Application of Omics Tools to Explore the Health of New Zealand Greenshell™ Mussels

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A thesis submitted to Auckland University of Technology in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

June 2023

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Thesis Abstract

The New Zealand Green-lipped mussel (*Perna canaliculus*) is an important corner stone of aquaculture and a major component of the country's coastal habitat systems. However, unexpected mortalities in farm and wild settings, especially during summer times (summer mortality) present a major ecological and economical challenge. The complex interactions between host, environment, and pathogens during these mortality events are poorly understood and require innovative diagnostic tools. Multi-omics applications are rapidly emerging as powerful tools to accurately and effectively assess the organism's stress and health condition, among others. This approach allows multi-faceted insights into complex biological processes and can provide insights into disease processes as well as the identification of molecular biomarkers for early warning systems. This thesis was designed to provide, for the first time, an integrative and multi-omics approach of the metabolic, protein and microbiome responses of *Perna canaliculus* to a range of stressors, such thermal, pathogenic and nutritional stress.

The bioinformatic integration of metabolomic and proteomic data revealed strong evidence of alterations in energy and immune-related metabolic pathways in mussels suffering from heat induced mortality in a mussel farm. Our resulted revealed indication of oxidative stress in unhealthy mussels as a result of perturbations in glutathione metabolism and protein glutathione S-transferases. In addition, degradation in the cytoskeleton structure and regulation of cilia/flagellum gill tissues of unhealthy mussels may be a contributing factor to undesired changes in gill membrane fluidity, permeability, and lipid composition impairing function. The integrative metabolome and proteome profile data provides new insight into molecular interactions associated with incidences of summer mortality in this species.

The application of microbiomics/microbiome analysis initially involved a baseline characterisation of bacteria and fungi within key wild *Perna canaliculus* tissues (gills, haemolymph, digestive gland, and stomach) using high-throughput amplicon sequencing of 16S rRNA gene and ITS1 region for bacteria and fungi, respectively. The study revealed that different mussel tissue types displayed distinctive bacterial profiles, which were dominated by phyla which reflected a fluid exchange between the circulatory system and surrounding aqueous environment, as well as a highly diverse digestive system microbiota. Along with a distinct pattern in microbiome structure, multiple significant phylum , including Gammaproteobacteria, Campylobacterota, Firmicutes, Cyanobacteria, and Bactroidota were identified in *Perna canaliculus* tissues. Among these biomarkers, Gammaproteobacteria, Bacteroidota and Cyanobacteria were shown to change in relative abundance when mussels were subjected to short term starvation periods in the laboratory. Further microbiome analysis of farmed mussels suffering from heat induced summer mortality revealed alterations in Gammaproteobacteria, Bacteroidota and Campilobacterota in the gill tissue and hepatopancreas tissue. Numerous significant bacterial genus signature was also identified in this thesis. The most interesting genus, *Endozoicomonas*, was found to be the most dominate member of the Phylum Proteobacteria in tissue types, such as gill tissue and haemolymph. Its variation in abundance within mussels exposed to different experimental conditions, suggests that this group may be a good biomarker for mussel condition and fitness. Specifically, changes in the relative abundances of *Endozoicomonas* bacteria were detected in the gill tissues of unhealthy mussels suffering from summer mortality. *Endozoicomonas* relative abundance was also altered in response to seasonal changes, potentially linked to temperature and salinity parameters. Based on these results, it is suggested that future studies focus on *Endozoicomonas* as a potential host health biomarker n *P. canaliculus*.

In conclusion, this thesis has successfully demonstrated the application of multiple omic approaches for the study of Green-lipped mussels, which contributes novel information regarding the animal's physiological and metabolic responses to stressors, such as temperature, pathogen and nutrition.

“From this instant on, vow to stop disappointing yourself. Separate yourself from the mob. Decide to be extraordinary and do what you need to do – now.”
-Epictetus

“We’re all gonna make it brah!”
-Aziz Shavershian

To my most beloved family and friends…

Acknowledgements

This PhD journey has truly been a life-changing experience for me. I am lucky and grateful to have met many amazing people who have shared this journey with me. I would like to take this opportunity to acknowledge the following wonderful people/organisation.

Firstly, my most sincere and deepest gratitude goes to my supervisor, Professor Andrea C. Alfaro, you have been the sources of my motivation and inspiration. Without you, I would not have been able to find the courage to complete this thesis. I am incredibly grateful to have met you, and fortunate to be your student. You have been a mentor, a friend and a mother to me in all the various times throughout my PhD journey. Under your guidance and support, I have grown into the scientist and man I am proud of. I look forward to continuing working with you throughout my career, you will always be an important part of my life.

Thank you to the Ministry for Business, Innovationand Employment for funding throughout my PhD, allowing me to achieve my dream of working with such a wonderful supervisor and university.

I would like to thank all the people who have helped me through all these years to complete my research. First to the postdocs from the Aquaculture Biotechnology Research Group, especially Tim and Emily, thank you so much for your help with the lab work, the science, the statistics and the writing of my project, you guys were always there to offer unconditional help, you are my role models and friends, I have nothing but utmost respect for both of you. Also, thank you to Leonie, for helping me with the mussel project, without you I would not have been able to obtain samples for this thesis. To Stephen Archer at the School of Science, thank you for teaching and sharing your knowledge and experience in the field of microbiome with me, your charisma and patience was a huge reason that I was able to learn this exciting new field of science in a fast manner, you have not only been a mentor but also a friend to me. A special thank you to Kevin Lee, Tim Layt, and Joe Chang from the School of Science for helping me where needed, I am thankful you guys have made my project run smoothly. To my fellow researchers from the Aquaculture Biotechnology Group past and present, including Awanis, Shaneel, Ronnie, Thao, Farhana, Natalia, Martin and Roffi, thank you all for the happy

moments we have shared together, most importantly thank you for being there to help me in times of difficulty.

I am especially grateful to the friends and colleagues who I have had the pleasure of knowing throughout my PhD, who have made these few years more colourful and vibrant. Thank you, Rahul, for being the best friend I could hope for, it was bittersweet I only got to meet you towards the end of my PhD, we shared many great moments and memories, as well as learnt a lot from each other, many of which are life changing, I hope we can still be bros after my time here at AUT. To my best friend Kevin Ma, thank you for being there for me during the good, the bad, and the ugly times of my thesis, it makes me feel at ease knowing that I got a true friend who will have my back, and who will put up with all my complaints and issues and provide help whenever I needed it the most, those video game nights were some of the most fun I had in recent years. To my best friend Louis Yin, you were always there for me, you looked out for my interests and brought the best out of me both mentality and physically, which contributed to the man I am today, thank you for being the brother I never had.

Finally, to my wonderful, dear family, my mother Mei and father Bin, you are the backbone of everything I am striving towards, words cannot express just how grateful I am for all the sacrifices and hardships you endured for me, thank you for tirelessly supporting me at every step of the way, thank you for the love, kindness, and care that you have showered over me, thank you for always believing in me and always guiding me to be a better person.

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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: _____

Date: 15/06/2023

Co-authors contribution

The co-authored literature review and experimental chapters in this thesis have been given a weighting (% time) to produce the completed output. As co-author, I hereby approve and declare that my role in this study, as indicated below, is representative of my actual contribution and I hereby give my consent that this work may be published as part of the PhD thesis of SiMing Li.

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Chapter 3: *Mapping the green-lipped mussel (Perna canaliculus) microbiome: A multi-tissue analysis of bacterial and fungal diversity (Li et. al 2022)*			
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Chapter 1.

General introduction and Literature review

1.1 New Zealand Aquaculture

New Zealand is home to a vast and diverse range of sea life, and is also world renowned for its high-quality seafood production. Taking advantage of New Zealand's sterling water quality and clean environment, the aquaculture industry has expanded exponentially from the 1980s to the current day. Three main species are exported globally to meet rising seafood demands in countries such as China. These include Greenshell™/Green-lipped mussels (*Perna canaliculus*), King salmon (*Oncorhynchus tshawytscha*), and Pacific oysters (*Crassostrea gigas*). Locally, aquaculture is a lifeline for many regional towns, supporting thousands of local families with job opportunities and services. It is apparent that aquaculture in a major and impactful component of New Zealand's economy and culture. With the rapid development of technology and new methods of scientific research, the New Zealand aquaculture industry is also rapidly growing, with the goal of reaching 3 billion dollars in exports by 2035 (FAO 2022).

The Green-lipped Mussel is the most vital aquaculture species in New Zealand (FAO, 2005c, 2005b), with a net export profit of 600 million USD in 2022 (FAO 2022). This mussel species is endemic to New Zealand (FAO, 2005b; "New Zealand Aquaculture Facts 2020," 2020), and it is cultivated mainly in the Marlborough Sounds and Coromandel areas ("New Zealand Aquaculture Facts 2012," 2012).

1.2 Challenges in New Zealand Aquaculture

While New Zealand aquaculture has experienced significant export successes, many hurdles and bottlenecks need to be overcome in order to reach the proposed 3 billion dollars in export income by 2035 (FAO 2022). Indeed, as industry intensifies, diversifies and expands in size and geographic locations, diseases have become an alarming constraint on production. These diseases limit not only the New Zealand aquaculture industry, but also the global industry in terms of production (Hedrick 1996; Subasinghe 2005; Whittington and Chong 2007; Oidtmann et al. 2011). According to the World Organisation for Animal Health, losses of cultured animal due to disease outbreaks are greater than US$3 billion annually in Asia alone (Bernoth 2008). For example, bacterial diseases, particularly infections related to *Vibrio* species, are considered to be a risk to the industry of Greem-lipped mussels. Mortalities and suboptimal product qualities resulting from these pathogenic infections can have devastating consequences on the expanding New Zealand mussel aquaculture industry. These massive mortality events predominate in the summer when shellfish experience thermal stress and potential pathogenic infections from the likes of *Ostreid herpesvirus 1* (OsHV-1) and *Vibrio*, among others, during summer months, often overwhelming their immune systems (Nguyen and Alfaro 2020). For example, recent reports have shown massive marine mussel mortality events in countries, such as Netherlands (Capelle et al. 2021), Turkey (Öndes et al. 2020), France (Lupo et al. 2021), and New Zealand (Nguyen and Alfaro 2020).

1.3 The Greenshell™ mussel

The New Zealand Greenshell™ mussel or Green-lipped mussel (*Perna canaliculus*) is an endemic bivalve commonly found within intertidal and subtidal coastal habitats. Mussel beds provide important ecological functions, such as removing suspended sediment and particulate

organic material, resulting in improved water quality. *P. canaliculus* is also a highly valued species for the New Zealand's growing aquaculture industry, which supports a mussel sector worth over NZ$300 million in export revenues (Aquaculture New Zealand 2017). Although *P. canaliculus* have experienced relatively few health issues compared to other cultured shellfish, there are several pathogens and parasites that affect farmed mussels from time to time (Castinel et al. 2013). *Vibrio* spp., a Gram-negative bacteria associated with numerous infectious diseases in marine bivalves has been reported in *P. canaliculus* (Webb 2008). Digestive epithelial virosis, caused by an unenveloped RNA virus, has been implicated in multiple moderate to severe mortality cases (Jones et al. 1996; Diggles et al. 2002; Renault and Novoa 2004; Renault 2006). Other pathogens, such as funguses, protozoa and platyhelminthes have also been recorded in *P. canaliculus* (Webb 2008; Castinel et al. 2019a). However, with the exception of digestive epithelial virosis (Jones et al. 1996), no major health impacts have been identified in cultured or wild New Zealand mussel populations (Castinel et al. 2013) until recently.

In the past decade, there have been reports of mass mortalities on mussel farms and coastal locations of the North Island of New Zealand during summer months (Nguyen and Alfaro 2020). In addition, there have also been reports of mass mussel spat fall on the beaches between the months of August and the end of December and sometimes a secondary supply in January, February, March, but varies from year to year, and the volume varies enormously (Dunphy et al. 2015). Despite significant industry loses, the reason for these events remain largely unknown. It is believed that these mortalities are potentially associated with thermal stress caused by increasing water temperatures due to climate change (Dunphy et al. 2015). The combination of thermal stress with pathogen loads which appear to proliferate during the summer may lead to physiological 'tipping points' during these events, also referred to as

summer mortality. Given the ecological and economic importance of Green-lipped mussel, the monitor, and maintenance of both wild mussels and domesticated stocks is of vital importance. Currently, host-pathogen interactions, host-microbiome interactions and stress response mechanisms of the Green-lipped mussel during summer mortality are poorly understood. However, in recent years, application of omics such as metabolomics have already been conducted. (2022; Nguyen and Alfaro 2020; Ericson et al. 2022; Azizan et al. 2022).

Although other omics approaches have been employed in the aquaculture field, their application on the Green-lipped mussel appears to be limited, but is likely to increase as the technologies develop (Osuna-jiménez 2010; Rodrigues et al. 2012a; Cerdà and Manchado 2013; Li 2014). Indeed, a literature review by Lokman and Symonds 2014 revealed that although older omics platforms have been utilised plentifully in aquaculture research, there are still considerable avenues for exploration via the application of novel platforms such as metabolomics, epigenomics and metagenomics (Table 1).

To improve product yield of the farmed mussels in New Zealand, spat hatchery, and to strive and flourish in the global market, studies are needed to elucidate health threats and immunological responses at the molecular level for the New Zealand Green-lipped mussel. A comprehensive study of an organism's biology can be effectively achieved by the application of modern biological analytical tools and development of innovative analytical methods. This entails examination of the mussel's genome (genomics), transcriptome (transcriptomics), proteome (proteotomics), metabolome (metabolomics) and microbiomics, among others. The knowledge, extremely lacking in the mussel industry, can be acquired from these technologies can lead to the identification of molecular components that can enable us to understand the interactions among them resulting from (dys)functioning of the system when exposed to various factors. This knowledge can also be utilised to develop biomarkers for diagnostics tools,

disease mitigation strategies, and development of optimal diet. Therefore, results generated from these technologies can measure elements of aquaculture animal's pathophysiology and understand diseases that affect their health and production.

Table 1. Uptake of omics technologies in aquaculture research.

omics technology	Keyword	No. papers
Genomics	genom*	755
Transcriptomics	Transcriptom*	139
Proteomics	proteom*	79
Matabolomics	metabolom*	5
Foodomics	foodom*	1
Nutrigenomics	nutrigenom*	7
Epigenomics	epigenom*	1
Metagenomics	metagenom*	11

The different omics approaches were submitted to Web of Science as abridged search terms, together with 'aquacult*', and the resulting numbers of retrieved items (10 January 2014) listed (Lokman and Symonds 2014).

1.4 Omics Technologies

The utilization of high-throughput sequencing technology has propelled biological sciences into a completely new realm of interrogation and investigation. The knowledge gained from these endeavours has delivered a more comprehensive understanding of biological systems (from genotype to phenotype) at an linear rate (Figure. 1). Complex datasets generated from). High-throughput analysis can then be analysed with bioinformatics tools to understand and infer a variety of molecular- and organism-level processes, as well as providing new opportunities for biological and medical research discoveries. These techniques have been

widely utilized to identify biological biomarkers, understand and model complex biochemical pathways and to study pathophysiological processes. This revolutionary biological study approach is often referred to as ‘omics’, consisting of genomics, transcriptomics, proteomics, and metabolomics, among others. Specifically, genomics studies the structure andfunction of all the genes (genome) in an organism, while transcriptomics studies the mRNA (transcriptome) within a cell or organism. Proteomics studies the proteins (proteome), including their structure and function, within a cell/system/organism, and metabolomics studies the molecules that are intermediary or end products of metabolic reactions known as metabolites (metabolome) (Horgan and Kenny 2011). Collectively these research fields fit within the area of integrative systems biology, which is based on the idea that proteins, via mRNA, and then metabolites are synthesized in a hierarchical manner when genes are activated (Alfaro and Young 2018) (Figure. 2).

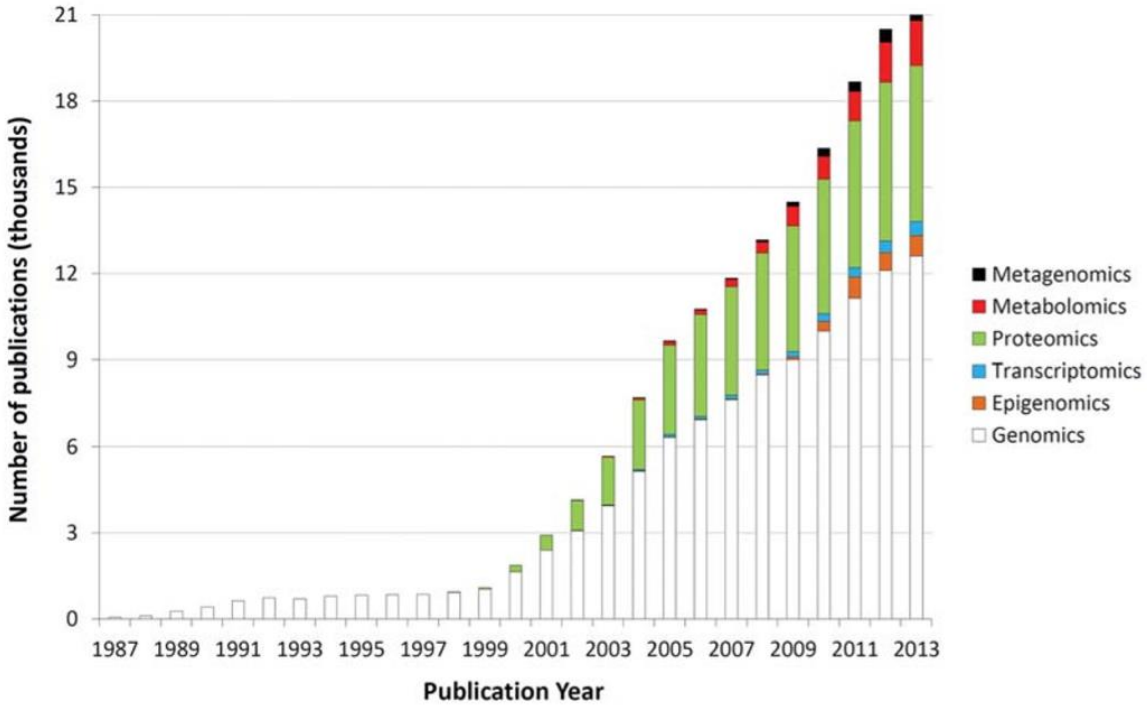


Figure 1. Growing use of omics techniques. There is a rapid increase in the number of studies published in which a range of omics techniques (shown in the legend on the right-hand side) haven been used. Obtain from Barnett et al., 2015 by using a PubMed search (carried out in October 2014) using the terms shown in the legend, and the 'results by year' were then exported from PubMed to generate the figure.

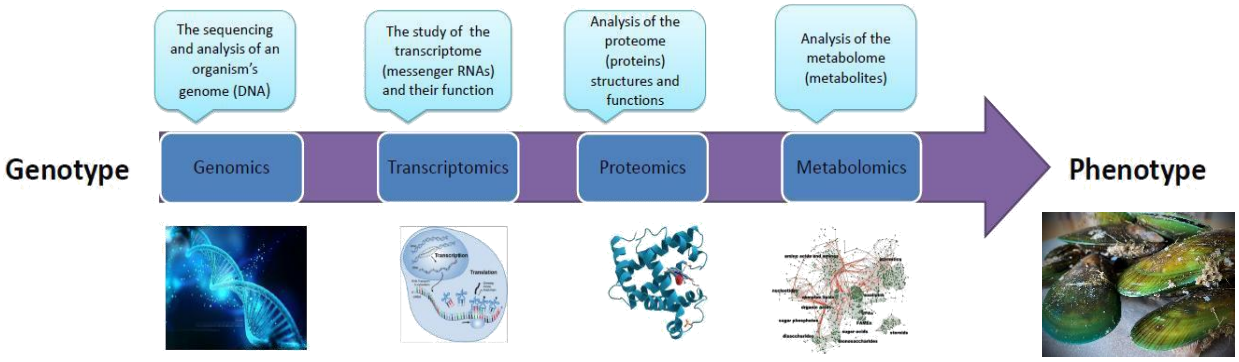


Figure 2. The 'Omics' cascade defining genomics, transcriptomics, proteomics and metabolomics, and depicting their position along the genotype to phenotype continuum.

'Omics' approaches allow for untargeted scientific studies (Ozdemir et al. 2009). This is enabled by the rapid emergence of advanced analytical platforms, statistical methods, and computational tools. An untargeted scientific approach allows for a global analysis of an organism's genome, transcriptome, proteome and metabolome with the ultimate aim to provide a more comprehensive picture of the biological context. The exploratory nature of untargeted approaches has the potential to generate novel hypotheses instead of simply validating a pre-identified hypothesis. At the same time, 'omics' provides the opportunity for unexpected information to be revealed, leading to high innovation and discovery in a very efficient manner (Young and Alfaro 2018). In general, omics studies are also more complex compared to traditional biology and involve multiple steps from molecule identification, raw data analysis to statistical analysis and validation (Horgan and Kenny 2011) .

1.5 Genomics and transcriptomics

Genomics is the study of structure, function, inheritance, and mappingof the genome (complete set of genetic material) of an organism. A major part of genomics is the determination of the

sequence of molecules that make up the genomic deoxyribonucleic acid (DNA) content of an organism. Compared to genetics, which refers to the study of individual genes and their part in inheritance, genomics aims at the collective characterization and quantification of genes, which are directly related to the production of proteins with the assistance of enzymes and messenger molecules (mRNA). Proteins in turn provide vital information on the structural make up of organism's tissues and organs, as well as regulating chemical reactions.

Genomics was the earliest form of omics study with the highest number of publications recorded at 2014 (Figure. 2), with application in many scientific fields, such as medicine, biotechnology and conservation genomics.

In the field of aquaculture, genetics is an extremely useful resource. For example, incorporation of genetic marker information can also be a useful asset to optimize genetic diversity and future genetic gain when establishing base populations for breeding programmes (Fernández et al. 2014). Furthermore, these genomic tools can be applied to investigate putative genomic signatures of selection during the domestication process of farmed fish species, thus potentially identifying genomic regions underlying variation in relevant phenotypes in wild and domestic fish populations (López et al. 2015a).

In New Zealand, the ability to improve aquaculture stock performance using genetic technologies has been established for fish and shellfish in New Zealand (Webb et al. 2007). For example, the bivalves, *Perna canaliculus*, *Spisula aequilatera*, *Pecten novaezelandiae*, *Austrovenus stutchburyi*, *Crassostrea gigas*, *Ostrea chilensis*, *Dosinia anus*, *Mactra discors*, *Paphies subtriangulata* and the gastropod *Haliotis iris* were surveyed in order to develop suitable primers for the PCR amplification of bivalve and viral DNA from OsHV -1. The study successfully established primers capable of detecting the presence of OsHV-1 via PCR (Webb

et al. 2007). Other applications of genomics involves the whole-genome assembly of farmed shellfish, such as *Mytilus coruscus* (Li et al. 2020a). Whole-genome assembly enables accurate reference genome of the targeted species and provides an essential resource with the advantage of enabling the genome-scale selective breeding. More importantly, it can also be used to assist in deciphering the speciation and local adaptation of the species in question.

Transcriptomics is a technique used to study an organism's transcriptome, the sum of all of its RNA transcripts including mRNA, rRNA, tRNA, and other non-coding RNA transcribed in one cell or population of cells (Wang et al. 2009). Here, mRNA serves as a transient intermediary molecule in the information network, whilst non-coding RNAs perform additional diverse functions (Lowe et al. 2017). Transcriptomic technologies provide a broad account of which cellular processes are active and which are dormant. Therefore, measuring the gene expression of an organism in different tissues or conditions, or at different times, yields information on how genes are regulated and subsequently reveal details of an organism's biology, condition or disease (Lowe et al. 2017). Transcriptomics enables the identification of genes and pathways that respond to and counteract biotic and abiotic environmental stresses (Batista et al. 2017; Balan et al. 2018). Another useful application of transcriptomics is gene function identification and functions that are responsible for certain phenotypes (Adams 2008).

