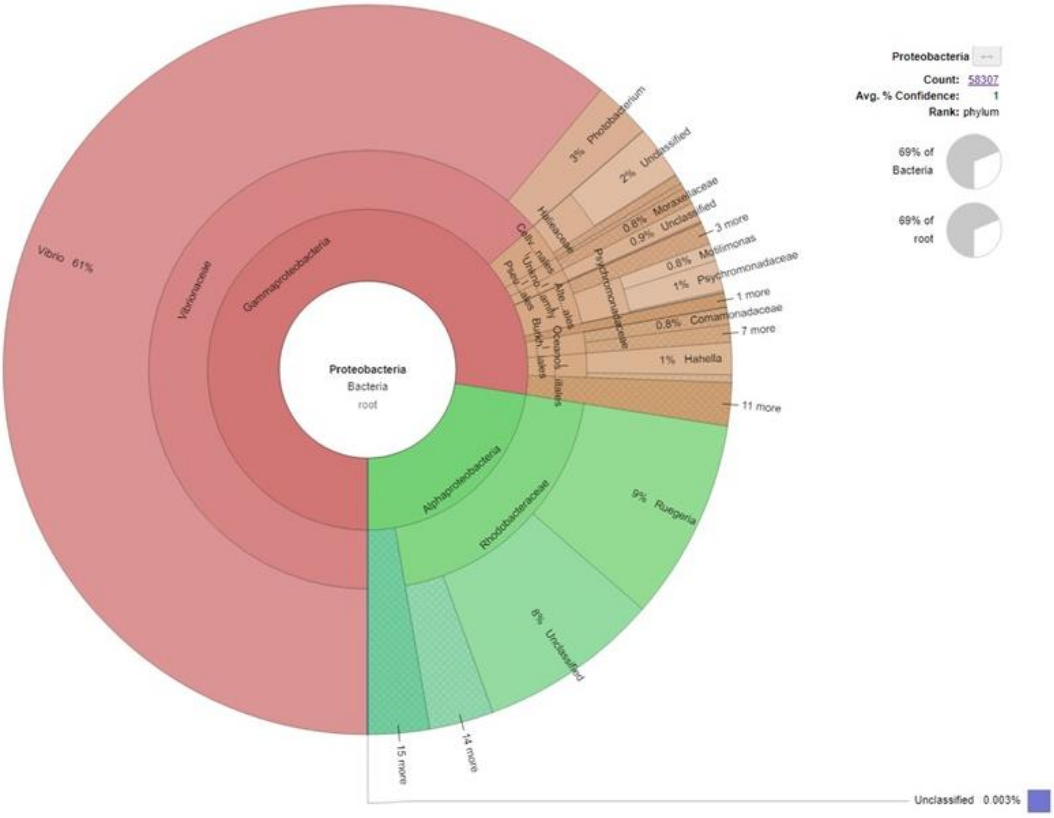

Figure 4. Bacterial composition at the genus level in the WFS infected shrimp guts

Opportunistic pathogens in diseased shrimp

Observing the differences in bacterial composition, notable patterns emerge when categorizing bacteria into red and green groups based on their relative abundance in healthy and diseased shrimp. In healthy shrimp, the composition of red bacteria is less than green bacteria groups. However, in diseased shrimp, red bacteria become more dominant. This observation aligns with previous studies indicating that certain bacterial groups might become opportunistically pathogenic under stress or unfavorable environmental conditions (Sampaio et al. 2022).

Based on the results, two prominent bacterial species were found in diseased shrimp, namely *Photobacterium damselae* and *Vibrio vulnificus*. Where *P. damselae* is known for its virulence, characterized by the presence of strong cytolysins and exotoxins that can disrupt cell performance, *V. vulnificus* is an opportunistic pathogen, growing in tropical salinity and temperature conditions such as the aquaculture environment in Indonesia. The aquaculture methods practiced in Indonesia such as high stocking density and limited biosecurity can cause water quality to deteriorate, thus creating ideal living conditions for opportunistic pathogens.

In addition to these two bacteria, *Vibrio coralliilyticus*, a bacterium that causes coral bleaching, can be a risk bacterium in crustacean species. Its occurrence in shrimp gut makes it a potential cross-environmental transmission of pathogens that can be affected by the same water resources. These findings confirm that environmental and management practices in aquaculture in Indonesia still require improved disease outbreak mitigation such as optimizing stocking densities, improving water quality management, providing probiotics, and increasing biosecurity to reduce the prevalence of pathogenic bacterial groups.