Trancriptomic approaches can have notable applicability to improve aquaculture profitability (Li 2014). For example, a previous study successfully utilised RNA-Seq technology to identify coding SNPs that were likely to affect protein bioactivity and/or function, when comparing gene expression profiles in muscle in fast-and slow-growing rainbow trout (Salem et al. 2012). Accordingly, functional genetic differences could be identified to explain growth differences that can assist in selection programmes. Likewise, expression profiles disparities of stress- or immune-related genes may assist in the selection of fish stocks for resistance to stresses and

diseases (Sánchez et al. 2011; Gao et al. 2012). In addition, differences in gene expression between gonads of male and female fish may prove valuable for sexing, as proposed on the basis of transcriptome information from Adriatic sturgeon, *Acipenser naccari* (Vidotto et al. 2013). Other applications of RNA-Seq have centred around ovarian lipid physiology; thus, RNA-Seq has indicated that the expression of a suite of lipolytic and lipogenic genes in the eel ovary is affected by the steroid hormone 11-ketotestosterone (Vidotto et al. 2013).

1.6 Proteomics

Proteomics is the study of proteins, particularly their composition, structures, functions, and interactions that direct the activities of cells (Chandrasekhar et al. 2014a). The proteome, therefore, is the complete set of proteins produced by the genome at any one time. Cells of a particular species contain the same genome irrespective of cell types, developmental stage or environmental conditions. The proteome, on the other hand, varies considerably due to diverse patterns of gene expression and different patterns of protein post-translational modification, such as phosphorylation, ubiquitination, and methylation. Most importantly, the proteome is constantly changing through its biochemical interactions with the genome and the environment (Abhilash 2009). Therefore, the proteome is very diverse and cannot be fully mapped by gene expression analysis alone, making proteomics an advantageous tool for characterizing cells and tissues of interest (Abhilash 2009).

In the field of aquaculture, proteomics has been applied to study many factors, such as welfare, safety, nutrition, and diseases, which are directly responsible for the end-product quality (Rodrigues et al. 2018). Although proteomics application on aquaculture topics is limited when compared to uptake of genomics and transcriptomics (Table 1), uptake may be accelerating. For example, proteomics approaches based on 2-dimentional gel electrophoresis or selected tandem mass spectrometry ion monitoring (SMIM) were used as complementary authentication

methodologies for the identification and authentication of commercially relevant shrimp species (Ortea et al. 2009; Pascoal et al. 2011). Another study utilised quantitative proteomics to elucidate the species-specific molecular responses to bacterial infection (Chaikeeratisak et al. 2012). The study utilised a gel-based quantitative analysis of differentially expressed proteins from the lymphoid organ of the shrimp *P. monodon* after *Vibrio harveyi* infection was reported. In New Zealand, a collaborative study (YY Kohn, T Kleffmann, S Nakagawa, M Lagisz, PM Lokman, University of Otago, Dunedin; and JE Symonds, NIWA, Ruakaka) adopted proteomics approaches to understand in what way 'good' and 'bad' newly fertilised eggs of zebrafih differed at the protein level. Even though the quality scores of the eight-cell embryos that were analysed were not dramatically different, this approach succeeded in identifying differences between (replicated) samples.

1.7 Metabolomics

Metabolomics is the analysis of biochemical intermediates known as metabolites (small molecules <1500 Da) (Muthubharathi et al. 2021). This relatively new field has received considerable attention in the past decade and has been considered to be one of the more powerful 'omics' in aquaculture application (Viant 2007). herefore, by profiling metabolites, we can capture a physiological snapshot of the metabolic state of an organism at a given time. This allows scientists to study the pathophysiological interaction between gene and protein downstream products and environmental factors and discover potential biomarkers in an organism that respond to various stimuli.

In the field of aquaculture, application of metabolomics has been shown to benefit product quality analysis of refrigerated Bogue (Boops boops) (Ciampa et al. 2012) and New Zealand surf clams (*Crassula aequilatera*) (Alfaro et al. 2019a) in response to different storage conditions. Another application highlights the potential use of metabolomics analyses to

identify altered metabolite fingerprints in the shrimp (*Litopenaeus vannamei*) from response to super-intensive farming. The study uncovered valuable metabolites for diagnostic purposes in the context of health monitoring (Schock et al. 2013). The metabolomic approach has also been applied to evaluate immune stimulatory products for whiteleg Shrimp (*Penaeus vannamei*), revealing alterations in biochemical pathway disturbance in energy metabolism and increases in key metabolites, such as itaconic and lactic acid (Alfaro et al. 2022). Aquaculture biotechnology research in NZ has made significant strides in utilising metabolomic approaches on research that directly benefits the NZ aquaculture industry. For example, metabolomics has been used to study the immune responses of Greem-lipped mussels (infected with pathogenic *Vibrio* sp.) (Nguyen et al. 2018b). The study uncovered sets of up-regulated and down-regulated metabolites in additional to perturbations of the host innate immune system following infection, including oxidative stress, inflammation and disruption of the TCA cycle, changes in amino acid metabolism and protein synthesis. Another metabolomics study by Young and Alfaro (2015) identified candidate biomarkers for quality assessment of hatchery-reared mussel larvae via GC/MS-based metabolomics. The study identified four metabolite–metabolite ratios involving levels of succinate, glycine, alanine, pyroglutamate and myristic acid that are involved in energy metabolism, osmotic regulation, immune function and cell–cell communication of the mussel larvae. These metabolites were proposed by the study to be candidate biomarkers for assessing mussel larval quality (Young et al. 2015). Furthermore, metabolomics was adapted to test multi-strain probiotics enhancement of immune responsiveness and alterations of metabolic profiles in the New Zealand black-footed abalone (*Haliotis iris*) (Grandiosa et al. 2018). Finally, haematological immune response of Chinook salmon (*Oncorhynchus tshawytscha*) to short-term poly (I:C) challenge was also examined via metabolomics (Lulijwa et al. 2020). Findings uncovered significantly altered biochemistry profile of 25 metabolites involved in the branched-chain amino acid/glutathione,

transsulphuration pathways and down-regulation of glycolytic and energy metabolism pathways. Alongside marine animals, untargeted GC-MS metabolomics have also been adapted to analyse halophytes, such as the New Zealand mangroves (*Avicennia marina*) subjected under multi-factorial abiotic stress conditions (Ravi et al. 2020). This innovative study successfully uncovered the presence of stress-protective phenolic compounds (syringic and sinapic acids) not previously reported in mangroves.

1.8 Microbiomics

Microbiomics is a rapidly growing area of scientific research wherein all the microorganisms of a given community are explored and analysed within environments, such as soil or water, a particular organ of an animal, such as gut or skin or a specific organism. Investigation of microbial communities can potentially lead to discovery of novel organism with exciting properties, such as production of natural products with antimicrobial properties, as well as understanding host-microbiome interactions (Santos et al. 2022). This information can potentially be utilised to develop medicine, nutritional optimisation, and diagnostic tools.

At its principle, microbiomics aims to investigate the composition of a microbial community and how it changes over time, or when said community is subjected to different level of alerting factors and/or stresses, such as increase in temperature or salinity. This is achieved via the utilization of 16sRNA amplification (via polymerase chain reactions) on extracted DNA samples. The resulting amplified DNA fragments are then sequenced and matched against sequence databases to identify the microorganisms present. Similar to other omics, this captures a snapshot of the communities present in a sample at any given time. Subsequently, these community structures can be used to conduct comparative analyses in samples or to

identify perturbations of species of bacteria within a sample when subjected to different conditions.

Microbiomics is a very suitable tool to provide information that can be applied in further research into host-microbe interactions as it plays a key role in maintaining aquatic animal health and organ-level functioning. Indeed, marine microbiota dynamically interacts with and/or modified by surroundings environment, such as temperature, nutrients, salinity and oxygen levels. Marine microbiota also have well established roles in pathogen exclusion and host immunity, including systemic and mucosal innate and adaptive immune responses and development of the immune system (Cui et al. 2019; Sehnal et al. 2021). For example, Meres et al. (2012) studied epizootic shell disease of the American lobster (*Homarus americanus*) and found that among the 170 bacterial taxa that were identified in the exoskeleton microbiota, 58 were helpful in discriminating diseased and healthy states, although no single causative agent was identified. The authors concluded that epizootic shell disease is caused by a dysbiotic shift in the exoskeleton microbial community, and may be the result of stress induced by environmental factors that cause opportunistic bacterial invasion of the carapace (Meres et al. 2012). Finally, Lokmer and Wegner (2015) studied bacterial diversity associated with the gill and gut of the invasive Pacific oyster *Crassotrea gigas* under non-stressed and thermally stressed conditions. The author discovered that the microbial dynamics and composition of communities in healthy animals (including infection survivors) were significantly affected by temperature and temperature stress, but not by infection.

1.9 Bioinformatics in Omics Analyses

High throughput analytical platforms must be paired with specialised analysis pipelines because large numbers of sets of genes, gene expression, metabolites, or proteins must be profiled in a single procedure or a combination of procedures. Computational omics analysis (i.e., the discipline now known as bioinformatics) is therefore a key requirement to explore the vast amounts of data generated (Schneider and Orchard 2011).

In classic biological studies, few pre-determined null-hypotheses are evaluated. However, 'omics' studies require large datasets and thousands of hypotheses need to be simultaneously tested, each based on very few independent replicates. As a result, bioinformatic analyses in the omics era necessitate a new standard in the design of omics experiments: the p>n paradigm. Under this new paradigm, the number of independent subjects (n = samples) is much smaller than the number of variables (p = number of genes, metabolites or proteins in an expression profile) that are analysed (Kirpich et al. 2018). The massive sample sizes and high dimensionality introduces unique computational and statistical challenges, including scalability and storage bottleneck, noise accumulation, spurious correlation as well as measurement error (Fan et al. 2014). However, while these big datasets are very heterozygous in nature, they hold great promise for discovering patterns, correlations and changes. Simultaneous consideration of many hypotheses, each prone to a decision error, require powerful mathematical adjustments for this multiple testing situation (Dunkler et al. 2011). From a computational point of view, to address the complexity of these data, knowledge discovery – the process of automatically searching large volumes of data for patterns – is a crucial step. The process of bioinformatics analysis in omics studies usually includes: (1) sample analysis of genes, mRNA, proteins, or metabolites via respective analytical platforms, (2) data processing, molecule (e.g. protein or metabolite) identification and quantification, (2) basic statistical data analysis (fold change, t-test, correlation analysis etc, (3) pathway analysis

and network based analysis and (4) data modelling in a system wide context (Schneider and Orchard 2011).

1.10 Omics Integration

The overview of individual omics technologies has highlighted the opportunities their use can give when aiming to identify markers which reflect or predict genotypes or phenotypes. Indeed, a specific technology may go a long way in meeting this aim. However, complex traits can often benefit from complementary omics approaches, an emerging area of biology known as 'systems biology'. In doing so, the genes, proteins and metabolites, etc. that contribute to complex traits are not merely identified, but their interaction, and the molecular pathways leading to the expression of these traits, can be more readily deciphered (Lokman and Symonds 2014), in turn generating a more comprehensive understanding of the link between phenotype and genotype. For example, liver, blood plasma, and white muscle samples of Chinook salmon (*Oncorhynchus tshawytscha*) were used to study feed efficiency via a combination of metabolomics and proteomics integrative analyses (Esmaeili et al. 2023). The omics integration analysis of this study revealed connection of metabolites related to lipids and amino acids with the catabolism of proteins in feed inefficient salmon.

To fully realise the omics potential, the development of bioinformatics expertise and integrated omics analysis approaches will be required to incorporate all available datasets into a single analysis framework. The integration of experimental data from multiple omics platforms is an emerging approach and is critically required for full application of omics studies. Integration of multiple omics data aims to help identify latent biological relationships that may become evident only through holistic analyses, which integrate measurements across multiple biochemical domains. Integration of large heterogeneous datasets collected from multiple

omics studies is a major challenge and fast becoming the main developmental point of integrative systems biology in the immediate future (Gomez-Cabrero et al. 2014). Therefore, as the omics fields grow in scope and complexity, so does the need for development of more sophisticated data analyses and bioinformatics methods/tools (Boccard and Rudaz 2014).

Despite, the accumulating number of integrative research, there are fewer such integrated studies in aquaculture. To list a few, integrated analysis of transcriptomics and metabolomics were used to identify whether the nutritional value of fish fillet related to fish maturation or fish age in Blunt Snout Bream (*Megalobrama amblycephala*) (López et al. 2015b). Integrated metabolomics and transcriptomic analysis were also used to examine brain energy metabolism in the male Oriental river prawn (*Macrobrachium nipponense*) in response to hypoxia and reoxygenation (Wei et al. 2018). Applications of integrative omics were used to understand fish immunity (Ye et al. 2018). Among the few multiple omics studies, one study examined the proteomic and metabolomics responses of Pacific oyster to elevated pCO_2 exposure separately (Wei et al. 2015). Another combined flatfish research project by “GenomaEspaña” and “Genome Canada” titled "Pleurogene" has been attempting to develop new technology to assess gene and protein expression during the reproduction and breeding of two flatfish, Senegalese sole and Atlantic halibut (Rodrigues et al. 2012a). The project also includes the development of E-mold, an integrative bioinformatics platform for genomic, proteomic and morphological information from flatfish. Although omics have been globally employed in the aquaculture field, their application is still few in members. This is likely to increase as the technological platform and bioinformatics develop (Prieto-Alamo et al. 2012; Rodrigues et al. 2012b; Cerdà and Manchado 2013; Chao Li 2014). To our knowledge, the application of integrative omics tools and platforms have huge potential to be extensively utilised into research that can benefit in New Zealand aquaculture industry.

As mentioned previously, integrative omics has the potential to generate simultaneous hypotheses from a single dataset. In the context of the New Zealand Green-lipped mussel (*Perna canaliculus*). These hypotheses could involves comprehensively discovering what molecular biomarkers at the metabolome, proteome and microbiome level, can potentially be indicative of mussels suffering from stressful events such as summer mortality. In addition, can these significant biomarkers be correlated via bioinformatic tools to elucidate potential altering molecular mechanisms as a result of substantial stress to the animal. Finally, are there perturbations in any molecular pathways that are a result of stressed animals due to environmental factors. With the information gathered by this thesis, diagnostic and intervention tools could be developed to monitor mussel and spat health as well as mitigate any potential outbreaks. There are also potentials to develop medicine and diet optimisations to further promote healthier mussel growth and development.

1.11 Research objectives and thesis structure

This research is based on 4 data chapters which aims to apply and integrate of multiple omics tools, such as metabolomics, proteomics and microbiomics on multiple tissues of the New Zealand Green-lipped mussel (*Perna canaliculus*) to explore underlying pathophysiological mechanisms, explore bacterial species associations, identify the microbiome differences among the groups, discover key patterns in microbiome alterations and host-microbiome interactions when subjected to various stresses, detrimental events as well as other seasonal time-points that are indicative of the effect of summer mortality. Knowledge gained from these chapters can potentially lead to the elucidation of stress response mechanisms and susceptibility or resilience of the Green-lipped mussel during the summer mortality in order to develop biomarkers, probiotics, mitigation strategies, and guide management decisions.

1.11.1 Chapter 1

This chapter introduce the thesis and reviews the literature surrounding the New Zealand aquaculture industry, the species *P. canaliculus* and its importance to said industry. Threats to the *P. canaliculus* and the industry include mass mortalities during the summer months on mussel farms and in natural habitats. This chapter then highlights the importance of exploring and understanding what is happening to mussels during these mortality events at a molecular level and microbiota, and follows on by reviewing omics technologies, its applications and potentials to study the underlying pathophysiological mechanism and host-microbiome interactions of *P. canaliculus* suffering from poor health as a result of changing environments and rising temperature in the summer months. Finally, this chapter presents the structure and aims of this thesis.

1.11.2 Chapter 2

This chapter addresses stress mechanisms and immunological responses occurring in *P. canaliculus* on a protein and metabolic level during summer months when potential pathogenic outbreaks and mortality threatens the industry. *P. canaliculus* were collected during a mortality event at a commercial aquaculture farm in Firth of Thames, New Zealand. Gill tissues from healthy and unhealthy mussels were excised and processed for metabolomic (GC-MS) and label-free proteomic (LC-MS) profiling. Univariate analyses were conducted separately on each data layer, with data being integrated via sparse multiple discriminative canonical correlation analysis. Pathway enrichment analysis was used to probe coordinated changes in functionally related metabolite sets.

1.11.3 Chapter 3

This chapter focuses on the microbiome community structure, including microbes and fungi of *P. canaliculus*. We conducted the first baseline characterization of bacteria and fungi within key host tissues (gills, haemolymph, digestive gland, and stomach) using high-throughput amplicon sequencing of 16S rRNA gene targeting the V3 – V4 region and ITS1 region for bacteria and fungi, respectively.

1.11.4 Chapter 4

This chapter investigates host-microbiome relationships occurring in the gut when *Perna canaliculus* experiences significant variations in food quantity and quality in coastal areas. Host gut microbiomes play an important role in animal health and resilience to conditions, such as malnutrition and starvation. Prolonged starvation may be a contributory factor towards incidences of mass mortalities in farmed mussel populations, resulting in highly variable production costs and unreliable market supplies. Here, we examine the gut microbiota of *P. canaliculus* in response to starvation and subsequent re-feeding using high-throughput amplicon sequencing of the 16S rRNA gene targeting the V3 – V4 region.

1.11.5 Chapter 5

This chapter focuses on host-microbiome relationships occurring in the gill tissue and hepatopancreases tissue of the *Perna canaliculus* when it experiences thermal stress and pathogen overload during the warmer months of summer, hence leading to summer mortality events. Here, we examine the gill tissue microbiota of healthy and unhealthy *P. canaliculus* samples collected after a mass summer mortality event using high-throughput amplicon sequencing of the 16S rRNA gene targeting the V3 – V4 region.

1.11.6 Chapter 6

This chapter investigates how seasons affect the microbiome profile and structure in *Perna canaliculus.* This chapter describes the alterations in microbiome signatures of the *Perna canaliculus* gill tissue microbiomes. We collected time-series data, including seawater parameters such as pH, temperature, chlorophyll *a* and salinity during the 3-year period in the Firth of Thames with a deployed SeaFET ISFET pH sensor and a MicroCAT salinity, temperature, pressure and oxygen sensor in a highly impacted coastal embayment. At the same time, we collected samples during every month of the season during the same 3-year time span and analysed the gill tissue microbiota using high-throughput amplicon sequencing of the 16S rRNA gene, targeting the V3 – V4 region.

1.11.7 Chapter 7

The General Discussion summarizes the findings of this research, including a general description of the metabolic and proteomics responses of *Perna canaliculus* to summer mortality, microbiome structure and microbiome changes that underpins mussels as they respond to short-term and long-term changes in environment. It also summarises knowledge gaps, challenges and key areas of new research essential to further understand pathophysiological mechanism of the *Perna canaliculus.* Moreover, it summarizes how the NZ aquaculture industry could use the information provided form the findings of this thesis to increase productivity.

Chapter 2.

An Integrated Omics Approach to Investigate Summer Mortality of New Zealand Greenshell™ Mussels

This work has been published as:

Li S, Alfaro AC, Nguyen TV, Young T, Lulijwa R. An integrated omics approach to investigate summer mortality of New Zealand Greenshell™ mussels. *Metabolomics*. 2020;16(9):100. Published 2020 Sep 11. doi:10.1007/s11306-020-01722-x

2.1 Introduction

The New Zealand Greenshell™ mussel (*Perna canaliculus*) industry is worth over $300 million in export revenues (Aquaculture New Zealand 2017). This is a well-established industry with sophisticated farming technologies that provide premium mussels to global markets. Although *P. canaliculus* have experienced relatively few health issues compared to other cultured shellfish, there are several pathogens and parasites that affect farmed mussels from time to time. *Vibrio* spp., a Gram-negative bacteria associated with numerous infectious diseases in marine bivalves has been reported in *P. canaliculus* (Webb 2008). Digestive epithelial virosis, caused by an unenveloped RNA virus, has been implicated in multiple moderate to severe mortality cases (Jones et al. 1996; Diggles et al. 2002; Renault and Novoa 2004; Renault 2006). Other pathogens, such as funguses, protozoa and platyhelminthes have also been recorded in *P. canaliculus* (Webb 2008; Castinel et al. 2019a). However, with the exception of digestive epithelial virosis (Jones et al. 1996), no major health impacts have been identified in cultured or wild New Zealand mussel populations (Castinel et al. 2013) until recently.

In the decade, there have been multiple reports of mass mortalities on mussel farms of the North Island of New Zealand during summer months. Despite significant industry loses, the reason for these events remains unknown. However, it is believed that these mortalities are associated with thermal stress caused by increasing water temperatures due to climate change (Dunphy et al. 2015). The combination of thermal stress with pathogen loads which appear to proliferate during the summer may lead to physiological 'tipping points' during these events. Currently, host-pathogen interactions and stress response mechanisms that occur during summer mortality are poorly understood. Such knowledge is needed to develop mitigation strategies, and guide management decisions during these events. Due to the complexity of this multi-factor health issue, an integrated omics approach may provide detailed information needed to help elucidate stress response mechanisms and susceptibility or resilience to summer mortality.

'Omics' is a relatively new approach that aims to collectively characterize and quantify biological molecules, such as metabolites, proteins, RNA and DNA to understand their structures, functions, and dynamic interactions within an organism. Among these omics, metabolomics focuses on low weight molecules (metabolites). Since metabolites are the end-products of cellular regulatory processes and are highly sensitive to environmental change, metabolomics captures a snapshot of the organism's physiological state at a given time. In recent years, a series of metabolomics studies conducted on *P. canaliculus* have significantly improved our understanding of shellfish health and immune responses. Such studies include copper-induced immunomodulation (Nguyen et al. 2018a), identification of itaconic acid as a biomarker of *Vibrio* infection (Nguyen et al. 2019a; Nguyen and Alfaro 2019b), and metabolic responses to *Vibrio* sp. infection (Nguyen et al. 2018b; Nguyen et al. 2018c; Nguyen et al. 2019c). These studies provide a solid foundation for holistic downstream 'omics' investigations of underlying pathogen invasion and stress response mechanisms as well as

molecular regulatory processes. Proteomics is the extensive study of proteins, including their composition, structures, functions, and interactions that direct the activities of cells (Chandrasekhar et al. 2014b). This approach enables the detection of functional and correlational alterations of an organism's proteome in response to various environmental and pathogenic factors. Proteomics approaches have been extensively applied to various marine mussels, including the response of Mediterranean blue mussels (*Mytilus galloprovincialis)* to acute heat stress (Tomanek and Zuzow 2010), the effect of marine pollutants on blue mussels (Mytilus edulis) (Apraiz et al. 2006) and the identification of azaspiracid toxin-binding proteins in blue mussels (Nzoughet et al. 2009).

While metabolomics has been extensively used to investigate P. canaliculus metabolic processes, this is the first application of proteomics in this species. Furthermore, the aim of this study is to integrate metabolomics and proteomics approaches to investigate stress mechanisms (e.g. thermal, pathogenic) in New Zealand *P. canaliculus* during a summer mortality event.

2.2 Methods

2.2.1 Sample Collection

A mussel summer mortality event was investigated at a farm in Kaiaua, Firth of Thames, North Island, New Zealand during April 2018. This mortality event coincided with an unprecedented, coupled ocean-atmosphere heatwave for the country, with record sea surface temperatures being recorded in the region (Salinger et al. 2019). High mortalities had been observed on some dropper lines by farmers, while other lines appeared to have 'healthy' mussels. Twenty-five mussels were collected from 'healthy' lines and 25 mussels were collected from 'unhealthy' lines (adults ca. 2 yrs old; weight 59.96 ± 8.56 g; shell length 9.4 ± 0.48 cm). Immediately after collection, mussels from each group were transported in separate cool and moist polyboxes to the Aquaculture Laboratory (Auckland University of Technology, Auckland, New Zealand; 3-hour transport time). Upon arrival, mussels were acclimated in two separate tanks with re-

circulating seawater at 17C° for 24 hours to recover from the transport stress. Mussels were examined for their behavioural responses to gentle manipulation. Mussels from lines experiencing high mortality were much slower to respond (shell closure) compared to the other group. Internal examination of a randomly selected subgroup revealed clear differences between healthy and unhealthy mussels. Most notably, unhealthy mussels had abnormal looking tissues and internal organs had extensive mucus (Figure. 3A&B). In addition, haemolymph samples were taken from another set of mussels from each group, placed on slides, Giemsa stained and microscopically examined. Numerous rod-shaped bacilli were observed in the haemolymph of unhealthy mussels, while no or very few bacteria were found in samples from the healthy mussels (Figure. 3C&D).

Six randomly selected mussels from each group were cleaned externally with fresh seawater. The gills of these mussels were excised and placed in 2 mL cryovials (BioStor™), then immediately snap-frozen in liquid nitrogen and stored at –80°C until further analyses.

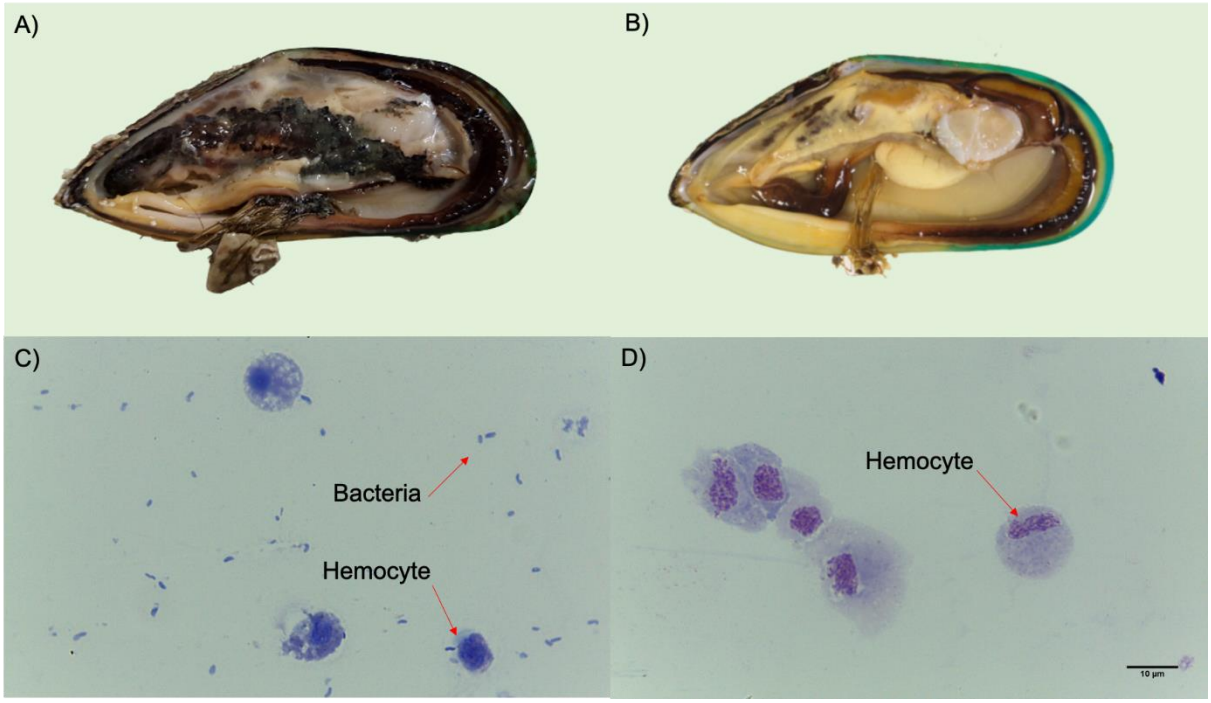


Figure 3. Internal organs of unhealthy mussels (A) and healthy mussels (B), and Giemsa slide staining of haemolymph samples from unhealthy mussels (C) and healthy mussels (D).

2.2.2 GC-MS Based Metabolomics

Gill tissues were lyophilised and ground under liquid nitrogen using a mortar and pestle. Metabolites were extracted from samples (5 mg) in cold methanol–water solution (MeOH:H_2O), according to Villas-Bôas et al. (2011) with minor modifications (Nguyen et al. 2018e). Briefly, dried samples were mixed with 20 µL of d4-alanine (10 mM) internal standard and extracted using 500 µL of cold (−20 °C) 50% MeOH:H_2O solution. The mixture was vortexed vigorously for 1 min, re-frozen on dry ice then thawed again. Extracts were cold (−6 °C) centrifuged at 2500 rpm for 10 min (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany), and supernatants were collected in 2 mL plastic vials placed on dry ice. A second extraction was performed using 500 µL of cold 80% MeOH:H_2O. Matched supernatants were combined and dried in a SpeedVac Concentrator (Savant™ SC250EXP; Thermo Scientific, Asheville, NC, USA) with a Savant™ RVT5105 Refrigerated Vapor Trap (Savant™ RVT5105) for 4 h (0°C, vacuum ramp 3, 42 torr/minute).

Extracted metabolites were derivatized via methyl chloroformate (MCF) alkylation, according to (Villas-Bôas et al. 2011), with minor modifications. Briefly, dried samples were re-suspended in 400 µL of 1 M sodium hydroxide and quantitatively transferred to Kimble™ silanized borosilicate glass tubes (12 × 75 mm) (ThermoFisher, Auckland, New Zealand) containing 334 µL of methanol and 68 µL of pyridine. 40 µL of MCF reagent Sigma-Aldrich, St. Louis, MO, USA) was added and the mixture vortexed for 30 sec. Another 40 µL of MCF was added, followed by vortexing for 30 sec. To separate the MCF derivatives from the mixture, 400 µL of chloroform (Merck, Darmstadt, Germany) was added, vortexed for 10 sec, then followed by addition of 800 µL of 50mM sodium bicarbonate (Merck, Darmstadt, Germany) solution and vortexed for a further 10 sec. The mixture was centrifuged at 2500 rpm on an Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) for 6 min. The upper aqueous layer was discarded and a small amount of anhydrous sodium sulphate (BDH Chemicals, Poole, UK) was added to remove residual H_2O. The chloroform phase containing the MCF derivatives was transferred to 2 mL amber CG glass vials fitted with inserts (Sigma-Aldrich, St. Louis, MO, USA).

MCF derivatives were injected into a GC-MS system (Agilent GC7890B coupled to a MSD5977A with an Agilent autosampler [Agilent Technologies, USA], with a quadrupole mass selective detector [EI] operated at 70 eV). The system was equipped with a ZB-1701 GC capillary column (30 m× 250 µm id × 0.15 µm with a 5 m guard column) (Phenomenex, Torrance, CA, USA). The instrumental setup parameters were conducted according to Smart et al. (2010) (Smart et al. 2010). Samples (1 µL) were injected under pulsed splitless mode with the injector temperature set to 260°C. The helium gas flow through the GC-column was set at a constant flow of 1 mL min^{-1}. The GC-oven temperature was initially held at 45°C for 2 min, and then raised with a gradient of 9°C min^{-1} to 180°C. After 5 min, the temperature was increased at 40°C min^{-1} to 220°C. After a further 5 min, the temperature was increased at 40°C

min^{-1} to 240°C and held for 11.5 min. Finally, the temperature was increased at 40°C min^{-1} until it reached 280°C, where it was held for a further 2 min. The interface temperature was set to 250°C, the source was set at 230°C and the quadrupole temperature was set at 150°C. The mass spectrometer was operated in scan mode (starting after 6 min; mass range 38–650 amu at 1.47 scans sec^{-1}). Identification of compounds was carried out using mass spectra acquired in scan mode from 38 to 550 amu, with detection threshold of 100 ion counts. A derivatized sample blank containing the internal standard, a derivatized standard amino acid mix, a non-derivatized standard alkane mix (C10–C40) and a sample of pure chloroform solvent were also injected to test for presence of contaminants and ensure reproducibility of GC-MS measurements. Alkane samples were used to check Kovats retention index and create the calibration file.

Deconvolution and identification of GC-MS data were performed using AMDIS (Automated Mass Spectral Deconvolution and Identification System) software (version2.66) (National Institute of Standards and Technology, USA). Subsequently, R (version 3.3.1) and inhouse MassOmics package (Aggio et al. 2011) were used to produce a summary report and to integrate peaks. Peak height data were manually checked with ChemStation software (Agilent Technologies) and AMDIS for the presence of contaminants. Repeats (based on ID number, match factor and retention time) and aberrant records were removed. Data were finally normalized by the peak intensity of the internal standard and by sample biomass.

2.2.3 Label-Free Proteomics Analysis (SWATH-MS)

Approximately 5 mg of dried powdered sample was sonicated on ice in 150 µL urea/thiourea/dithiothreitol (DTT) buffer (7 M urea, 2 M Thiourea, 10 mM DTT in 50 mM ammonium bicarbonate) using a Soniprep 150 sonicator (MSE, UK) for 4 x 30 sec at approximately 0-15 microns. Disulphides were reduced via incubation at 56°C in a Discover chilled microwave (CEM Corp, Matthews, SC, USA) at 30 W power. Cysteines were

alkylated by addition of iodoacetamide (IAM) to 50 mM final concentration and incubated in the dark at room temperature for 30 min, followed by the addition of 3 µL 1M DTT to quench residual IAM. Total protein content for each sample was quantified via EZQ assays (Life Technologies, Auckland, New Zealand) as per the manufacturer's instructions, using an Enspire plate reader (PerkinElmer, Waltham, MA, USA). 30 µg of protein from each sample was diluted 10-fold in 50 mM ammonium bicarbonate to permit trypsin digestion with 1 µg sequencing grade modified porcine trypsin (Promega, Madison, WI, USA), and samples incubated at 45°C for 2 hr in a chilled microwave at 15 W power. Samples were acidified to pH 3 via addition of 50% formic acid, centrifuged for 3 min at 16,000 g, and desalted using 10 mg OASIS HLB SPE cartridges (Waters, Milford, MA, USA) as per the manufacturer's instructions. Purified peptides were eluted in 300 µL 50% acetonitrile in 0.1% formic acid, and then concentrated to a final volume of 25 µL in a vacuum concentrator (ThermoSavant, Holbrook, NY, USA).

For Ion library generation, a pooled digest was applied to an SCX MicroSpin column (The Nest Group, Inc.) and fractionated using 25 mM, 50 mM, 100 mM, 150 mM, 200 mM and 300 mM NaCl. LC-MS/MS analysis of these fractions was performed on an Eksigent 425 nanoLC chromatography system (Sciex, Framingham MA, USA) connected to a TripleTOF 6600 mass spectrometer (Sciex). Samples were injected onto a 0.3 x 10 mm trap column packed with Reprosil C18 media (Dr Maisch) and desalted for 5 min at 5 µL/min before being separated on a 0.075 x 200 mm picofrit column (New Objective) packed in-house with Reprosil C18 media. The following gradient was applied at 250 nL^{-1} using a NanoLC 400 UPLC system (Eksigent): 0 min 5%B; 2 min 5%B ; 105 min 35%B; 110 min 98%B; 115 min 98%B; 116 min 5%B; 125 min 5%B where A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The picofrit spray was directed into a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (Sciex) scanning from 350–1600 m/z for 200ms, followed by 30 ms MS/MS

scans on the 60 most abundant multiply-charged peptides (m/z 350–1600) for a total cycle time of ~2 seconds. The mass spectrometer and HPLC system were under the control of the Analyst TF 1.7 software package (Sciex).

The resulting data were searched against a database comprising the available oyster (*Crassostrea Gigas*) sequences from Uniprot and an in-house library translated from a Green-lipped mussel transcriptome, appended with a set of common contaminant sequences (99,814 entries in total) using ProteinPilot version 5.0 (Sciex). Search parameters were as follows: Sample Type, Identification; Search Effort, Thorough; Cys Alkylation, Yes; Digestion, Trypsin. The resulting group file exported from ProteinPilot was transferred to PeakView v2.2 for use as an Ion Library for SWATH analysis.

LC-MS/MS was then conducted for each sample using a SWATH acquisition method using the same LC conditions as above, which had a sequential isolation window width of 23.6 m/z (with 1 m/z overlap) covering a mass range of 400–1250 m/z, resulting in 36 overlapping windows. The accumulation time was 50 ms for the MS scan and 50 ms for the MS/MS scans, thereby making a total cycle time of ~1.9 sec. Resulting fragment ion areas were calculated by PeakView v2.2 using the SWATH MicroApp 2.0 tool (Sciex).

Raw results from SWATH MicroApp were further filtered using an in-house developed Excel macro to refine the final dataset. The raw reported peptide and protein areas were assigned a 0 value if the FDR score was more than 0.05 or the fragment areas were less than 3000 or peptides with less than 3 transitions. Following this, normalization was performed using the sample with highest summed value as a reference, and individual normalization factors were calculated for all the samples. After this step, all peptide transitions which had retention time less than 25 mins were removed, as these features appeared to have peak widths that were narrower or wider than ideal. Following this, if a peptide was not found (with a non-zero value) in 3 or more

samples in that group (i.e., either healthy or unhealthy), it was deleted. Finally, the sum of all remaining peptide areas was calculated for each protein.

2.2.4 Statistical Analysis and Bioinformatics

Metabolite and protein data were autoscaled prior to statistical analysis. Univariate significance analysis of microarrays/metabolites (SAM) and fold change analysis were performed via Metaboanalyst 4.0 (Chong et al. 2018) to screen for differentially regulated/expressed metabolites and proteins between healthy and unhealthy mussels. Multivariate projection to latent structures discrimination analysis (PLS-DA) was employed as a supervised method to minimize the possible contribution of intergroup variability and identify discriminating features between healthy and unhealthy groups. PLS-DA model performances were assessed and validated using Monte-Carlo based permutation testing (prediction accuracy during model training; permutated 1000 times; α=0.05) and Leave One Out Cross Validation (LOOCV) to provide accuracy, multiple correlation coefficient (R^2), and cross-validated R^2 (Q^2) values. The important classifiers were identified via their variable importance in projection (VIP) scores.

Integration of the multiple omics data was performed using the R package "mixOmics" (Rohart et al. 2017). We applied 'data integration analysis for biomarker discovery using latent variable approaches for omics studies' (DIABLO) to examine underlying structure, Pearson's correlation and data variability. DIABLO implements dimension reduction techniques that extends both sparse PLS-DA (sPLS-DA) and sparse generalized canonical correlation by projecting the data into a smaller subspace while capturing the correlation structure and highlighting the largest sources of variation from the data, resulting in reliable sample classification and powerful explanatory capacity (Singh et al. 2016). sPLS-DA results of the associations between selected significant metabolites and proteins was visualized via circos plots showing the strongest positive and negative Pearson's correlations ($|r| > 0.8$). The

performance of DIABLO model was assessed using 10-fold cross-validation repeated 50 times to ensure reliable evaluation via the balanced error rate.

Quantitative enrichment analysis (QEA) (Xia et al. 2011) and network topology analysis (NTA) (Nikiforova and Willmitzer 2007) were also performed to investigate functional relationships among metabolites. Pathways involving two or more annotated metabolites that matched entries within the Kyoto Encyclopaedia of Genes and Genomes database (Kanehisa 2000) with simultaneous QEA p-values < 0.05, QEA false discovery rates (FDRs) < 0.1 and with NTA pathway impact (PI) scores > 0.1 were considered as potential primary pathways of interest, according to Young et al. (2017).

2.3 Results

2.3.1 Metabolomic and proteomic profiles

A total of 182 spectral peaks were identified by GC-MS after filtering, with 79 metabolites being reliably annotated (Supplementary Data S1). Of the proteomics data, 5524 peptides were quantified via LC-MS and contributed to identification of 1418 proteins. 907 of these proteins had at least two unique peptide hits, and were annotated using NCBI BLASTp (Altschul et al. 1990) against the *C. gigas* protein database and an in-house *P. canaliculus* transcriptome database. The list of identified proteins with their respective % coverages and e-values are provided (Supplementary Data S2).

2.3.2 Univariate Analyses

SAM analysis (Figure. 4A) revealed 25 metabolites with significantly different ($p < 0.05$) levels between the profiles of healthy and unhealthy mussels (Figure. 5) (Supplementary Data S3). Of these, 11 metabolites were up-regulated in unhealthy mussels and 14 metabolites were down-regulated in unhealthy mussels compared to healthy ones. SAM analysis (Figure. 4B) also identified 17 differentially expressed proteins that were all down regulated in the unhealthy mussel group ($p < 0.05$) compared to the healthy mussel (Figure. 6).

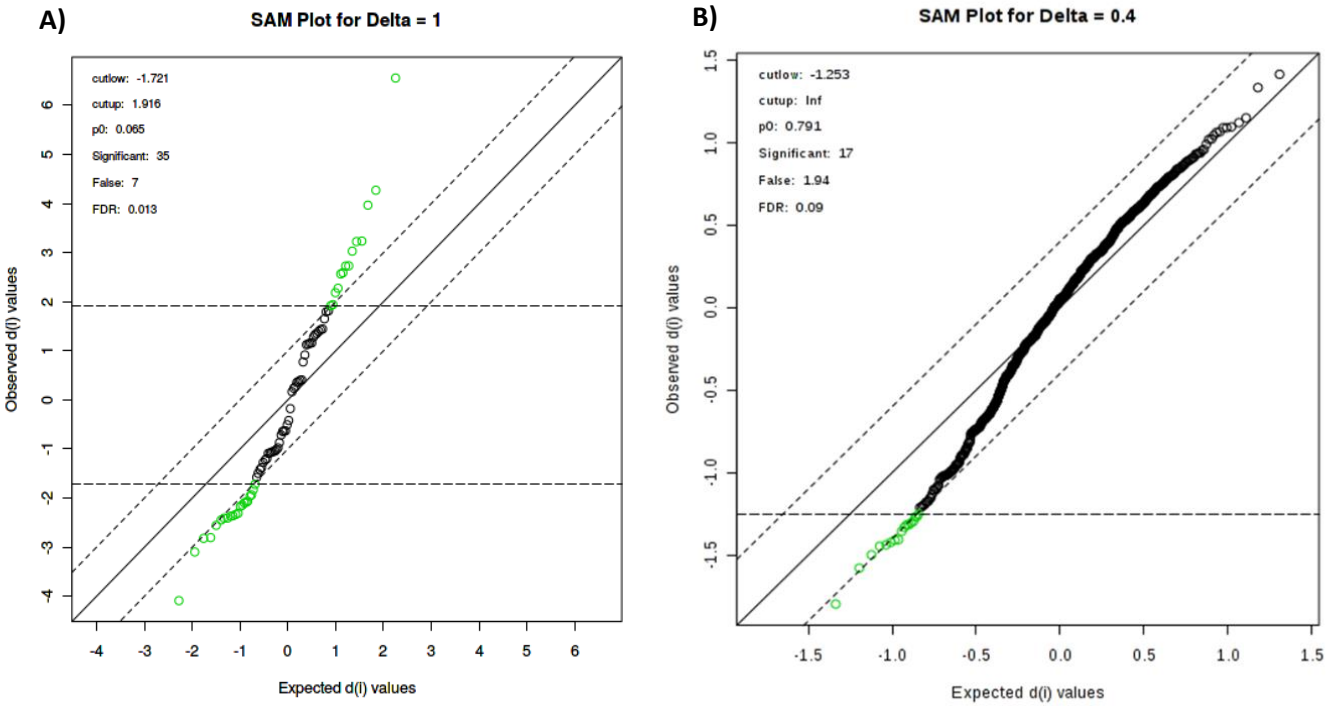


Figure 4. Significant Analysis of Metabolites (SAM) plot to reveal A) differently regulated the metabolites and B) differently expressed proteins profiles of the gill tissues in the unhealthy mussels.

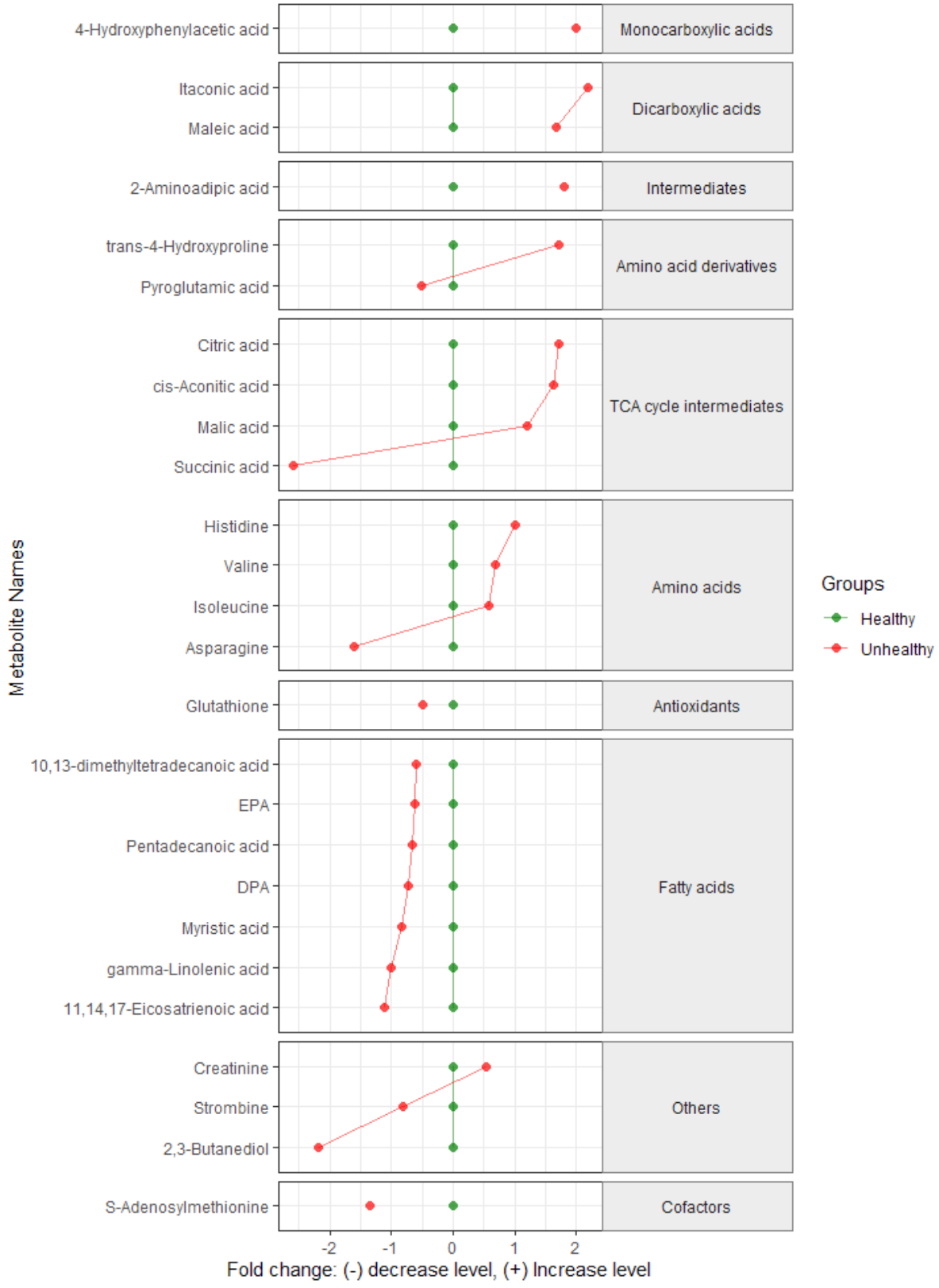


Figure 5. Summary of statistically different metabolites between healthy (green) and unhealthy (red) groups with their respective Log_2 fold change values.