The *Vibrio* sp. is a type of bacteria capable of surviving in a wide range of salinities, specifically between 20 and 40 ppt, and thrives at pH 7.0-7.5 (Hikmawati et al. 2019). This genus is dominant in brackish water and estuarine environments and is commonly associated with marine organisms, such as crustaceans (Sampaio et al. 2022). Heenatigala and Fernando (2016) and Rungrassamee et al. (2016) suggested that *Vibrio* is an opportunistic pathogen in white shrimp (*Litopenaeus vannamei* (Boone, 1931)). The shift of *Vibrio* from nonvirulent to virulent is influenced by two factors: significant environmental changes and bacterial cell density reaching quorum sensing levels (Sampaio et al. 2022). Infections caused by *Vibrio* sp. can affect shrimp at all stages of development, from the nauplius, zoea, mysis, and post-larval stages to adult shrimp in grow-out ponds (Sarjito et al. 2015). *Vibrio* spp. are Gram-negative facultative anaerobic bacteria with the potential to cause mass mortality in shrimp farming. *Vibrio* has been identified as a pathogen in various aquatic organisms, particularly in larval and juvenile crustaceans (De Souza Valente and Wan 2021).

Species-level analysis of pathogenic bacteria

To provide more detailed information regarding the causative agents of WFS, an analysis of the composition of white shrimp intestinal bacteria at the species level was performed. Classification of bacteria using NGS was performed based on a collection of 16S rRNA gene sequences expressed in operational taxonomic units (OTUs) and sorted based on prevalence. The results of the analysis of the intestinal bacterial composition of healthy shrimp and those with WFS at the species level are shown in Figures 5 and 6.

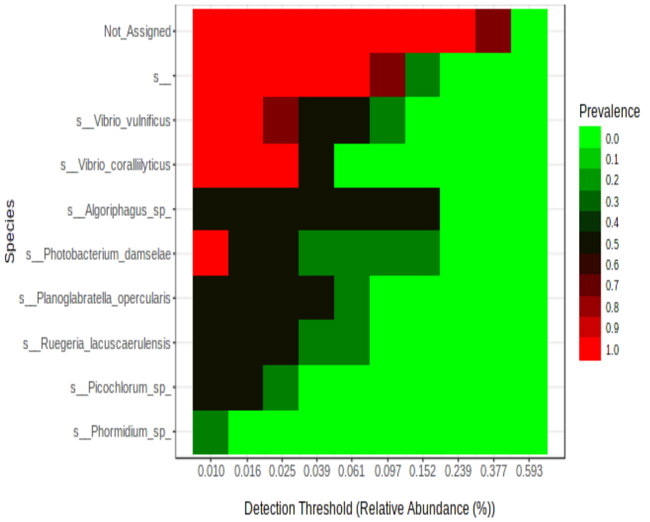

Figure 5. Composition of bacteria at the species level in WFS infected shrimp guts

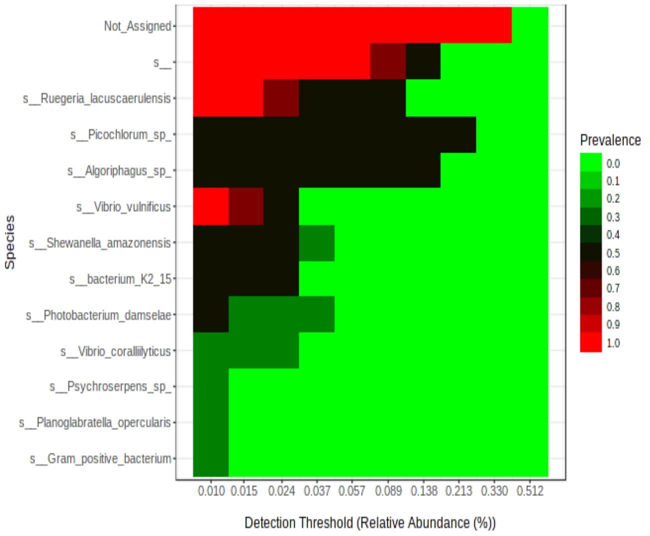

Figure 6. Composition of bacteria at the species level in healthy shrimp guts

Based on the analysis, three species differed significantly in their abundances between the two groups of shrimps evaluated (with WFS and healthy shrimps), in the following order of dominance: *Photobacterium damselae, Vibrio coralliilyticus,* and *Vibrio vulnificus*. *P. damselae* was most abundant in shrimp with WFS. First, bacterial sequences of *P. damselae* in the gut of shrimp with WFS had 23,730 OTUs (Operational Taxonomic Unit), OTUs represent clusters of closely related sequences, often used as proxies for species in microbiome studies. The significance of OTU abundance should be elaborated upon. Higher OTU counts typically indicate greater microbial diversity or dominance of specific taxa, whereas lower OTU counts may suggest reduced diversity or a shift towards pathogenic dominance. Whereas that in the healthy shrimp gut was only 4,694 OTUs. Second, *V. vulnificus* whose total number of bacteria in the WFS and healthy shrimp gut samples was 19,463 and 7,271 OTUs, respectively. Third, *Vibrio coralliilyticus* with a total number of sequences in WFS and healthy shrimp guts was 12,947 and 4,129 OTUs, respectively. Overall, *P. damselae* contributed the most to shrimp abundance, as indicated by the WFS.

Many strains of *Photobacterium damselae* have been identified as significant pathogens (Gouife et al. 2022). This Gram-negative marine bacterium comprises two distinct subspecies: *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* (Moric et al. 2023). The pathogenicity of *P. damselae* is largely attributed to its ability to produce potent cytolysins and exotoxins (von Hoven et al. 2019).