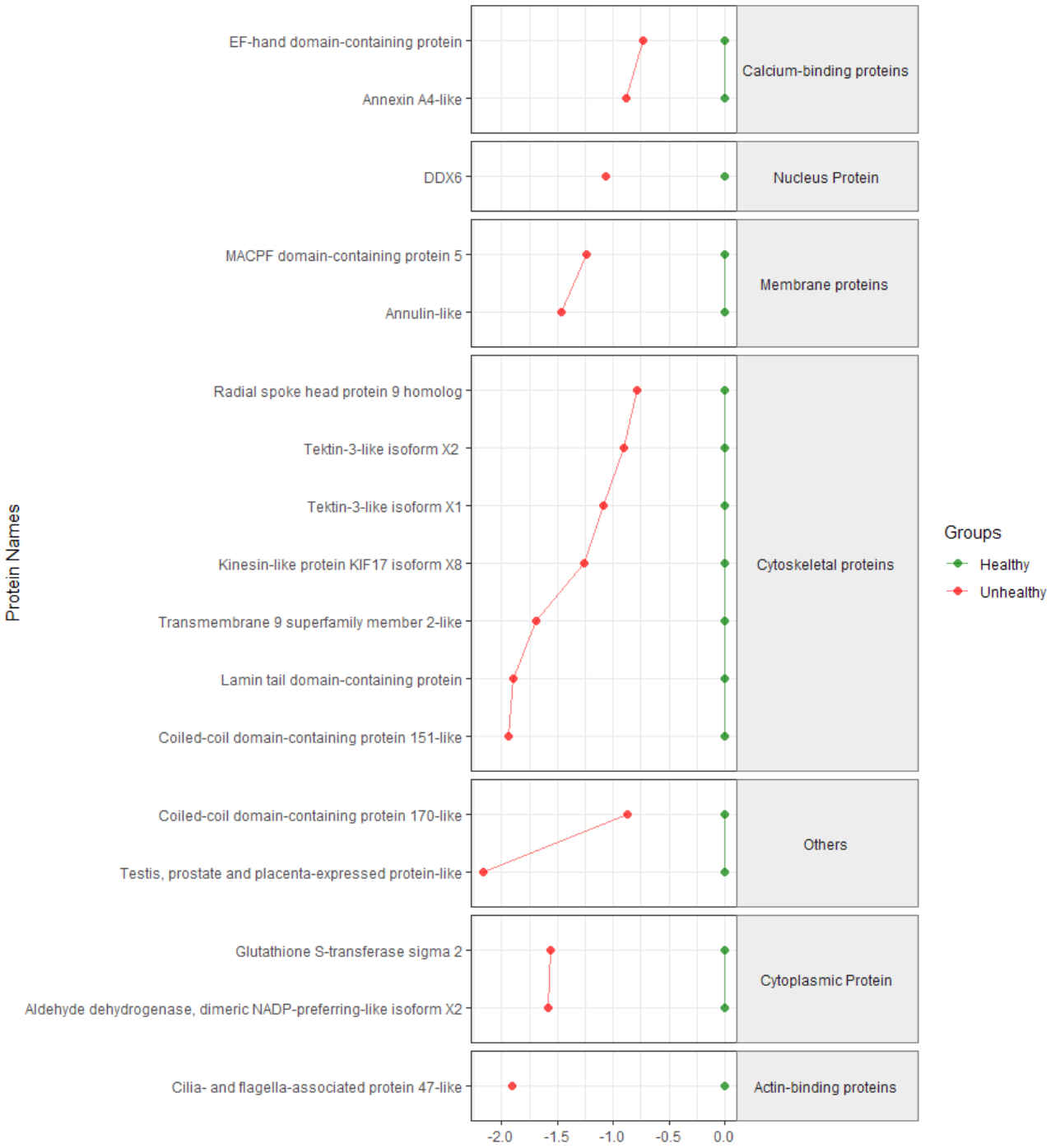


Figure 6. Summary of statistically different protein expressions between healthy (green) and unhealthy (red) groups with their respective Log_2 fold change values.

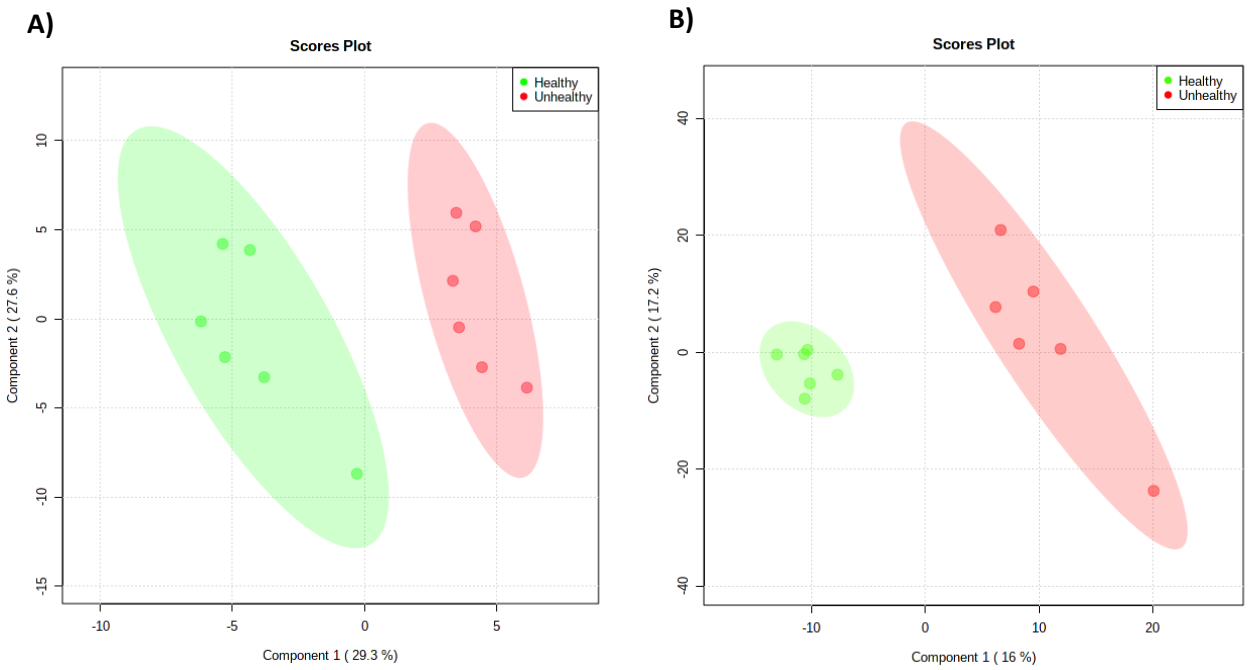


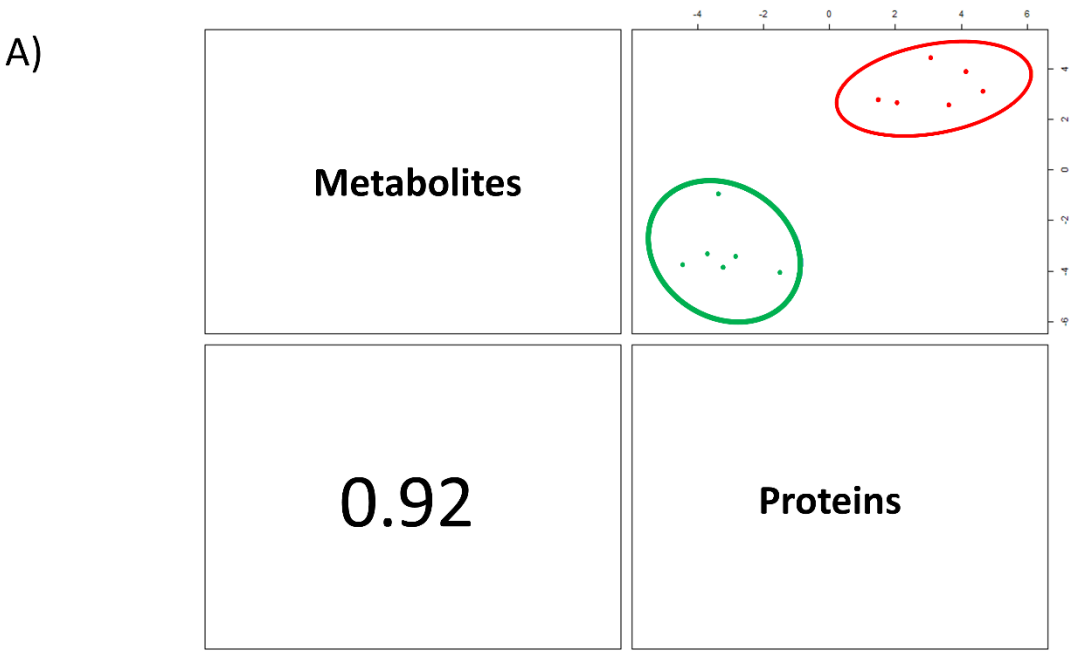
Figure 7. Multivariate PLS-DA analyses of metabolite (A) and protein (B) profiles of the gill tissues from healthy and unhealthy mussels.

2.3.3 Multivariate Analyses

PLS-DA of gill tissue metabolite (annotated) profiles demonstrated good separation between samples of healthy and unhealthy mussels (Figure. 7A). The performance of the PLS-DA model was assessed via its accuracy (0.92), R^2 (0.98) and Q^2 (0.73) values, with the metrics suggesting a good fit with reasonable predictive power. PLS-DA analysis of the gill tissue proteomic (annotated) profiles also revealed two distinct clusters (Figure. 7B). The PLS-DA model indicated a reasonable fit and predictive power (accuracy = 0.75, R^2 = 0.99, Q^2 = 0.44).

Integration of the metabolomics and proteomics datasets performed using sPLSDA from DIABLO revealed that first components from the protein and metabolite set were highly correlated to each other (Pearson coefficient = 0.92). The model also indicated a clear separation between healthy and unhealthy mussels using the metabolite and protein data, based

on the first component of the sPLSDA model (Figure. 8A). The 6-fold cross validation revealed an 8.3% overall classification error rate for metabolites and 0% overall classification error for proteins. In addition, the circos plot showed that 2-Aminoadipic acid has a strong negative correlation with multiple structural proteins such as TEKT3s and CCDCs (Figure. 8B). Protein CCDC 151 and protein ANXA4 like was shown to have a strong positive correlation with multiple fatty acids including pentadecanoic acid, dimethylteradecanoic acid and myristic acid. (Figure. 8B).


A)
Metabolites
0.92
Proteins

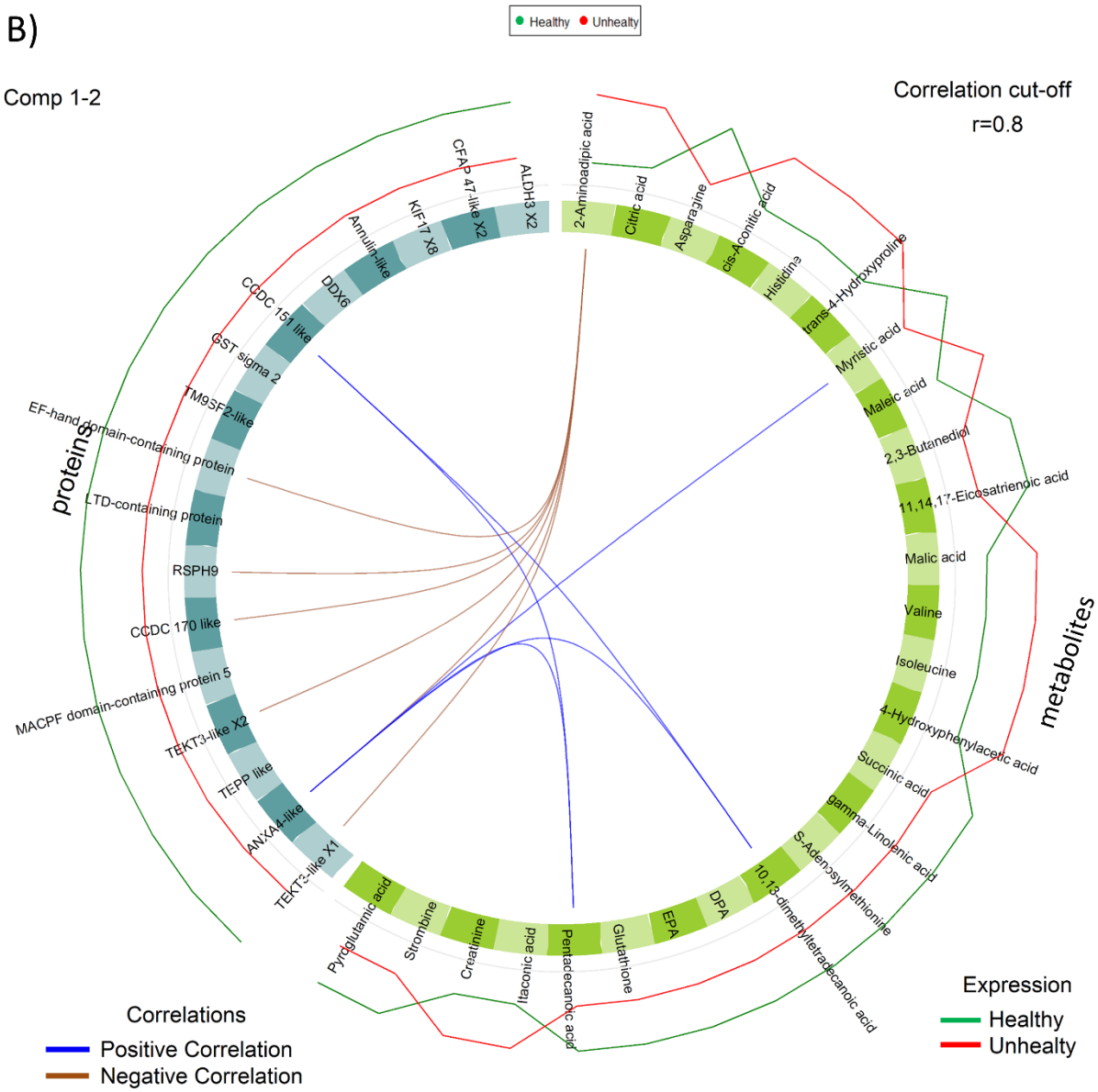

B)
Healthy
Unhealty
Comp 1-2
Correlation cut-off
r=0.8
proteins
metabolites
ALDH3 X2
CFAP 47-like X2
KIF17 X8
Annulin-like
DDX6
CCDC 151 like
GST sigma 2
TM9SF2-like
EF-hand domain-containing protein
LTD-containing protein
RSPH9
CCDC 170 like
MACPF domain-containing protein 5
TEKT3-like X2
TEPP like
ANXA4-like
TEKT3-like X1
2-Aminoadipic acid
Citric acid
Asparagine
cis-Aconitic acid
Histidine
trans-4-Hydroxyproline
Myristic acid
Maleic acid
2,3-Butanediol
11,14,17-Eicosatrienoic acid
Malic acid
Valine
Isoleucine
4-Hydroxyphenylacetic acid
Succinic acid
gamma-Linolenic acid
S-Adenosylmethionine
10,13-dimethyltetradecanoic acid
DPA
EPA
Glutathione
Pentadecanoic acid
Itaconic acid
Creatinine
Strombine
Pyroglutamic acid
Correlations
Positive Correlation
Negative Correlation
Expression
Healthy
Unhealty

Figure 8. A) Sample discrimination plot from plotDiablo displaying the first component in each data set (upper diagonal plot) and Pearson correlation (0.92) between each component (lower diagonal plot) The scatterplot (upper diagonal plot) also indicated the discriminative power of the first component to separate the different types (healthy and unhealthy). B) Circos plot of horizontal sPLS-DA displaying correlations among significantly different metabolites and proteins between healthy and unhealthy mussels. Positive correlations (correlations cut-off $r = 0.8$) between metabolite and proteins are indicated by the blue lines. Negative correlations (correlations cut-off $r = 0.8$) between metabolites and proteins are indicated by the brown lines. Relative expression of each feature is represented by green (healthy) and red (unhealthy) lines.

2.3.4 Pathway Analysis

Metabolic pathway analyses based on the collection of annotated metabolites of the gill tissues highlighted metabolic pathways most likely to be associated with mussel health status (Table 1). Of the 40 pathways in the KEGG database which were matched to our sample metabolite profiles, nine of them were labelled as primary pathways of interest according to our selection criteria.

Table 2. List of significantly altered metabolic pathways identified as pathways of interest in the gill tissues of unhealthy mussels.

Pathway Name	Hits	p-value	FDR	Impact
Glyoxylate & dicarboxylate metabolism	7	<0.001	0.012	0.186
Citrate cycle (TCA cycle)	3	<0.001	0.012	0.173
Aminoacyl-tRNA biosynthesis	19	0.008	0.072	0.250
Phenylalanine, tyrosine & tryptophan biosynthesis	2	0.023	0.115	1.000
Phenylalanine metabolism	2	0.023	0.115	0.500
Alanine, aspartate & glutamate metabolism	7	0.037	0.115	0.818
Arginine & proline metabolism	7	0.042	0.115	0.524
Butanoate metabolism	3	0.043	0.115	0.133
Glutathione metabolism	7	0.048	0.119	0.509

2.4 Discussion

This study provides the first multi-omics dataset for *P. canaliculus,* with initial proteome characterization and further metabolome identification. This study also provides detailed information on mussel summer mortality, revealing key health perturbations including general

metabolism (e.g., energetics, oxidative stress, fatty acid biosynthesis), degradation of structural components (e.g., cytoskeleton and membrane proteins), and potential health biomarkers in key protein–metabolite correlations.

Distinct differences in energy-related pathways between healthy and unhealthy mussels were identified with metabolite signatures indicating disruption of the tricarboxylic acid (TCA) cycle in unhealthy mussels. Central to aerobic respiration, the TCA cycle releases stored energy by oxidation of acetyl-CoA derived from carbohydrates, fats, and proteins into adenosine triphosphate (ATP) and carbon dioxide. In a previous study, notable increases in TCA intermediates (e.g., succinic acid, fumaric acid, malic acid) were detected in mussels (*P. canaliculus*) experimentally infected with pathogenic *Vibrio* sp (Nguyen et al. 2018a). Accumulation of TCA cycle metabolites indicates interruption to this central metabolic pathway (Jha et al. 2015). It is emerging that citric acid in particular plays a major role during pro-inflammatory activation, with functions in cytosolic fatty acid biosynthesis for membrane expansion, production of pro-inflammatory mediators (e.g., nitric oxide, reactive oxygen species), and as a substrate for the synthesis of itaconic acid via the intermediate cis-aconitic acid (Infantino et al. 2011; Michelucci et al. 2013). Itaconic acid is a key immune-responsive metabolite stemming from diversion of TCA cycle flux, and our research group recently identified it as a molluscan non-specific immunological biomarker of viral and bacterial infections in *C. gigas* and *P. canaliculus* (Young et al. 2017a; Nguyen et al. 2019a; Van Nguyen and Alfaro 2019b). The altered TCA cycle-related metabolites in this study (i.e., succinic, malic, citric, cis-aconitic and itaconic acids) establishes strong evidence of TCA cycle disruption and reaffirms itaconate as a biomarker for poor health in marine molluscs.

Upregulation of glutathione (GSH) alongside a relative high coverage of metabolites within the glutathione pathway (i.e., cysteine, glutamate, 5-oxoproline, and ornithine) was also revealed. Altered glutathione metabolism in unhealthy mussels may indicate animals were

under oxidative stress. Oxidative stress is caused by an imbalance between production and accumulation of reactive oxygen species (ROS) in cells and tissues, and the inability of a biological system to detoxify them. ROS interacts with many biological molecules including proteins, lipids and nucleic acids, potentially causing tissue damage, impairing cellular function, lowering immune capacity, altering the physico-chemical properties of cell membranes, and ultimately undermining vital functions (Manduzio et al. 2005; Bartosz 2009). Therefore, levels of ROS within cells and tissues are kept in check by GSH, a metabolite that quenches ROS production and prevents cellular damage(Pompella et al. 2003). Thus, GSH can be used as a biomarker of susceptibility to mortality in marine bivalves with induced oxidative stress (Pena-Llopis et al. 2002). The role of GSH and its synthesis pathway in regulation of oxidative stress has also been recently demonstrated in *P. canaliculus* following a *Vibrio* sp. challenge (Nguyen et al. 2018c). In addition, significant under-expression of glutathione s-transferase (GST) in unhealthy mussels from the proteomics results provides evidence of oxidative stress. GSTs are a family of evolutionarily conserved detoxification enzymes that catalyse the conjugation of electrophilic substrates to GSH (Oakley 2011; Smith et al. 2013). Inhibition of GST activity has been reported in mussels (*M. galloprovincialis*) during toxin-induced stress (Gomes et al. 2013). GSTs are a major contributor to antioxidant defences in *M. edulis* mussels (Manduzio et al. 2004), and alteration in GST activity has been observed in the mussel *Mytella guyanensis* experiencing oxidative stress from trace metal contaminated sites (Aloísio Torres et al. 2002). In this study, a reduction in free fatty acids (FFA), such as the essential long-chain polyunsaturated fatty acids docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and gamma-linolenic acid were identified in the gills of unhealthy mussels. FFAs are key components of lipids and various metabolic pathways. For example, EPA serves as a precursor of short-lived hormone-like eicosanoids which have a wide range of physiological functions in immunity,, inflammation, neurons, reproduction, and enhancement of an organism's adaptation

to environmental stress (Vance and Vance 2008; Parrish 2009). DHA and EPA are both high-energy storage lipids and required in maintaining homeostasis (Fokina et al. 2015). Since EPA and DHA cannot be de novo synthesized at high rates in marine mussels (De Moreno et al. 1976; de Moreno et al. 1977), their incorporation and elimination in membrane and storage lipids are strongly regulated (Delaunay et al. 1993; Soudant et al. 1998). We also observed a decrease in saturated fatty acid myristic acid and pentadecanoic acid. Myristic acid is acquired from dietary sources in mussels and stored inside gills and digestive glands (Fokina et al. 2015). A study by Li et al. (2017) showed that low expressions of FFAs (including myristic acid and pentadecanoic acid) in Pacific oysters are associated with decreased energy metabolism and decreased resistance to stress.

Out of the 17 significantly under-expressed proteins in the unhealthy mussels, six were cytoskeletal proteins. Among these cytoskeletal proteins, three are related to the structure complex of the cilia and flagella. The first one is a radial spoke head protein 9, a multi-unit protein that plays a role in the mechanical movement of the flagellum/cilium (Pigino and Ishikawa 2012). However, its structure and mode of action remains poorly understood. The other two structural proteins are tektin-3 isoforms (tektin-3 X1 and tektin-3 X2). Tektins are integral microtubule proteins in cilia and flagella found within the gill tissues (Pirner and Linck 1994; Amos 2008). Decreased tektin expression is noteworthy because the numerous cilia of mussel gills are responsible for creating the water currents that allow the mussel to feed and respire. Any damage to these cilia, which are directly exposed to the external medium, would require rapid repair to maintain feeding and metabolic processes (Fields et al. 2014). Since gills from bivalves are one of the most metabolically active tissues (Wang et al. 2018), we hypothesise that stresses caused by heat or pathogenesis in unhealthy mussels led to the changes in cytoskeletal protein abundance and gill structural changes. However, more work is needed to examine changes in gill structure of *P. canaliculus* under various stressed conditions.

Two other decreased cytoskeletal proteins in the unhealthy mussels, namely coil-coil domain-containing protein 151 (CCDC151) and kinesin-2 (KIF17), present evidence of altered regulation, assembly and motile mediation of the gill tissue cilia/flagella. CCDC151 is an evolutionary conserved protein responsible for controlling motile intraflagellar transport (IFT) dependent dynein arm (inner and outer rows of arms associated with the doublet microtubules of motile cilia) assembly (Jerber et al. 2014), and is critical for flagellar beat frequency and cilia waveform (King 2012). IFT is the bidirectional transport of multi-subunit protein complexes along axonemal microtubules beneath the ciliary membrane (Hao and Scholey 2009), and is responsible for the assembly of structural and functional components of cilia and flagellum in the cytoplasm (Kozminski et al. 1993). Previous studies have shown CCDC151 gene knockout in planarians (flatworms which move on a ventral ciliated epithelium) resulted in cilia functional and characteristic loco-motion defects (Rompolas et al. 2010; Jerber et al. 2014). Therefore, faults in IFT machinery of mussel gills would potentially have substantial consequences on gill function. KIF17 on the other hand is the anterograde microtubule-based motor that carries out movement of IFT particles along the microtubule (Rosenbaum and Witman 2002; Scholey 2008). Although KIF17 is a human gene encoded protein, it has been identified as a homodimer in invertebrates and functions to mediate anterograde IFT along the distal singlets of microtubules within cilia, and required for the assembly of this singlet only region (Snow et al. 2004). Thus, the identification of KIF17 in our study requires further targeted studies to verify its specific function in *P. canaliculus* and how it affects IFT in the cilia/flagella.

A single ‘omics’ such as metabolomics can be used to study metabolite profiles and recognize a biosystem’s physiological and biochemical status in relation to phenotype (Alfaro and Young 2018; Young & Alfaro 2018). However, the regulation of the phenotype and the underlying pathophysiological mechanisms occur on multiple levels that can be better understood when

multiple omics are analysed holistically. Our integrative results show a distinctive correlation between the metabolic and proteomic profiles of the mussels. One major contributor to this correlation is an observed positive relationship between the calcium binding protein Annexin A4 and structural protein CCDC151 (both significantly under-expressed in the unhealthy mussels) and three down-regulated saturated fatty acids (pentadecanoic acid, 10,13 dimethyltetradecanoic acid and myristic acid) in unhealthy mussels. Annexins A4 belongs to the annexin family of proteins that utilizes their calcium dependent ability to bind to and organize phospholipid bilayers and have been shown to play specific roles in membrane repair (Boye et al. 2017; Simonsen et al. 2019). Although Annexin A4 belongs to the family of human annexin proteins it has previously been reported to be down-regulated in oysters under pH stress (Dineshram et al. 2015). In this context, and based on our integrative findings, we suggest that lowered expression of Annexin A4 may be associated with lowered saturated fatty acid content within the gill tissue cell membranes of unhealthy mussels. Temperature changes can induce changes in membrane fatty acid compositions in marine mussels (Fokina et al. 2015), and this novel link highlights Annexin A4 as an important entry point for future pathophysiological mechanistic research into heat stress responses of *P. canaliculus*.

Another finding from our integrative analysis was the negative relationship between 2 aminoadipic acid (significantly upregulated in unhealthy mussels) and lowered expression of cytoskeletal proteins tektin-3 (both isoforms), CCDC170 like, radial spoke head protein 9, EF-hand domain containing-protein, and annexin A4. 2-aminoadipic acid (2-AAA) is a component of the lysine degradation pathway and proposed as a small-molecule marker of oxidative stress (Sell et al. 2007; Zeitoun-Ghandour et al. 2011). Elevated levels of 2-AAA have been reported in unhealthy Pacific oyster larvae infected with OsHV-1 virus (Young et al. 2017b), and can be induced in shellfish by exposure to stressors (Chen et al. 2015; Koyama et al. 2015). Additionally, 2-AAA is a marker of protein carbonyl oxidation resulting from ROS-induced

protein damage (Sell et al. 2007; Kriebardis et al. 2007; Smerjac and Bizzozero 2008; Suzuki et al. 2010; Wilson and González-Billault 2015). Previous studies also have reported that carbonylation of cytoskeletal proteins cause loss of function. For instance, actin filaments and microtubules both destabilize and disassemble upon oxidation of their protein components (Dalle-Donne et al. 2001; Neely et al. 2005). We suspect that gill cytoskeletal proteins of unhealthy mussels may have experienced degradation due to cytoskeletal protein carbonylation.

2.4 Conclusions

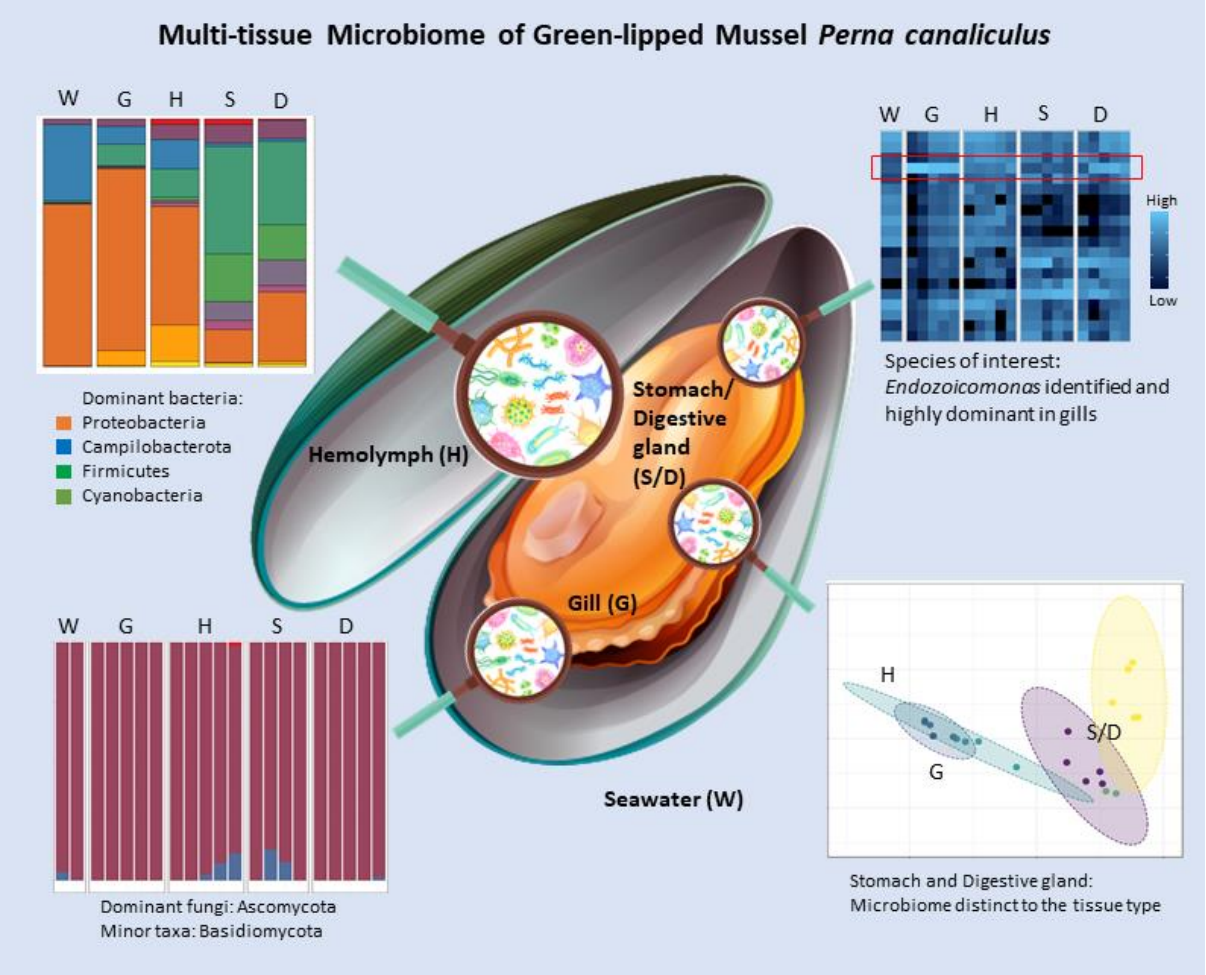
This study presents the first integrated investigation of metabolite and protein expression responses of *P. canaliculus* mussels during a period of high mortality in summer. The profiling of the gill tissues revealed strong evidence of alterations in energy and immune-related metabolic pathways in unhealthy mussels. Perturbations in glutathione metabolism and protein glutathione S-transferases also provide evidence for oxidative stress in unhealthy mussels. Degradation in the cytoskeleton structure and regulation of cilia/flagellum gill tissues of the unhealthy mussels may be a contributing factor to undesired changes in gill membrane fluidity, permeability, and lipid composition impairing function. This study provides novel findings that highlight the value and potential of applying a multi-omics approach to explore metabolite–protein interactions that underpin the pathophysiological state of mussels in farm conditions.

Chapter 3.

Mapping the green-lipped mussel (*Perna canaliculus*) microbiome: A multi-tissue analysis of bacterial and fungal diversity

This work has been published as:

Li S, Young T, Archer S, Lee K, Sharma S, Alfaro AC. Mapping the Green-Lipped Mussel (Perna canaliculus) Microbiome: A Multi-Tissue Analysis of Bacterial and Fungal Diversity. *Curr Microbiol*. 2022;79(3):76. Published 2022 Jan 29. doi:10.1007/s00284-021-02758-5



3.1 Introduction

The New Zealand green-lipped mussel (*Perna canaliculus*) is an endemic bivalve commonly found within intertidal and subtidal coastal habitats. Mussel beds provide important ecological functions, such as removing suspended sediment and particulate organic material, resulting in improved water quality. *P. canaliculus* is also a highly valued species for the New Zealand's growing aquaculture industry, which supports a mussel sector worth over NZ$300 million in export revenues (Aquaculture New Zealand 2017).

Given their ecological and economic importance, monitoring health of wild mussels and maintaining the health of domesticated stocks is of utmost importance. While infections from pathogenic microbes may lead to deleterious outcomes, host-microbe interactions are also thought to play a key role in maintaining mussel health and organ-level functioning. Filter-feeding marine mussels are in continuous and direct contact with a dynamically shifting microbial environment (Glasl et al. 2016; Pita et al. 2018). Indeed, the high filter feeding capability of marine mussels allows them to filter large volumes of seawater, while capturing different types of particulate water-borne pollutants as well as microorganisms (Neori et al. 2004; Pagano et al. 2016). Growing evidence suggests that microbes can offer their host organisms probiotic functions, such as enhanced pathogen defence, immunological regulation, and improved digestion efficiency and nutrient uptake, among other factors (O'brien et al. 2019; Rausch et al. 2019; Simon et al. 2019). Thus, characterizing the microbial structures of host compartments can inform the underlying functionalities of host-microbiota interactions.

Although a previous report of microbiota characterizations of marine mussels (*Mytilus galloprovincialis*) revealed that different tissues harbour unique microbial communities which serve specific functional purposes (Musella et al. 2020), the functions of microbial communities in the gut, stomach, and digestive gland tissues are less explored among the diverse range of marine molluscs. Additionally, there have been no microbiota characterization

studies for the endemic New Zealand green-lipped mussel *Perna canaliculus*. The aim of this study was to profile the microbiota associated with different tissues of farmed green-lipped mussels using high-throughput sequencing. The main objectives were to: 1) profile marine bacteria and fungi in different mussel tissues and the surrounding seawater, 2) describe microbiome variability among individual samples, 3) determine bacterial and fungal community similarity/dissimilarity among the different tissue types, and 4) Identify key dominant host-associated taxa across the tissue types.

3.2 Material and Methods

3.2.1 Sample Collection

Five healthy adult mussels (length = 95.8 mm ± 6.6; weight = 66.3 g ± 9.9) and a sample of seawater (1 L) were collected in September 2020 (autumn) from a mussel farm located in Kaiaua, Firth of Thames, New Zealand (GPS coordinate: -37.0610, 175.3002). Mussels were cleaned and washed externally with fresh filtered seawater to remove biofouling. Haemolymph was extracted from the abductor muscle using sterile disposable 1 mL syringes and transferred to sterile 2 mL cryovials (BioStor™) containing 20 µL RNA stabiliser (Qiagen, Germany). The digestive gland, stomach and gill tissues were dissected and the samples were placed in 2 mL cryovials with RNA stabiliser (200 µL), then immediately snap-frozen in liquid nitrogen and stored at –80°C until further analyses. Sub-aliquots of seawater were filtered through single use 25 mm diameter Whatman filters with *0.2 µm* pore size (Cytiva, USA) using 20 mL syringes flooded with RNA stabiliser. Filters were sealed in parafilm and stored at 4°C for 2 weeks before DNA extractions.

3.2.2 Microbial DNA extraction

Total microbial DNA was extracted from tissue samples (each 20-30 mg) and haemolymph (200 µL) using the DNeasy PowerSoil kit (Qiagen, Germany) according to the manufacturer's instructions and the adapted protocol of Musella et al. (2020). Tissues were lysed using a

FastPrep system (MP Biomedicals; Irvine, California) at six movements per second for one minute prior to extraction. The elution step from the DNeasy PowerSoil kit was repeated twice with 50 μL Tris elution buffer, incubating the columns for five minutes at room temperature before centrifugation. DNA samples were stored at −20°C before subsequent processing. To extract microbial DNA from seawater filters, samples were flooded with 1mL of extraction buffer 1, incubated at 60°C for 30 minutes. Then, the fluid was pushed into a clean 2mL bead tube for processing with the DNeasy PowerSoil kit according to the manufacturer's instructions. Multiple tubes of seawater were pooled at the column stage.

3.2.3 PCR amplicon and sequencing

Purified DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen; USA). MiSeq (Illumina, USA) libraries were prepared as per manufacturer's protocol (16S Metagenomic Sequencing Library Preparation; Part # 15044223; Rev. B [Illumina; San Diego, CA, USA]) and as previously described (Archer et al. 2020). PCR was conducted with primer sets targeting the V3-V4 regions of the bacterial 16S rRNA gene: PCR1 forward (5' CCTACGGGNGGCWGCAG 3') and PCR1 reverse (5' GACTACHVGGGTATCTAATCC 3') and the internal transcribed spacer region (ITS) between the fungal 18S and 5.8S rRNA genes: ITS1 forward (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 reverse (5' GCTGCGTTCTTCATCGATGC 3').

3.2.4 Bioinformatics and statistical analysis

Data were pre-processed using our established workflow (Archer et al. 2020). Briefly, 16S rRNA gene and fungal ITS1 amplicons were processed using the R package DADA2 v1.8 (Callahan et al. 2016) and cutadapt v3.4 (Martin 2011) to remove forward (CCTACGGGNGGCWGCAG) and reverse (GACTACHVGGGTATCTAATCC) primer sequences for 16S rRNA gene, and forward (CTTGGTCATTTAGAGGAAGTAA) and reverse (CTTGGTCATTTAGAGGAAGTAA) primer sequences for fungal ITS1 region. High quality bacterial reads (forward base reads < 230 and reverse base reads < 220 were trimmed

and removed) and then filtered by removing reads exceeding maximum expected error of 2 for forward reads and 5 for reverse reads or reads containing ambiguity N symbol. Fungal reads were untrimmed (due length hyper-variability of the region) and reads exceeding maximum expected error of 5 for forward reads and 8 for reverse reads, or those with ambiguity N symbols were removed. The reads were used to train the error model and then dereplicated to acquire unique sequences, which were used to infer sequence variants with the trained error model. The forward and reverse reads were merged and chimeric sequences were removed. For bacteria we used amplicon sequence variants (ASVs) to assign taxa since this has been shown as the most robust 5 method currently available for bacterial 16S rRNA gene-defined taxa identification. ASVs were then clustered into and assigned assigned taxonomic ranks using SILVA nr v132 database (Quast et al. 2013). Fungal reads were taxonomically classified using the UNITE v7.2 database (Nilsson et al. 2019). R v3.5.2 (R Core Team 2020) and the R packages MicrobiomeAnalyst (Dhariwal et al. 2017), phyloseq (McMurdie and Holmes 2013), and ggplot2 (Wickham 2011) were used for downstream statistical analysis and data visualisation (i.e., relative bacterial/fungal abundances, ordination [principal coordinates analysis; Bray-Curtis], hierarchical cluster analysis [Bray-Curtis distance; Ward linkage]), alpha diversity [Chao1 index], heatmap [data scaled by pseudo-log]).

3. 3 Results & Discussion

Bacterial communities were distinct by tissue type (PERMANOVA; F-value = 6.1784; R^2 = 0.59246; p-value < 0.001) (Figure 9a & 9b) with the exception of stomach and digestive gland tissues that were highly similar to one another, but distinct from the gill tissues and haemolymph. The seawater samples were also clustered closely with the gill tissue and haemolymph, indicating similar bacterial communities. The gills of bivalves perform respiratory, excretory, and feeding functions, which require them to interact directly with seawater. Therefore, our results suggest that close contact between gills and haemolymph

allows waterborne microbiota from the external environment to be transferred to the haemolymph via the gills as has been shown previously (Brito et al. 2018). Higher species richness was observed in digestive gland and stomach tissues compared to seawater, haemolymph and gill tissues (Figure 10b). This is most likely reflective of the difference in functions and selection of a host-associated microbiota of symbionts with nutrition-related roles (Burgos-Aceves and Faggio 2017; Ikuta et al. 2019). These findings indicate a highly selective host recruitment of the mussel microbiome which aligns with previous studies in fish; gill microbial communities tend to be more associated with interactions and communication processes involving the circulatory system, signal transduction, and cell motility, whereas gut microbiota are associated with metabolism and genetic information processing (Kuang et al. 2020).

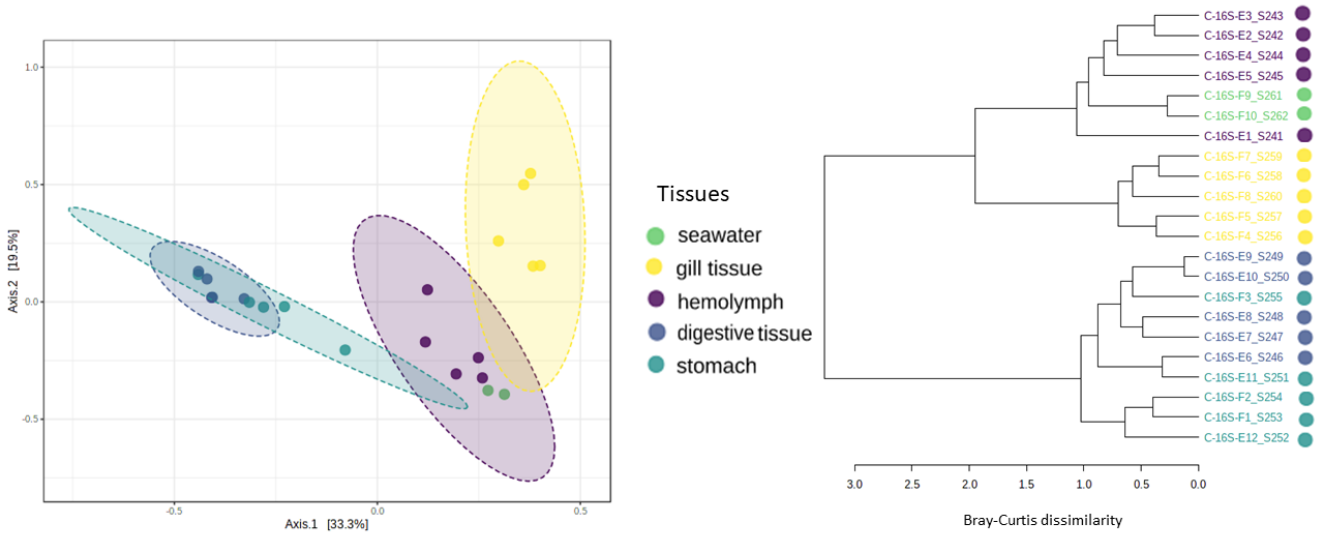


Figure 9. Cluster analyses of *P. canaliculus* tissues and seawater based on bacterial profiles (ASV) and Bray-Curtis distances: (a) Principal coordinates analysis (PCoA); (b) Hierarchical clustering dendrogram (Ward algorithm). Both constructed based on Bray-Curtis distances.

Profiling the microbiome of *Perna canaliculus* at multiple taxonomic levels revealed distinct bacterial structures (Figure 10a) (for Family, Class, Order, Genus levels refer to Supplementary Figures S1-S5). The dominance of Proteobacteria, specifically gamma, and Campilobacterota (a new phylum that contains Epsilonproteobacteria based on Genome Taxonomy Database) in the gill tissues and haemolymph are consistent with prior findings in other mussel species (Li et al. 2018, 2019a), oysters (Lokmer and Mathias Wegner 2015), and abalone (Mizutani et al. 2020). These findings suggest that host-associated bacterial communities in distantly-related marine molluscs may be more tightly linked with general tissue types, as a potential consequence of organ-level function and/or environmental interaction. Proteobacteria have been found to dominate fish gill tissues where they are thought to play crucial roles in supporting the mucosa's microbial barrier, and, with many being opportunistic pathogens, they may even contribute to the development and maintenance of the host immune system through stimulatory mechanisms (Gomez et al. 2013; Wu et al. 2021). However, their functional roles, if any, are yet to be established in molluscan gill mucosa.

In regard to the bacterial profile of digestive gland and stomach tissues, the high abundances of anaerobic Phyla Firmicutes and Bacteroidota, as well as Cyanobacteria were to be expected. Microbes within the Phylum Firmicutes can produce short-chain fatty acids from complex polysaccharides, which provide nutrition for the intestinal mucosal cells (Muegge et al. 2011; Koh et al. 2016). High levels of Firmicutes may also contribute to the maintenance of the normal function of the intestinal mucosa and the regulation of the intestinal microbial environment (Koh et al. 2016; Hao et al. 2017). Bacteroidetes participate in carbohydrate transport and protein metabolism, which are involved in digestive processes (Karlsson et al. 2011). Interestingly, Bacteroidota/Firmicutes ratios have been extensively researched as markers for gut health and dysbiosis in humans and mice (Mariat et al. 2009; Jami et al. 2014;

Nguyen et al. 2015; Magne et al. 2020), however, their significance in mussel gut functioning is yet to be explored. The high abundances of Cyanobacteria in digestive gland and stomach tissues in the present study are most likely derived from the environment (ingested food and seawater). However, the absence of this group of bacteria in the gills and haemolymph could be due to said tissues' undesirable conditions for proliferation of this type of bacteria.

In contrast to bacterial communities, the fungal profiles in this study were more ambiguous due to the large amounts of unmatched/unidentified ASVs. Furthermore, the relative abundances of identified fungi did not reveal any specific patterns or tissue-specific associations (supplementary information S6 – S10). Identified fungal phyla were almost entirely dominated by Ascomycota, except for seawater, which contained a large proportion of unidentified phyla. The alpha diversity for fungal species revealed a slightly higher diversity in the digestive gland, and lower diversity in the gills compared to stomach, seawater and haemolymph (Figure 10c). The lack of clear trends in the distribution of fungi found in this study is not surprising given the stochastic nature of fungal dispersion (Bahram et al. 2016; Tipton et al. 2019) and the lack of studies on marine fungi (Amend et al. 2019).