P. damselae, previously known as *Vibrio damsela,* is an important pathogen that poses a serious threat to fish and shellfish aquaculture. It has been reported to cause wound infections and fatal diseases in humans and various marine species (Osorio et al. 2018). Zhou et al. (2024) determined that the LD50 of *Penaeus monodon* as approximately 3×10^5 CFU/g. Given its high abundance, this bacterium is likely to be the primary cause of shrimp diseases. Wang et al. (2020a) identified *P. damselae* as the main bacterial agent responsible for wound infections in tiger prawns (*P. monodon*) and black gills. Rivas et al. (2013) have indicated that this bacterium plays a significant role as the leading pathogen responsible for the mass mortality of cultured tiger shrimp in Taiwan. Additionally, *P. damselae* sp. *piscicida* causes pasteurellosis in fish (Mohamad et al. 2019). According to Gulla et al. (2019), infected fish typically present with symptoms such as darkened skin, reduced appetite, and abnormal swimming behavior, including lingering near the water surface or around waste discharge areas. Externally, wounds may develop, and internally, hemorrhage may be observed in organs such as the liver and kidneys.

V. vulnificus was the second most predominant bacteria. *V. vulnificus* is an opportunistic pathogenic bacterium isolated from various seafood, including fish, shrimp, oysters, and shellfish (Rodgers et al. 2014). In addition to infecting shrimp, *Vibrio* sp. can infect grouper fish and cause acute death in 17-46 hours (Rahardjo et al. 2023) and Asian sea bass (Manchanayake et al. 2023). The development and survival of *V. vulnificus* in its natural habitat are strongly influenced by salinity and water temperature. *Vibrio vulnificus* requires salt for growth. Conditions favorable for the growth of *V. vulnificus* are a salinity range of 5-25 ppt and temperature >20°C.

Vibrio coralliilyticus is the third most predominant bacterial species. *Vibrio coralliilyticus* is a rod-shaped Gram-negative bacterium that is a well-known coral pathogen responsible for tissue lysis, bleaching, and drastic coral reef loss worldwide (Kim et al. 2020). In addition to infecting corals, these bacteria have been shown to infect fish, such as rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)), brine shrimp larvae, and *Artemia* sp.

In 2015, a continuous mass death took place at a Pacific oyster hatchery in Korea, which was confirmed for the pathogen *V. coralliilyticus*. The LD50 of *V. coralliilyticus* against Pacific oysters had similar test results, namely 1.2×10^4 to 4.0×10^4 CFU/mL. Due to its ability to infect several aquatic organisms, *V. coralliilyticus* is considered one of the main pathogens responsible for economic losses, while the LD50 of *V. coralliilyticus* against Eastern oysters for six days ranged from 1.1×10^4 to 3.0×10^4 CFU/mL sea water (Kim et al. 2020).

In this study, two types of *Vibrio* bacteria were found in diseased shrimp gut samples, namely *V. vulnificus* and *V. coralliilyticus*. These results by several previous studies have also reported that *Vibrio* bacteria are often identified in WFS cases, including *V. parahaemolyticus* (green colonies), *V. fluvialis* (yellow colonies), *V. vulnificus* (green colonies), *V. alginolyticus, V. mimicus* (green colonies), *Vibrio cholera* (Saraswati and Wijaya 2019), and *V. anguillarum* (Jayadi et al. 2016). In recent years, WFS has been reported in several Asian regions. The results of this study revealed that the dominant bacteria in shrimp fed a WFS are *V. harveyi* (Anjaini et al. 2018; Sumini and Kusdarwati 2020), which is also thought to be caused by *V. parahaemolyticus* and *V. sinaloensis* (Wang et al. 2020b).

When infected with WFS, there is damage to the cells that make up the hepatopancreatic tubules, which undergo a transformation and then aggregate to become like gregarines or what is commonly known as Aggregated, Transformed Microvilli (ATM). ATM originates from the exfoliation of shrimp hepatopancreatic tubular epithelial cells and accumulates between the hepatopancreas and midgut before being excreted with the midgut. When ATM is severe, it can cause white feces to form on the shrimp (Sriurairatana et al. 2014).

Environmental and management factors affecting disease outbreaks

The emergence of WFS outbreaks as a result of MDS according to Wang et al. (2014) who demonstrated that the cultivation environment greatly influences the composition of the bacterial community in healthy and sick shrimp. The same statement was also put forward by Luan et al. (2023), who found that intestinal bacteria are influenced not only by the host but also by their environment.

In conclusion, the findings revealed a distinct difference in the bacterial composition between healthy shrimp and those fed a WFS. The finding of pathogenic bacteria such as *P. damselae, V. vulnificus,* and *V. coralliilyticus* in WFS-infected shrimp emphasizes their role in disease progression.

In contrast, many *Algoriphagus* sp. bacteria can be found in healthy shrimp that contribute to the maintenance of gut health. This information can be used to develop strategies for disease prevention and management by farmers.

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