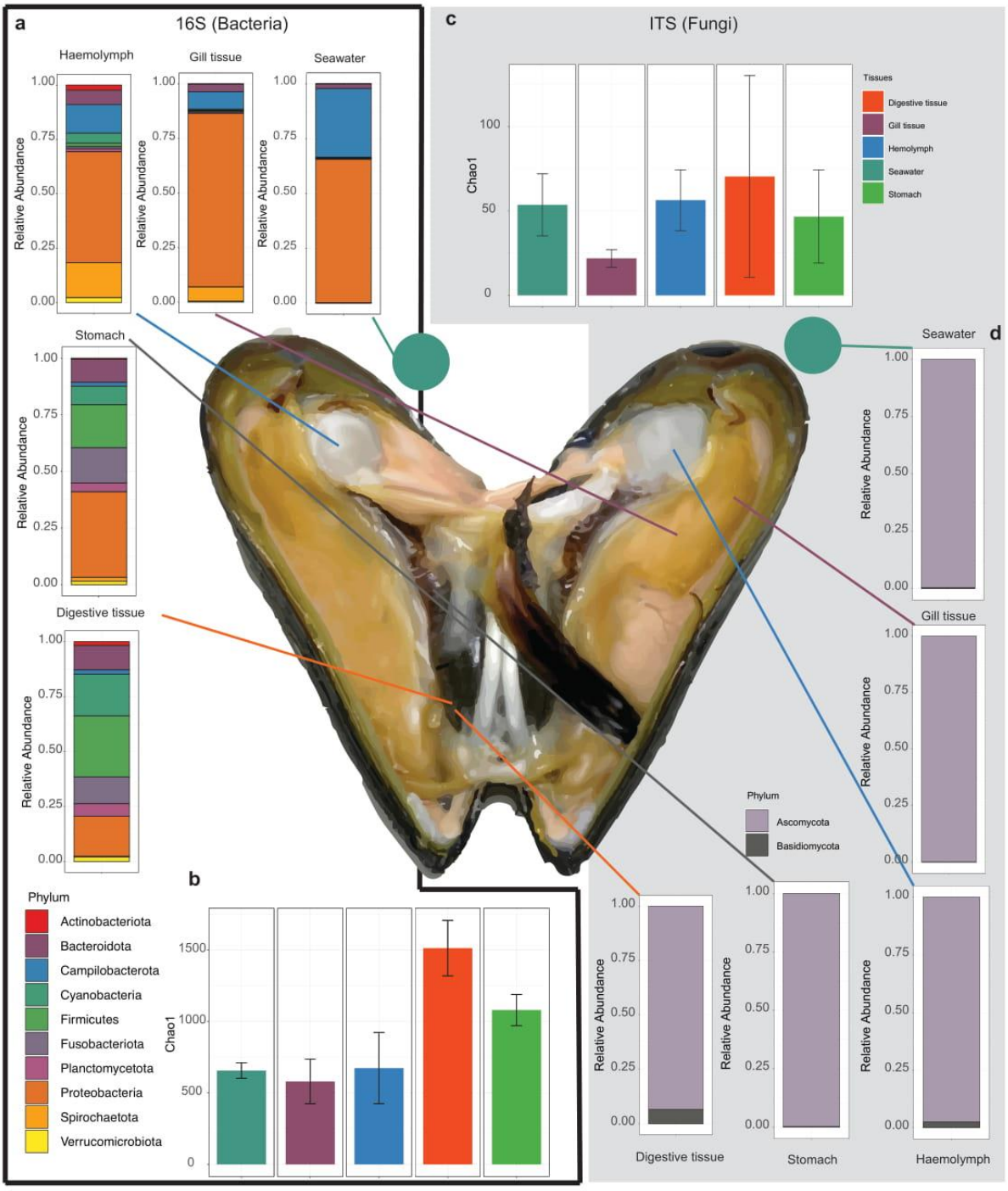


Figure 10. Microbiome profiling of *P. canaliculus*: (a) Bacterial relative abundances at the phylum level in different tissue types and seawater; (b) Bacterial alpha diversity; (c) Fungal alpha diversity; (d) Fungal relative abundances at the phylum level in different tissue types and seawater

Finally, to describe microbiome variability among individuals, and to identify key dominant host-associated taxa across tissue types, the top 20 bacterial genera were ranked from highest to lowest in terms of abundances across all samples (Figure 11). The results visualized via a heatmap revealed that bacterial genera, such as an unclassified genus of Families Spirochaetaceae, *Moritella* and *Poseidonibacter* were more abundant in gill tissue, haemolymph and/or seawater. Bacteria, such as *Mycoplasma*, *Synechococcus* and *Psychrilyobacter* were elevated in digestive gland and stomach tissues. Interestingly, high relative abundance of *Vibrio* spp. was observed across seawater and all tissue types. The presence of *Vibrio* spp. is to be expected as they are ubiquitous in marine and estuarine environments, and on surfaces and intestinal contents of marine animals (Thompson et al. 2004a). Although many *Vibrio* species are harmless, several can be highly pathogenic for humans and/or marine animals (Castinel et al. 2019; Froelich and Noble 2016; Petton et al. 2015). Warm temperature favours the proliferation of *Vibrio* spp. and has contributed to mass mortalities in shellfish farms (Eiston et al. 2008; Le Roux et al. 2016). Higher abundances of *Moritella* and *Poseidonibacter* in the gill tissues were expected because these bacteria are of marine origin (Kautharapu and Jarboe 2012; Guo et al. 2019; Kim et al. 2021).

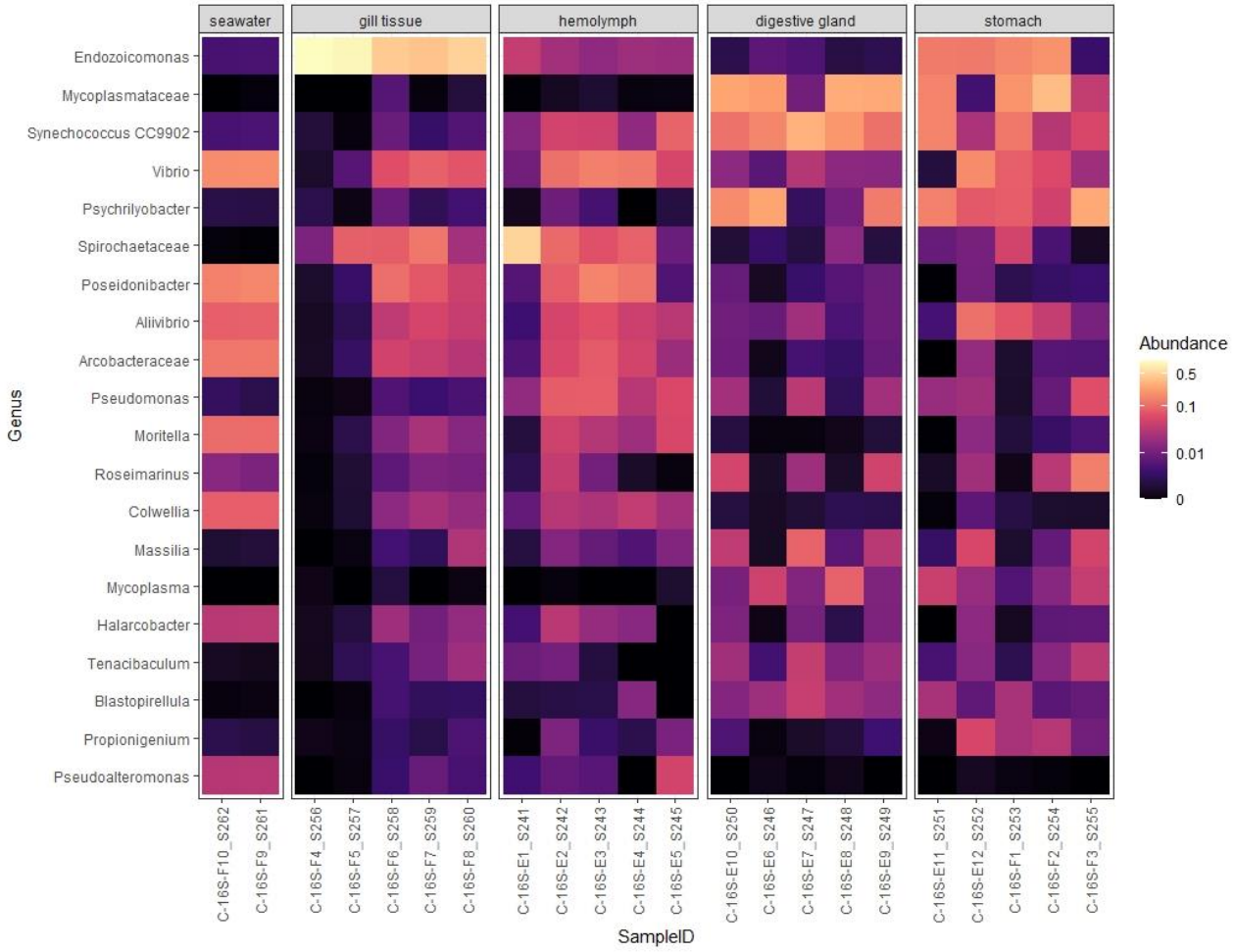


Figure 11. Top 20 relative abundant bacterial genera (ranked from most abundant to least abundant) across different tissue of P. canaliculus and seawater. Bacterial genus is shown row-wise, samples are shown column-wise and coloured by relative abundances. The range of the scale has been transformed via a pseudo log transformation. The few family names represent all the genera within that family merged into a single taxon.

Higher abundances of *Mycoplasma* in the stomach were not surprising as they are common members of the intestinal bacterial flora of many marine species (e.g., fish, abalone) where they may provide nutrients to their hosts (Tanaka et al. 2004; Bano et al. 2007; Huang et al. 2010). *Synechococcus* cyanobacteria are one of the most important components of photosynthetic picoplankton (Partensky et al. 1999; Flombaum et al. 2013; Sohm et al. 2016),

and their presence in digestive samples of *P. canaliculus* represents their dietary origin. *Psychrilyobacter* is a marine member of the Phylum Fusobacteria. This genus is an obligate anaerobic halophile that is able to grow well in low temperatures, and it has been recently isolated and described from marine sediments and marine animals (Navarrete et al. 2009; Zhao et al. 2009; Schuett and Doepke 2010). Interestingly, the most abundant genus identified (*Endozoicomonas*) across samples was elevated in gill and stomach tissues. A study using comparative analysis revealed that *Endozoicomonas* likely participate in nutritional symbiosis and their genomes may be enriched for transport and secretion processes, such as transfer of carbohydrates, amino acids, and proteins between the symbiont and host (Neave et al. 2017a). In addition, *Endozoicomonas* species seem to have symbiotic relationships with the host by producing antimicrobial substances to deter potential invading microbes (Bourne et al. 2008). Previous reports have also shown that *Endozoicomonas* dominates the gut of *Mytilus galloprovincialis* in response to thermal stress (27°C), suggesting that the microbes from this genus play a crucial role in maintaining health (Li et al. 2019b). Contrary to these reports, the presence of *Endozoicomonas has been associated with mortalities of shellfishes, such as* green-lipped mussels, clams and scallops *in New Zealand (Howells et al. 2021), and infecting the gill tissues of king scallop (Hooper et al. 2019).* The identification of major microbial genera in *P. canaliculus* microbiomes demonstrates key associations and similarities with other marine organisms. These taxa also represent targets for future microbial-host interaction research in *P. canaliculus* for the potential development of host health biomarkers.

3.4 Conclusions

Bacteria and fungi were profiled in different tissues of *P. canaliculus* and surrounding seawater. Distinct compositional patterns of microbes were identified at various taxonomic levels. Seawater, gills, and haemolymph contained proteobacterial groups, while digestive gland and

stomach tissues were dominated by common anaerobic gut microbes involved in fatty acid synthesis, carbohydrate digestion and gut maintenance. Fungal profiles in all samples were dominated by taxa within the Phylum Ascomycota, but could not be identified beyond this taxonomic level. This study also highlights the open association between the circulatory physiology (gills and haemolymph) of mussels and surrounding seawater, and the high selectivity of microbiomes in the digestive system (digestive gland and gut). Furthermore, by comparing individual sample variability, we identified key genera of interest, such as *Endozoicomonas*, which could potentially be used as markers for mussel health in the future. Our study represents the first detailed characterization of microbiome profiles of *P. canaliculus* within different tissues, hence providing a baseline for future physiological and health studies of this important aquaculture species.

Chapter 4

Gut microbiome resilience of green-lipped mussels, *Perna canaliculus*, to starvation

4.1 Introduction

New Zealand Green-lipped mussels (*Perna canaliculus*) are commonly found within intertidal and subtidal coastal habitats. This species is also cultivated on marine farms which support a growing aquaculture industry worth over NZ$300 million in export revenues (Aquaculture New Zealand 2020). Given their ecological and economic importance, monitoring the health of wild populations and maintaining the health of cultivated stocks is of utmost importance. These filter-feeding bivalve molluscs are in constant and direct contact with a dynamically shifting aquatic microbial environment (Glasl et al. 2016; Pita et al. 2018). Food availability may vary in quantity and quality within short- (hours) and long-term (seasons) temporal scales, resulting in variable host nutritional states and potential susceptibility to pathogens. Indeed, periods of starvation may play an important role in determining 'tipping points' for mussel populations to survive or succumb to other factors thought to be involved in incidences of mass mortalities (e.g., disease, marine heatwaves). Mussels filter large volumes of seawater, capturing different types of particulate matter and microorganisms (Neori et al. 2004; Pagano et al. 2016). Host-microbe interactions are thought to play a key role in maintaining mussel health and organ-level functioning, but exposure to pathogenic microbes in the environment may lead to deleterious outcomes. However, little is known about the composition of the mussel's microbiome under various health states.

Animals can adapt to sudden short-term (<7 days) changes in diet or starvation via metabolic and physiological adjustments, as well as community shifts in their gut microbiomes (Furet et al. 2010; Xia et al. 2014). For example, some aquatic organisms can survive with limited food resources by utilizing alternative energy sources, such as ketone bodies, fatty acids and nitrogenous compounds produced by microorganisms in their gut (Kohl et al. 2014; Kohl and Carey 2016; Barreto-Curiel et al. 2017; Egerton et al. 2018). However, prolonged (>7 days) absence of food and nutrients may lead to shifts in microbial diversity and composition in the gut (Kohl et al. 2014). Alterations in structure and function of the microbial community affects the intestinal immune system, and can stimulate inflammatory responses in aquatic animals (Bailey 2014). In turn, a weakened immune system may lead to increased susceptibility to pathogen infections and other stresses, such as temperature, salinity, and pollutants, which compounded, may result in lower mortality thresholds (Dehler et al. 2017; Karl et al. 2018; Kers et al. 2018; Zha et al. 2018; Mir et al. 2019). Indeed, previous studies have shown that food limitations in juvenile *Perna canaliculus* mussels reduce their ability to cope with heat stress (Delorme et al. 2020). Additionally, starvation results in lowered oxygen consumption in blue mussels (*Mytilus edulis*) leading to significantly reduced behavioural activity (Tang and Riisgård 2018). However, little is known about the long-term impacts of starvation on the microbiome and health of mussels in general. Thus, the aims of this study were to: 1) profile the gut microbiome of healthy mussels under normal feeding, starvation and post-starvation recovery states, 2) identify the microbiome differences among these mussel groups, and 3) identify key patterns in microbiome alterations indicative of the effect of prolonged starvation and post-starvation recovery.

4.2 Material and Methods

4.2.1 Sample Collection

Approximately 100 healthy adult mussels (length = 92.8 mm ± 5.9; weight = 63.1 g ± 8.7) were collected from a rocky shore near Kaiaua, Firth of Thames, New Zealand (GPS coordinate: -37.0610, 175.3002) in September 2020 (autumn) (Figure. 12). Only mussels that were fully submerged during the lowest tide point (subtidal) were collected and immediately transported while submerged in a container with seawater to the marine laboratory at the Auckland University of Technology (AUT), Auckland, New Zealand (approximately 1-hour travel time). The mussels were then placed in a static 50 L seawater tank. The water temperature in the tank was maintained at 14°C, and oxygenated with air stones connected to an air pump. Five mussels were randomly selected (Beach group) and dissected to collect microbiome samples from the gut contents (see below). The rest of the mussels were left in the tank without any food (food deprived animals). The water in the tank was changed every two days to maintain good water quality parameters (pH = 8.2, Temperature = 14°C, Salinity = 35 ppt, DO = 9mg/L). The seawater was filtered through a 0.5-micron filter bag to remove any potential food particles before introduction into the tank where animals were held. After 14 days of starvation five additional mussels were randomly selected (Starvation group) for microbiome sampling in the same manner as the initial group. On the same day (day 14), another five mussels were collected from the same rocky shore location near Kaiaua, Firth of Thames, New Zealand, transported to the marine laboratory at AUT and sampled (as above) to serve as an in-situ control group (In-situ control 1 group). Additionally, on day 14, the left-over mussels in the tank were then transported from the laboratory back to the rocky shore where they had been collected from, and placed in a mesh bag (2cm wide openings) that was submerged and secured to the benthos. These mussels were allowed to recover from the starvation period. After 14 days of recovery time in the wild, five mussels randomly selected from the mesh bag (Recovery group) and

another five mussels from the surrounding area where the mesh bag was (In-situ control 2 group) were collected and transported back to the marine laboratory and sampled for gut microbiome analyses.

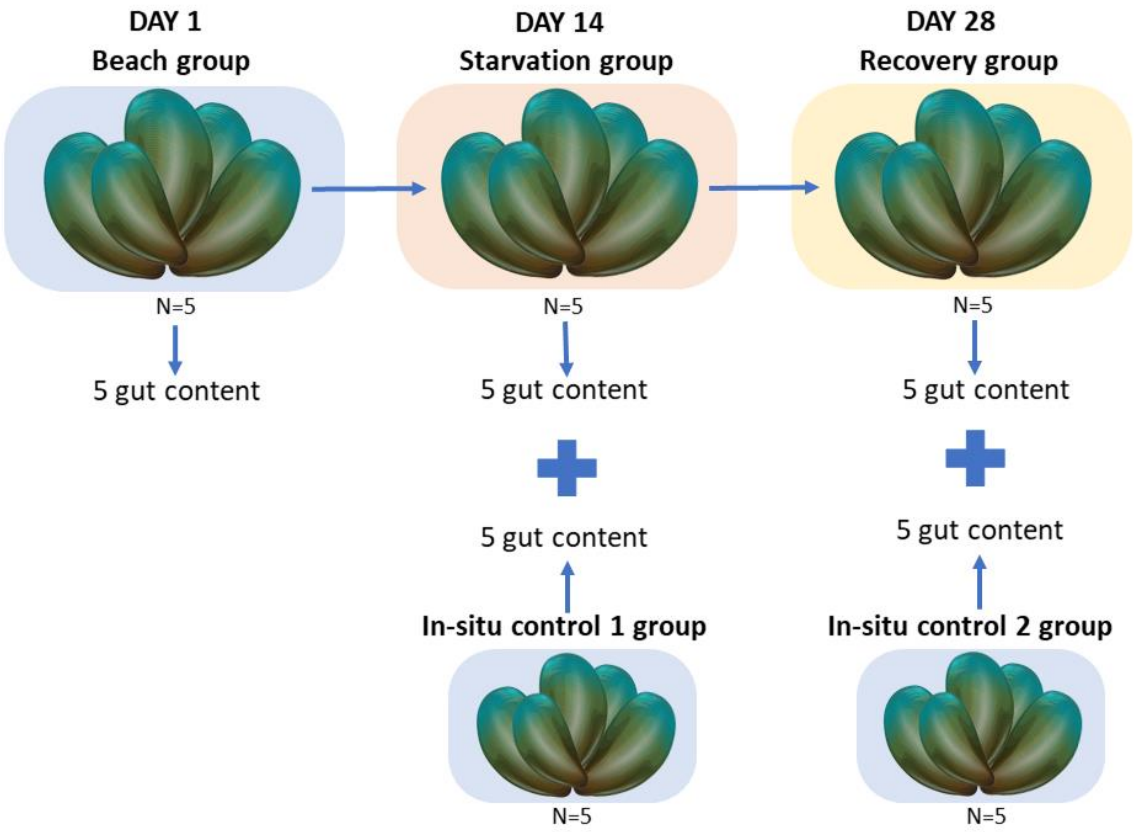


Figure 12. Schematic figure of experimental design: 100 mussels were collected from a natural coastal beach (Beach group). Mussels were then subjected to a 14 day starvation in a laboratory controlled static tank system. After 14 days 5 mussel gut were sampled from the 100 starved mussels (Starvation group) alongside 5 more mussels sampled from the original natural location to serve as controls (In-situ control 1 group. The rest of the starved mussels were placed back into the original natural beach in a mesh bag and were left to recover. After 14 days of recovery, 5 more mussels were sampled from the mesh bag (Recovery group) alongside 5 mussels sampled outside the mesh bag to serve as another control (In-situ control 2 group).

In order to collect gut microbiome samples, mussels were dissected by cutting the posterior and anterior adductor muscles using a sterile dissection knife. The mantle was peeled open with sterile forceps revealing the inner cavity. A cut was then made anteriorly near the oesophagus followed by another cut made posteriorly near the gastro-intestinal tract segment to isolate the

main stomach and the digestive gland. Then, the gut content (20–30 mg) was carefully removed from the mussel using sterile disposable forceps and transferred to sterile 2 mL cryovials (BioStor™) containing 20 µL RNA stabiliser (Qiagen, Germany), and immediately snap-frozen in liquid nitrogen and stored at –80°C until further analyses.

4.2.2 Microbial DNA extraction, PCR amplicon and sequencing

Frozen samples of gut contents were thawed and then homogenised using a FastPrep 24 system (MP Biomedicals; Irvine, California) at six movements per second for one minute prior to sub-sampling of uniform 250 µL volumes for DNA extraction. The total microbial DNA was extracted from the gut content samples using the DNeasy PowerSoil kit (Qiagen, Germany) following the manufacturer's instructions with the elution step repeated twice with 50 µL Tris elution buffer. Extracted DNA samples were stored at −20°C before subsequent processing.

Purified DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen; USA). MiSeq (Illumina, USA) libraries were prepared as per manufacturer's protocol (16S Metagenomic Sequencing Library Preparation; Part # 15044223; Rev. B [Illumina; San Diego, CA, USA]) and as previously described (Archer et al. 2020). PCR analyses were conducted with primer sets targeting the V3-V4 regions of the bacterial 16S rRNA gene: PCR1 forward (5' CCTACGGGNGGCWGCAG 3') and PCR1 reverse (5' GACTACHVGGGTATCTAATCC 3').

4.2.3 Bioinformatics and statistical analysis

The total bacterial sequence library size was 12,027,554 before filtering, and 4,727,038 read pair sequences passed quality filtering. Data were pre-processed using our established workflow (Archer et al. 2020). Briefly, 16S rRNA gene were processed using the R package DADA2 v1.8 (Callahan et al. 2016) and cutadapt v3.4 (Martin 2011) to remove forward (CCTACGGGNGGCWGCAG) and reverse (GACTACHVGGGTATCTAATCC) primer sequences for 16S rRNA genes. Bacterial reads were uniformly trimmed to 280 bp (forward) and 250 bp (reverse) and then filtered by removing reads exceeding maximum expected errors

of 2 for forward reads and 5 for reverse reads or reads containing ambiguity N. High quality bacterial reads were then clustered into amplicon sequence variants (ASVs), The resulting taxa were subsequently process using R v3.5.2 (R Core Team 2020). A total of 1386 amplicon sequence variants (ASVs) were inferred from high quality bacterial reads. which were assigned taxonomic ranks using R package DADA2 v1.8 (Callahan et al. 2016) and SILVA nr v132 database (Quast et al. 2013). MicrobiomeAnalyst (Dhariwal et al. 2017) was used to calculate alpha diversity and beta diversity; differences in group means/median were tested via Kruskal-Wallis. Multivariate interrogation of bacterial profiles were conducted using principal coordinates analysis (Bray-Curtis dissimilarity; tested using permutational MANOVA), hierarchical cluster analysis of samples (Bray-Curtis dissimilarity; Ward linkage), Heatmap with combined hierarchical cluster analysis of bacterial genera (Euclidean distance; Ward linkage), and co-occurrence network analysis based on sparse correlations for compositional (SparCC) data and using 100 permutations (correlation inclusion criteria: absolute r-value > 0.6, p-value < 0.05). The R packages phyloseq (McMurdie and Holmes 2013) and ggplot2 (Wickham 2011) were used to compare and visualise relative bacterial abundances differentials, and the R package ANCOM-BC (Lin and Peddada 2020) was used to test statistical significance of differential taxon abundances between sample groups.

4.3. Results

4.3.1. Richness and diversity of microbial communities

Amplicon sequencing of gut contents from all mussel samples generated a total sequence library size of 12,027,554 with 4,727,038 paired-end sequences passing quality filtering. High quality reads were clustered into 1386 bacterial ASVs. There was no significant difference in microbial richness or diversity between the sample groups for Chao1 (Kruskal-Wallis p-value: 0.40567) and Shannon (Kruskal-Wallis p-value: 0.15804) indices, respectively (Figure. 13).

However, a principal coordinate ordination analysis (PCoA) at the ASV level highlighted that the bacterial profiles of the Starvation group were distinctively clustered apart from the other sample groups (F-value: 2.567; R-squared: 0.34; p-value < 0.001) (Figure. 14). The permutational MANOVA yielded an R-squared value of 0.46 and p-value < 0.001.

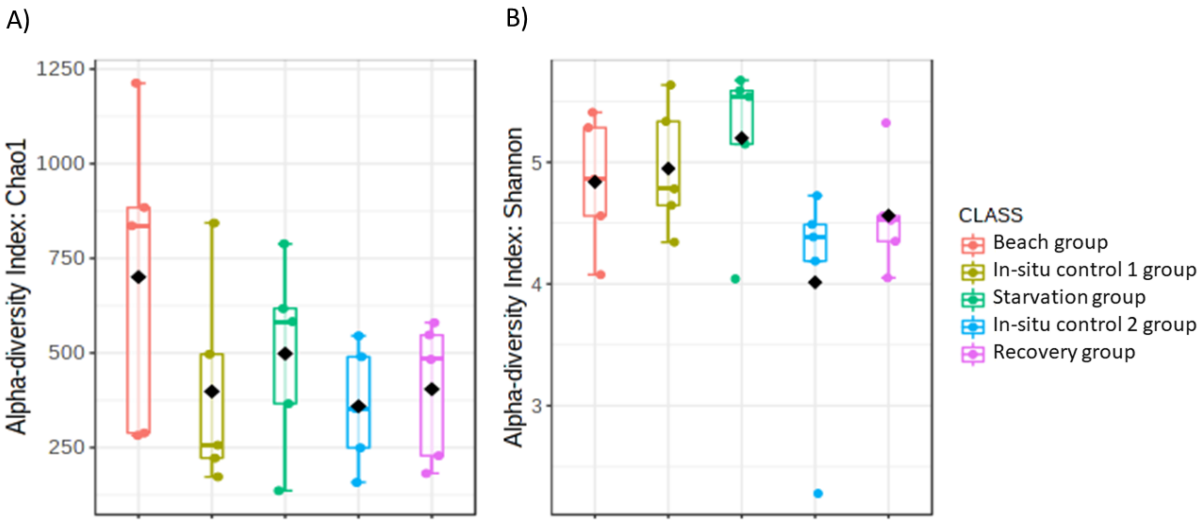


Figure 13. Richness and diversity indices for a Chao1 and b Shannon at ASV level. Black diamonds represent the mean of the data.

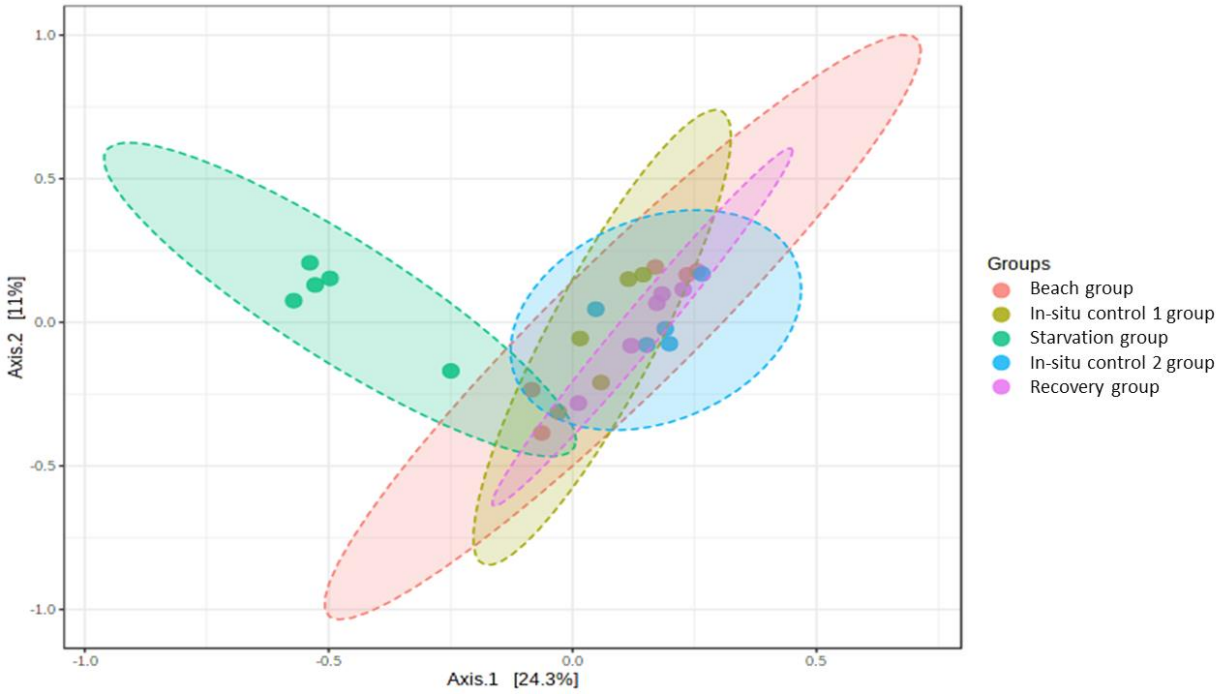


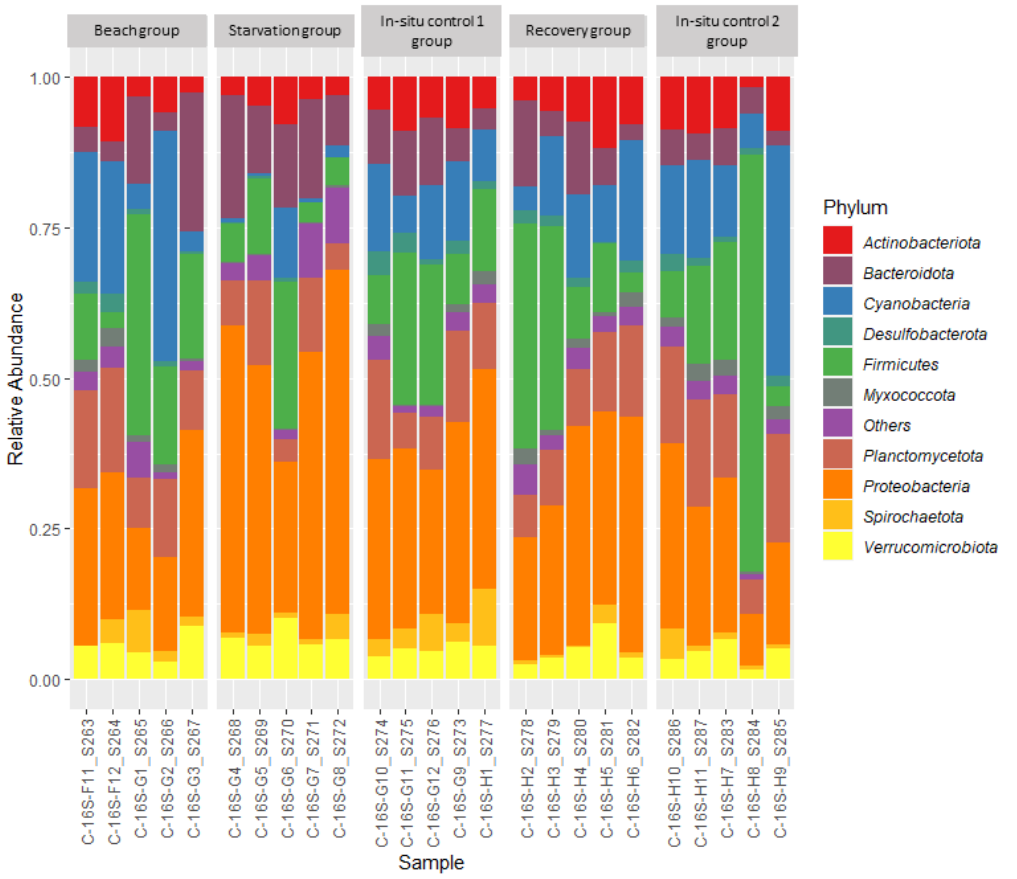
Figure 14. Principal coordinates analysis (PCoA) of mussels in the different groups based on gut bacterial profiles (ASV). Eclipses represent 95% confidence.

4.3.2. Microbial community structures analysis

Phylum-level community profiles at the different experimental points revealed distinct bacterial compositional patterns, particularly separating the starvation group from the others (Figure. 15a). Most noticeably, an elevation in dominance of Proteobacteria (Beach group = 24%, Starvation group = 47%, Recovery group = 25%; ANCOM-BC p-value < 0.001) and Bacteroidota (Beach group = 5%, Starvation group = 12%, Recovery group = 6%; ANCOM-BC p-value = 0.04) and lower relative abundance of Cyanobacteria (Beach group = 24%, Starvation group = 4%, Recovery group = 19%; ANCOM-BC p-value = 0.01) were observed in the starvation group compared with all other sample groups. Further analysis of the most abundant genera across the groups revealed that in the Starvation group, *Halioglobus* were richer (p-value = 0.001 and had higher abundance compared to the other groups, whereas, the

relative abundance of *Synechococcus* CC9902, a photosynthetic marine plankton (Kim et al. 2018) were lower (p-value = 0.04) in the Starvation group (Figure. 15b).

A)


Beach group
Starvation group
In-situ control 1 group
Recovery group
In-situ control 2 group
Relative Abundance
1.00
0.75
0.50
0.25
0.00
C-16S-F11_S263
C-16S-F12_S264
C-16S-G1_S265
C-16S-G2_S266
C-16S-G3_S267
C-16S-G4_S268
C-16S-G5_S269
C-16S-G6_S270
C-16S-G7_S271
C-16S-G8_S272
C-16S-G10_S274
C-16S-G11_S275
C-16S-G12_S276
C-16S-G9_S273
C-16S-H1_S277
C-16S-H2_S278
C-16S-H3_S279
C-16S-H4_S280
C-16S-H5_S281
C-16S-H6_S282
C-16S-H10_S286
C-16S-H11_S287
C-16S-H7_S283
C-16S-H8_S284
C-16S-H9_S285
Sample
Phylum
Actinobacteriota
Bacteroidota
Cyanobacteria
Desulfobacterota
Firmicutes
Myxococcota
Others
Planctomycetota
Proteobacteria
Spirochaetota
Verrucomicrobiota

B)

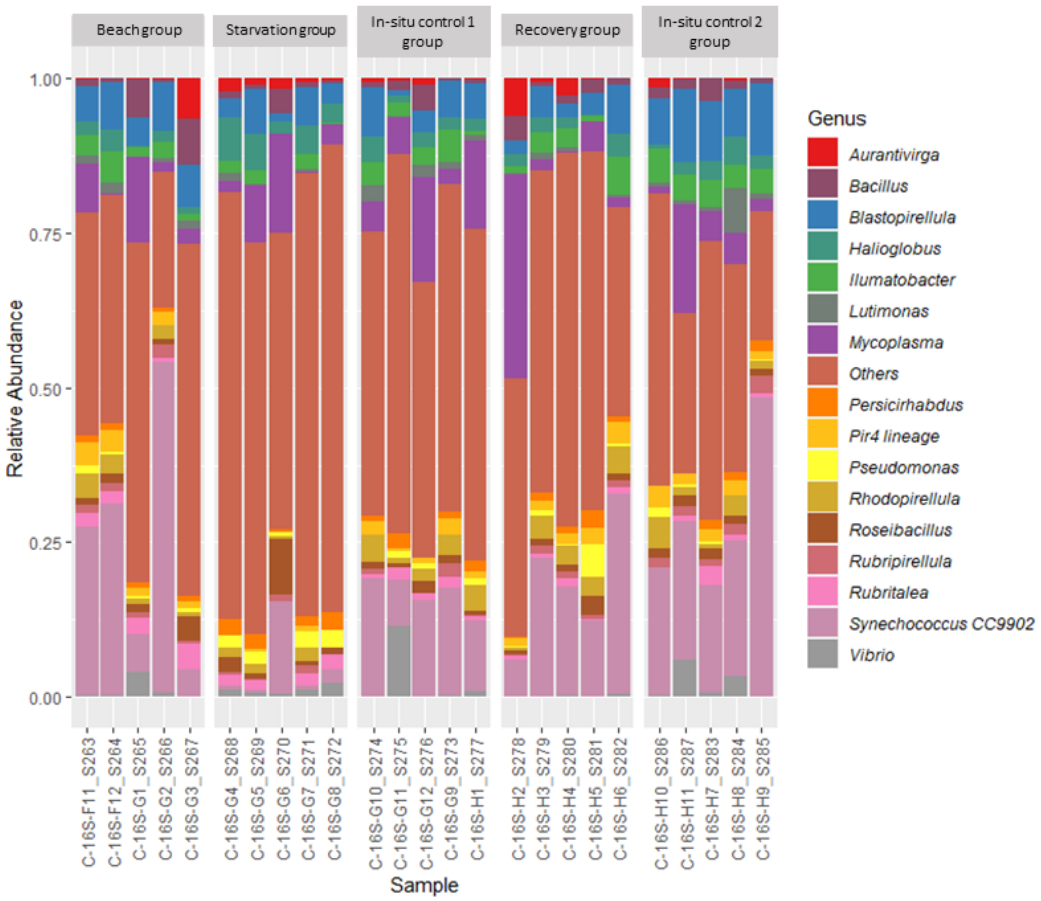

Beach group
Starvation group
In-situ control 1 group
Recovery group
In-situ control 2 group
Relative Abundance
1.00
0.75
0.50
0.25
0.00
C-16S-F11_S263
C-16S-F12_S264
C-16S-G1_S265
C-16S-G2_S266
C-16S-G3_S267
C-16S-G4_S268
C-16S-G5_S269
C-16S-G6_S270
C-16S-G7_S271
C-16S-G8_S272
C-16S-G10_S274
C-16S-G11_S275
C-16S-G12_S276
C-16S-G9_S273
C-16S-H1_S277
C-16S-H2_S278
C-16S-H3_S279
C-16S-H4_S280
C-16S-H5_S281
C-16S-H6_S282
C-16S-H10_S286
C-16S-H11_S287
C-16S-H7_S283
C-16S-H8_S284
C-16S-H9_S285
Sample
Genus
Aurantivirga
Bacillus
Blastopirellula
Halioglobus
Ilumatobacter
Lutimonas
Mycoplasma
Others
Persicirhabdus
Pir4 lineage
Pseudomonas
Rhodopirellula
Roseibacillus
Rubripirellula
Rubritalea
Synechococcus CC9902
Vibrio

Figure 15. Phylogenetic classification of the bacterial communities: a at the phylum level, and b at the genus level.

Clustering of the top 20 most abundant genera revealed four bacterial genera which belonged to Proteobacteria (Figure. 16a). These genera, including *Halioglobus*, *Amylibacter*, *Sulfitobacter*, *Vicingus* were clustered together and had higher relative abundances in the Starvation group. Finally, the bacterial profile dissimilarity at the genus level between the starved mussels and those in other groups were further highlighted via a dendrogram (Figure. 16b), with most members of the Starvation group being clustered separately. Additionally, the SparCC network analysis returned a complex structure depicting the interactive associations of the prevalent genera with others (Figure 17). Interestingly, we observed that *Synechococcus* CC9902 had significant negative correlations with 14 genera (r-values > 0.6 and p-values < 0.01). The colour of the nodes in the lattice indicated the occurrence of these specific 14 genera are elevated in starved mussel, forming a co-occurrence cluster.

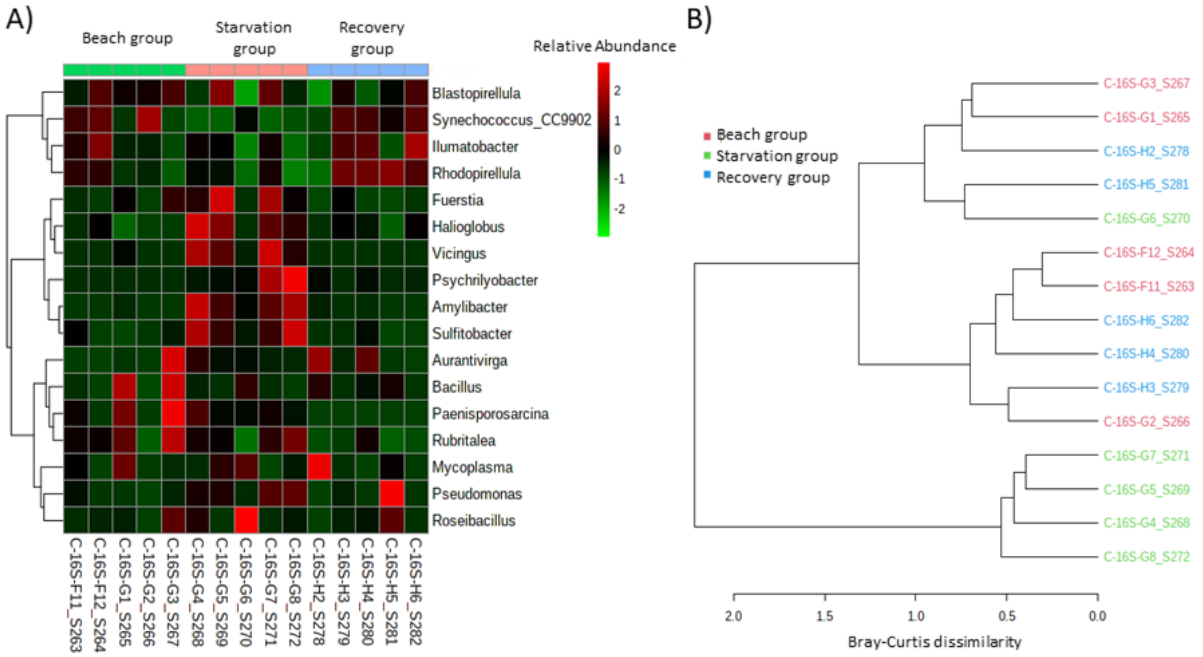


Figure 16. a Heatmap and cluster analysis of the top 20 (3 unclassified genera were removed) most abundant genera. Bacterial genera are shown row-wise, samples are shown column-wise and coloured by scaled relative abundance. b Hierarchical clustering dendrogram (Ward algorithm) constructed via Bray-Curtis distances of mussel samples based on genera.

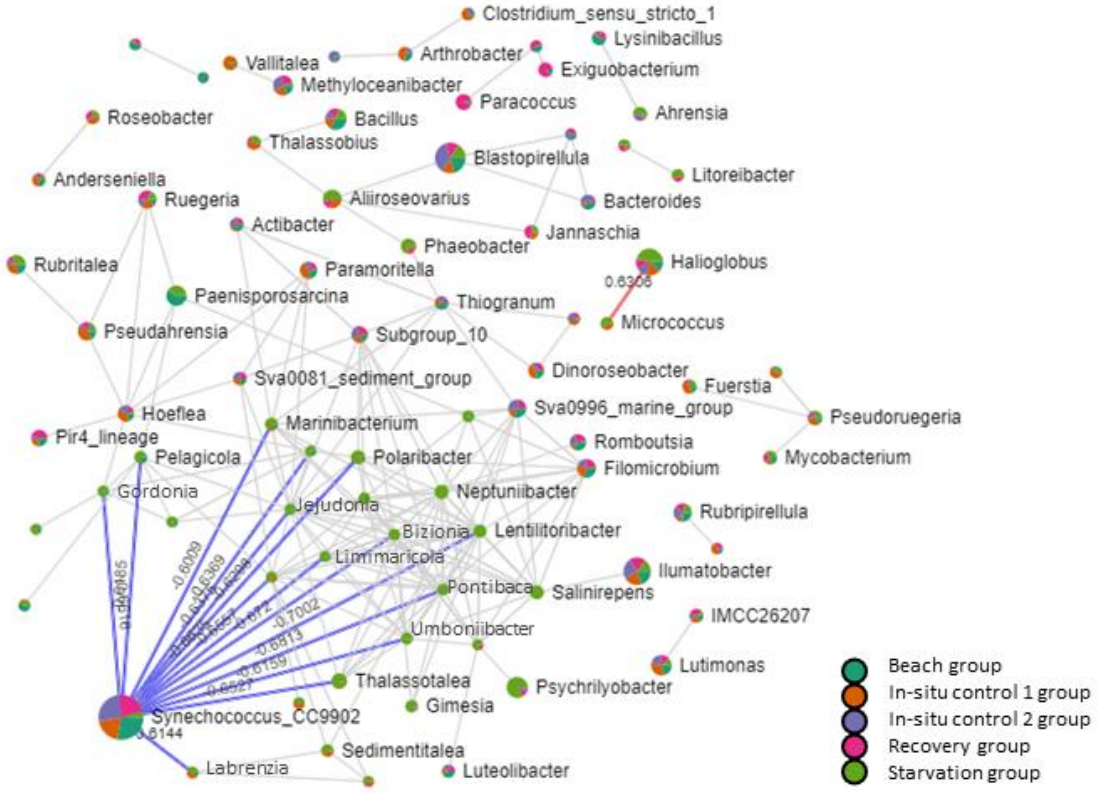


Figure 17. Co-occurrence network analysis of significant correlations (SparCC; absolute r-values > 0.6; p-value < 0.05) between genera. Each node represents a bacterial genus, and the edges represent the correlation coefficients between the genera. Synechococcus (CC9902) were selected as a significant hub genus and the blue edges from this node represents negative correlations between Synechococcus_CC9902 and other genera. The values on the edges signify the correlation coefficients. Nodes are coloured according to the groups (Beach group, In-situ control 1 group, In-situ control 2 group, Recovery group, and Starvation group).

4.4 Discussion

Shellfish such as mussels can experience long periods of food-limitation which may lead to starvation. However, knowledge on how the mussel gut microbiota responds to starvation remains scarce. This study provides the first exploratory analysis into the effect of starvation on the gut microbiome of *P. canaliculus*. The microbiome profile of mussels prior and post-starvation was compared and analysed to identify microbiome structural changes, key taxon and taxon interactions, and host-microbiome interactions indicative of stress levels and scope for recovery.

Richness and diversity of gut microbial communities were similar among groups of mussels before, during and after starvation. This is interesting since an artificial marine environment was created in the laboratory to starve the mussels, and previous studies have shown that artificial environments rarely maintain the same bacterial diversity as the original or natural environments (Patin et al. 2018). However, our results revealed no statistically significant shifts in alpha diversity between the starvation and control groups. The stability of bacterial richness may not be a good indicator for the effect of starvation on bacterial counts as opposed to other effects, such as seasonal (Häder et al. 1998) and temperature (Tiefenthaler et al. 2009; Vargas et al. 2021) changes. A potential reason for this result could be the re-stabilisation capacity of *P. canaliculus* microbiome towards the effects of starvation. Some aquatic animals can utilise nitrogenous compounds produced by various microorganisms in their gut as an alternative energy source during short-term starvation (Kohl et al. 2014; Kohl and Carey 2016; Barreto-Curiel et al. 2017; Egerton et al. 2018), hence promoting the diversity of such microorganisms. However, mussels which were starved had a distinct microbiome profile with a short-term effect on gut microbiome composition that was indicative of the starvation state. The mussel microbiome reverted to that of pre-starvation state after only 14 days, matching the microbiome profile of the in-situ controls. These findings suggest a fast recovery and further highlights the potential inherent resilience of the species to intermittent food supplies or starvation stresses during periods of low food availability.

The microbiome communities largely altered in response to host starvation were relative abundances of Proteobacteria (gamma), Bacteroidetes, Firmicutes and Cyanobacteria phyla. The most indicative change in phyla associated with the starvation period was an increase in the abundance of Proteobacteria (gamma). These shifts are consistent with studies on the effect

of starvation of zebrafish (*Danio rerio*) (Semova et al. 2012) and grass carp (*Ctenopharyngodon idellus*) (Tran et al. 2018). Previous studies have also reported that Proteobacteria were associated with unstable gut microbiota, energy instability, and inflammation (Shin et al. 2015; Tran et al. 2018). The increased Proteobacteria in post-starvation mussels followed by its decreased abundance in post-recovery mussels suggests the association of Proteobacteria with starvation state, and the mussel's ability to rapidly restore its intestinal microbiota and energy cycle. The Phylum Bacteroides was also found to have higher relative abundance in starved *P. canaliculus*. However, there are contradicting reports on whether this group of bacteria is commonly associated with starvation, and may highlight Bacteroides as a poor signature of the starvation condition. For example, Nile tilapia (*Oreochromis niloticus*) were shown to have lower abundance of Bacteroidetes after 14 days of starvation (Sakyi et al. 2020), whereas starvation in loach (*Paramisgurnus dabryanus*) revealed a higher abundance of Bacteroidetes (Peter et al. 2020). A large part of the proteins made by the Bacteroides genome are able to break down polysaccharides and metabolize their sugars (Xu et al. 2003). They play a fundamental role in the processing of complex molecules to simpler ones in the host intestine. Their ability to harvest alternative energy sources from food might allow Bacteroides to be more competitive than other bacteria in the *P. canaliculus* gut during starvation. (Flint et al. 2012; Lapébie et al. 2019). Finally, a significantly lower relative abundance of Cyanobacteria was detected in the starved mussels. This result was not surprising considering that the Phylum Cyanobacteria is a dominant group in the water column where they form an important part of the phytoplankton (Moore et al. 2019). Cyanobacteria are rich in proteins and contain carotenoids, vitamins, minerals, and essential fatty acids (Wells et al. 2017), and contribute significantly to the diet of most bivalve filter feeders. Some bivalve species, such as the swan mussel (*Anodonta cygnea) have been shown to preferentially select Cyanobacteria from the water column to boost their nutritional state and contribute to the*

accumulation of energy reserves during host gametogenesis (Lopes-Lima et al. 2014). Therefore, a decrease in Cyanobacteria in starved *P. canaliculus* can potentially be reflective of the mussel's energy deprivation and subsequently hinder its reproductive function. Another area for potential future work is to perform functional analysis on these community changes as only specific taxa within these groups have known functions.

At the genus level, the most obvious microbiome community alterations can be seen in the increase in *Halioglobus* and decrease in *Synechococus* (strain CC9902) in the starvation group. These species are involved in various chemical processes and have mostly been recorded in ocean and coastal areas. For example, *Halioglobus* was previously isolated from the coast of Japan and has been shown to have 18 genes related to denitrification (Park et al. 2012). *Synechococcus* cyanobacteria are one of the most important components of photosynthetic picoplankton (Partensky et al. 1999; Flombaum et al. 2013; Sohm et al. 2016), and their lowered abundance is reflective of this food source being unavailable to the mussels in the starvation group. However, information on host-microbiome interactions of theses genera and how they are modulated by host starvation remains scarce in molluscs. Therefore, in this study we performed exploratory investigations on bacterial co-occurrence via SparCC at the genus level to provide novel information on the underlying correlations of microbial community structure with regards to mussel starvation and post-starvation recovery. Bacteria interact extensively within and among species while responding to external stimuli. The dynamics of bacterial communities are determined by pairwise interactions that occur between species in the community (Stubbendieck et al. 2016). In the present study, our results further highlight the genus *Synechococus* (strain CC9902), and its association to multiple genera during mussel starvation. Indeed, this subset of genera has highly elevated community abundance during starvation. Although functional insights of these genera are currently lacking, our network

association analysis offers a starting point to seek metabolic implications for these correlations and their roles on host physiology.

4.5 Conclusions

We identified a microbial community dominated by Proteobacteria, Bacteroidota, and Cynaobacteria that rapidly shifted during mussel starvation then quickly returned to their natural state as mussels recovered from starvation. Specific shifts in bacterial communities appear to be a response to starvation, potentially associated with the host's energy stability and absorption from lack of food source. Our results highlight lowered community composition of *Synechococus* (strain CC9902) and its co-occurrence with multiple genera which were elevated during mussel starvation. These results offer insights into the effect of starvation on subsets of genus-level compositions and associations with one another. The findings of this study provide new knowledge on host–bacteria interactions within the gut microbiome in *P. canaliculus* during starvation stress and recovery and also serve as a foundation for further functional analysis of environmental effects on host-microbiome interactions and mussel physiology.

Chapter 5

Effect of Summer Mortality on the Gill Tissue Microbiome of Green-Lipped Mussels, *Perna canaliculus*

5.1. Introduction

Bivalves are a major export cornerstone in the aquaculture industry, which contributes 89% of global aquaculture production (FAO 2021). These valuable cultured species, consisting of mussels, clams, scallops and oysters reached a combined production value of 5.7 million tonnes (valued at 26.6 billion USD), and accounted for 90% of molluscan production and 14% of all aquaculture production in 2017 (FAO 2021). This well-established industry with sophisticated farming technologies produces premium shellfish in countries such as China, Republic of Korea, Chile, Vietnam, Spain, Thailand, U.S.A, France, Italy and New Zealand. However, massive mortality outbreaks have significantly threatened global cultivation of many bivalve species, such as Pacific oysters (*Crassostrea gigas*) (Alfaro et al. 2019b; Clerissi et al. 2020; Kim et al. 2020), European flat oysters (*Ostrea edulis*) (Charles et al. 2020; Fleury et al. 2020), blue mussels (*Mytilus edulis*) (Mydlarz et al. 2006; Seuront et al. 2019), and New Zealand flat oysters (*Ostrea chilensis*) (Lane et al. 2016). These massive mortality events predominate in the summer when shellfish experience thermal stress and pathogen loads often overwhelming their immune systems. Different pathogens and parasites have been predominantly linked with different shellfish hosts. For example, reports have shown massive Pacific oyster mortality events during summer months in more than 12 countries associated with the *Ostreid herpesvirus 1* (OsHV-1) and its variants (Alfaro et al. 2019b).

The endemic New Zealand Greenshell™ mussel or (*Perna canaliculus*) industry is worth over $300 million in export revenues (Aquaculture New Zealand 2017). Although *P. canaliculus* have experienced relatively few health issues compared to other cultured shellfish, there are several pathogens and parasites that affect farmed mussels from time to time. *Vibrio* spp., a group of Gram-negative bacteria associated with numerous infectious diseases in marine bivalves has been reported in *P. canaliculus* (Webb 2008). Digestive epithelial virosis, caused by an unenveloped RNA virus, has been implicated in multiple moderate to severe mortality cases (Jones et al. 1996; Diggles et al. 2002; Renault and Novoa 2004; Renault 2006). Other pathogens, such as funguses, protozoa and platyhelminthes have also been recorded in *P. canaliculus* (Webb 2008; Castinel et al. 2019a). In the last two years, there have been multiple reports of mass mortalities on mussel farms of the North Island of New Zealand during summer months. Despite significant industry loses, the reason for these events remains unknown. However, it is believed that these mortalities are potentially associated with thermal stress caused by increasing water temperatures due to climate change (Dunphy et al. 2015). The combination of thermal stress with pathogen loads which appear to proliferate during the summer may lead to physiological 'tipping points' during these events. Given the ecological and economic importance of *Perna canaliculus*, maintaining health of wild and domesticated stocks are of utmost importance. Host-microbe interactions are thought to play a key role in maintaining mussel health and organ-level functioning, but exposure to thermal stress and pathogenic microbes in the environment may lead to deleterious outcomes.

Marine microbiota dynamically interact with surroundings environmental conditions modulated by temperature, nutrients, salinity and oxygen levels and have well established roles in pathogen exclusion and host immunity, including systemic and mucosal innate and adaptive immune responses and development of the immune system (Cui et al. 2019; Sehnal et al. 2021).

Therefore, alterations of the *Perna canaliculus* microbiome might be indicative of stressful environmental conditions and pathogen loads during summer mortality. However, little is known about the composition of the mussel's microbiome under various health states. Studies of the relationships of resident bacteria, stress, and disease in other marine organisms have yielded some insights into the interactions among these factors. Egan and Gardiner (2016) have proposed that many diseases of marine organisms of unknown causes may be the result of multiple microorganisms contributing to disease progression (Egan and Gardiner 2016). Some of these diseases may also be the result of a proliferation of opportunistic pathogens when microbial imbalance (dysbiosis) occurs as a result of environmental changes or declining host resistance due to stress or senescence (Althani et al. 2016). Meres et al. (2012) studied epizootic shell disease of the American lobster and found that among the 170 bacterial taxa that were identified, 58 were helpful in discriminating diseased and healthy states, although no single causative agent was identified. The authors concluded that epizootic shell disease is caused by a dysbiotic shift in the shell microbial community, and may be the result of stress induced by environmental factors that cause opportunistic bacterial invasion of the carapace (Meres et al. 2012). Finally, Wegner et al. (2013) studied bacterial diversity associated with the gill and gut of the invasive Pacific oyster *Crassotrea gigas* under non-stressed and thermally stressed conditions. The authors also observed shifts in the composition of the microbiome after stress, with *Mycoplasma* becoming dominant notably in the gut, whereas the numbers of other bacteria such as *Flavobacteria* decreased in the gut (Wegner et al. 2013).

The aim of this study was to investigate the microbiome from gill and hepatopancreas tissues of farmed *Perna canaliculus* collected during summer mortality events using 16S rDNA amplicon sequencing. Specifically, microbiome differences between healthy and unhealthy mussels, such as distinct perturbations of bacterial species, alterations in bacterial diversity and

structure were used to identify potential interactions between host and significant bacterial species and their contribution to the detrimental health effects of summer mortality.

5.2. Methods

5.2.1. Sample Collection

A mussel summer mortality event was investigated at a mussel farm in Kaiaua, Firth of Thames, North Island, New Zealand during April 2018. This mortality event coincided with an unprecedented heatwave for the country, with record sea surface temperatures for the region (Salinger et al. 2019). High mortalities were observed on some dropper lines by farmers, while other lines appeared to have 'healthy' mussels. Twenty-five mussels were collected from 'healthy' lines and 25 mussels were collected from 'unhealthy' lines (adults ca. 2 yrs old; weight 59.96 ± 8.56 g; shell length 9.4 ± 0.48 cm). Immediately after collection, mussels from each group were transported in separate cool and moist polystyrene boxes to the Aquaculture Laboratory (Auckland University of Technology, Auckland, New Zealand; 3-hour transport time). Upon arrival, mussels were acclimated in two separate tanks with re-circulating seawater at 17C° for 24 hours to recover from the transport stress. Mussels were examined for their behavioural responses to gentle manipulation. Mussels from lines experiencing high mortality were much slower to respond (shell closure) compared to the other group. Internal examination of a randomly selected subgroup revealed clear differences between healthy and unhealthy mussels. Most notably, unhealthy mussels had abnormal looking tissues and internal organs had extensive mucus. In addition, haemolymph samples were taken from another set of mussels from each group, placed on slides, Giemsa stained and microscopically examined. Numerous rod-shaped bacilli were observed in the haemolymph of unhealthy mussels, while no or very few bacteria were found in samples from the healthy mussels (Figure 3).

Eight randomly selected healthy and 10 unhealthy mussels were cleaned externally with fresh seawater. The gills and hepatopancreas of these mussels were excised and placed in 2 mL cryovials (BioStor™), then immediately snap-frozen in liquid nitrogen and stored at –80°C until further analyses.

5.2.2. Microbial DNA extraction

Gill and hepatopancrease tissues were lyophilised and grounded under liquid nitrogen using a mortar and pestle. Total microbial DNA was extracted according to the adapted protocol of Musella et al. (2020). Briefly, 20–30 mg of mussel tissue sample was used by the DNeasy PowerSoil kit (Qiagen, Germany) according to the manufacturer's instructions. Tissues were lysed using a FastPrep system MP Biomedicals; Irvine, CA) at six movements per second for one minute prior to extraction. The elution step from the DNeasy PowerSoil kit was repeated twice in 50 µL Tris elution buffer, incubating the columns for five minutes at 4°C before centrifugation. DNA samples were stored at −20°C before subsequent processing.

5.2.3. PCR amplicon and sequencing

Purified DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen; USA). MiSeq (Illumina, USA) libraries were prepared as per manufacturer's protocol (16S Metagenomic Sequencing Library Preparation; Part # 15044223; Rev. B [Illumina; San Diego, CA, USA]) and as previously described (Archer et al. 2020). PCR was conducted with primer sets targeting the V3-V4 regions of the bacterial 16S rRNA gene: PCR1 forward (5' CCTACGGGNGGCWGCAG 3') and PCR1 reverse (5' GACTACHVGGGTATCTAATCC 3').

5.2.4. Bioinformatics and statistical analysis

Data were pre-processed using our established workflow (Archer et al. 2020). 16S rRNA gene were processed using the R package DADA2 v1.8 (Callahan et al. 2016) and cutadapt v3.4

(Martin 2011) to remove forward (CCTACGGGNGGCWGCAG) and reverse (GACTACHVGGGTATCTAATCC) primer sequences for 16S rRNA genes. Bacterial reads were uniformly trimmed to 280 bp (forward) and 250 bp (reverse) and then filtered by removing reads exceeding maximum expected errors of 2 for forward reads and 5 for reverse reads or reads containing ambiguity N. High quality bacterial reads were then clustered into amplicon sequence variants (ASVs), The resulting taxa were subsequently process using R v3.5.2 (R Core Team 2020) and were assigned taxonomic ranks using R package DADA2 v1.8 (Callahan et al. 2016) and SILVA nr v132 database (Quast et al. 2013). MicrobiomeAnalyst (Dhariwal et al. 2017) was used to calculate alpha diversity and beta diversity. Differences in group means/median were tested via Kruskal-Wallis. Multivariate interrogation of bacterial profiles were conducted using principal coordinates analysis (Bray-Curtis dissimilarity; tested using permutational MANOVA), hierarchical cluster analysis of samples (Bray-Curtis dissimilarity; Ward linkage), and co-occurrence network analysis based on sparse correlations for compositional (SparCC) data and using 100 permutations (correlation inclusion criteria: absolute r-value > 0.5, p-value < 0.05). Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to determine the taxa contributing to the effect size between healthy and unhealthy gill tissue and hepatopancreas tissue microbial communities. This analysis incorporated the non-parametric Kruskal-Wallis sum-rank test for significant differential abundance set at a significance of $p = 0.05$, followed by LDA to estimate effect size at log (10) values. The R packages phyloseq (McMurdie and Holmes 2013) and ggplot2 (Wickham 2011) were used to compare and visualise relative bacterial abundances differentials, and the R package Analysis of compositions of microbiomes with bias correction (ANCOM-BC) (Lin and Peddada 2020) was used to test statistical significance of differential taxon abundances between sample groups.

5.3. Results

5.3.1 Richness and diversity of microbial communities

Amplicon sequencing of gill and hepatopancreas tissues from all mussel samples generated a total sequence library size of 2,393,362 with 1,453,538 paired-end sequences passing quality filtering. High quality reads were clustered into 2385 bacterial ASVs. There were significant differences in microbial richness and diversity between healthy and unhealthy gill tissue groups for Chao1 (Kruskal-Wallis p-value: 0.004) and Shannon (Kruskal-Wallis p-value: 0.003) indices, respectively (Figure 18A). For hepatopancreas tissues, no significant diversity differences were observed between healthy and unhealthy groups (Chao1 Kruskal-Wallis p-value: 0.97 and Shannon Kruskal-Wallis p-value: 0.9) (Figure 18B). NDMS ordination analysis at the ASV level highlighted that the bacterial profiles of the healthy group were distinctively clustered apart from those of the unhealthy group (Figure. 19A). The permutational MANOVA yielded an R-squared value of 0.46 and p-value < 0.001. The bacterial profile dissimilarity at the ASV level between the healthy and unhealthy gill tissue groups were further highlighted via a dendrogram (Figure 19B), with most members of the healthy group being clustered separately from the healthy group. Regarding the hepatopancreas tissue samples, no distinctively clustering was observed between healthy and unhealthy tissues (Figure 19C&D).

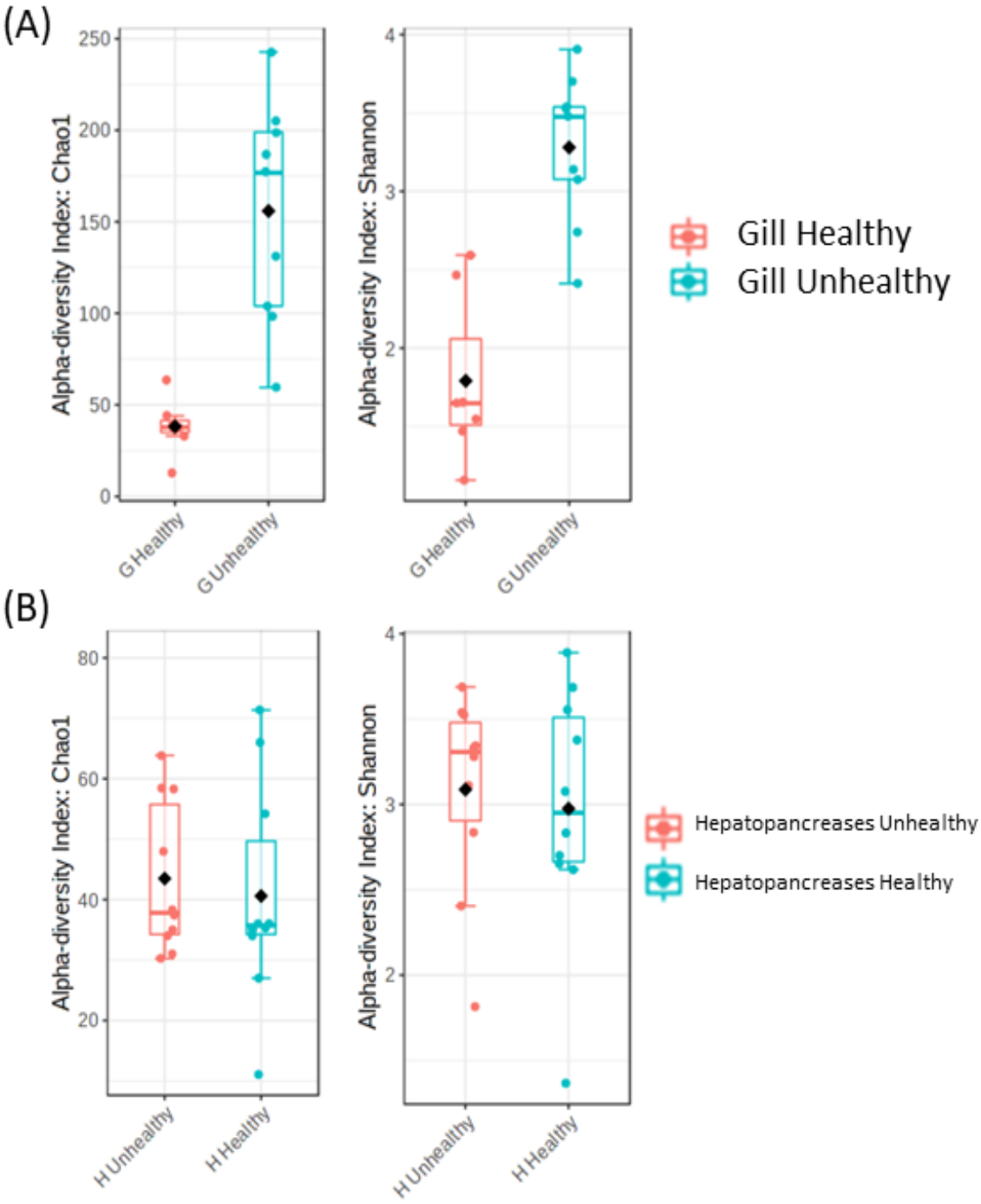


*Figure 18. Non-metric multidimensional scaling (*NMDS*) of (A) gill tissue samples and (C) hepatopancreases samples in the different groups based on gut bacterial profiles (ASV). Eclipses represent 95% confidence. (B) Hierarchical clustering dendrogram (Ward algorithm) constructed via Bray-Curtis distances of gill tissue samples based on genera. (D) Hierarchical clustering dendrogram (Ward algorithm) constructed via Bray-Curtis distances of hepatopancreas samples based on genera.*

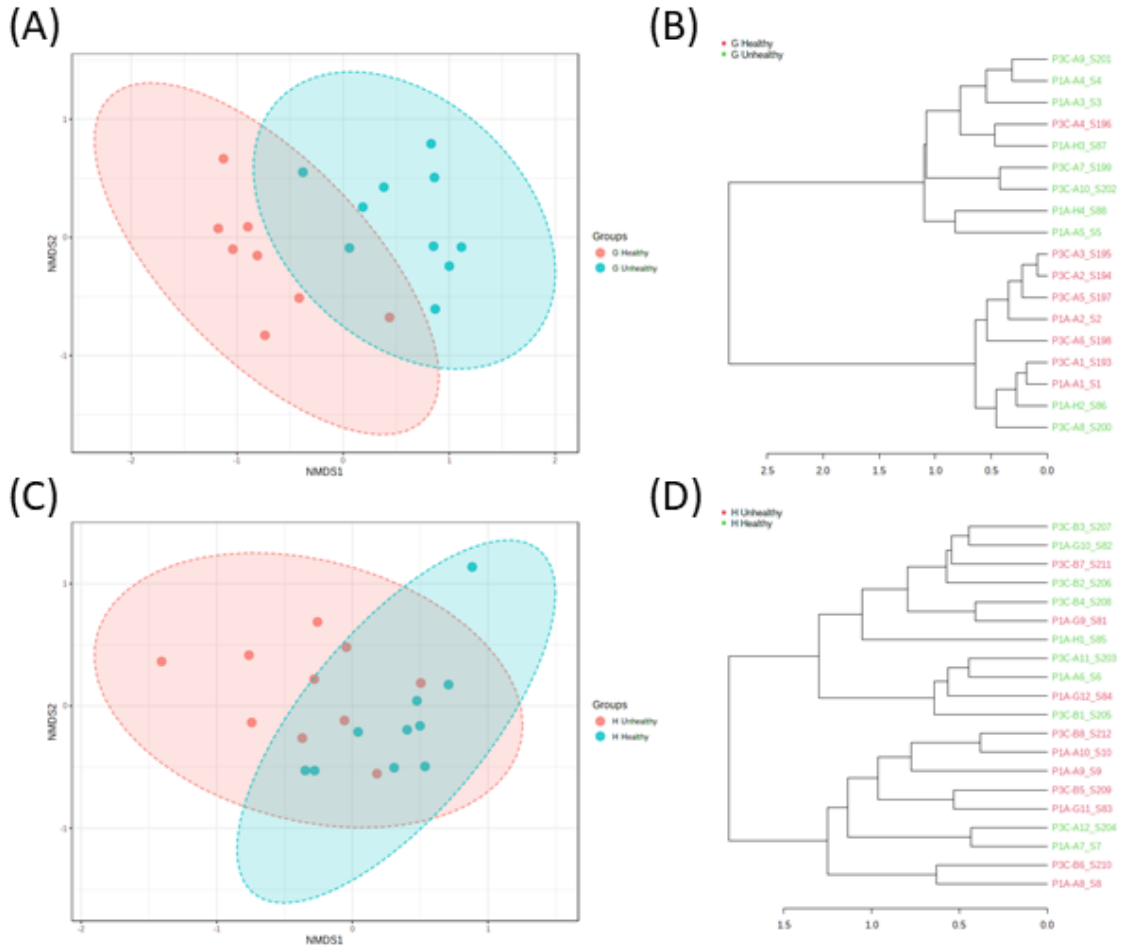


Figure 19. Non-metric multidimensional scaling (*NMDS*) of (A) gill tissue samples and (C) hepatopancreas samples in the different groups based on gut bacterial profiles (ASV). Eclipses represent 95% confidence. (B) Hierarchical clustering dendrogram (Ward algorithm) constructed via Bray-Curtis distances of gill tissue samples based on genera. (D) Hierarchical clustering dendrogram (Ward algorithm) constructed via Bray-Curtis distances of hepatopancreas samples based on genera.

5.3.2. Microbial community structures analysis

Phylum-level community profiles between healthy and unhealthy gill tissues revealed distinct bacterial composition patterns (Figure 20A). Most noticeably, healthy and unhealthy gill tissues were dominated by the Phylum Gammaproteobacteria with 94% and 82%, respectively (ANCOM-BC p-value = 3.8e-3, q value = 4.9e-2). Additionally, there was a significantly elevated dominance of Bacteroidota (healthy group = 2.4%, unhealthy group = 9%; ANCOM-

BC p-value = 0.02; q-value = 0.003), Campilobacterota (healthy group = < 0.1%, unhealthy group = 4%; ANCOM-BC p-value = 3.1e-6; q value = 5.3e-3) and Desulfobacterota (healthy group = < 0.1%, unhealthy group = 0.9%; ANCOM-BC p-value = 2e-4; q-value = 3.3e-3) in the gill tissue of both groups. Further examination of the gill tissue community structure at the genus level revealed alterations of numerous genera (Figure 20B). Particularly, and elevation abundance of *Endozoicomonas* (healthy group = 36%, unhealthy group = 80%; ANCOM-BC p-value = 1.7e-7; q value = 2.6e-5), *Vibrio* (healthy group = 5%, unhealthy group = 15%; ANCOM-BC p-value = 1.6e-3; q value = 0.02), *Aliivibrio* (healthy group = < 1%, unhealthy group = 7%; ANCOM-BC p-value = 2.2e-7; q value = 3.4e-5), and *Roseimarinus* (healthy group = 1.3%, unhealthy group = 15%; ANCOM-BC p-value = 7e-12; q value = 1e-10). In addition, Linear discriminant analysis Effect Size (LEfSe) of the gill tissues at a logarithmic discriminant analysis (LDA) score of ±2 showed relevance or effect size of differential genera of the gill tissue to be *Endozoicomonas*, *Aliivibrio*, *Vibrio*, and *Roseimarinus* (Figure 21A).

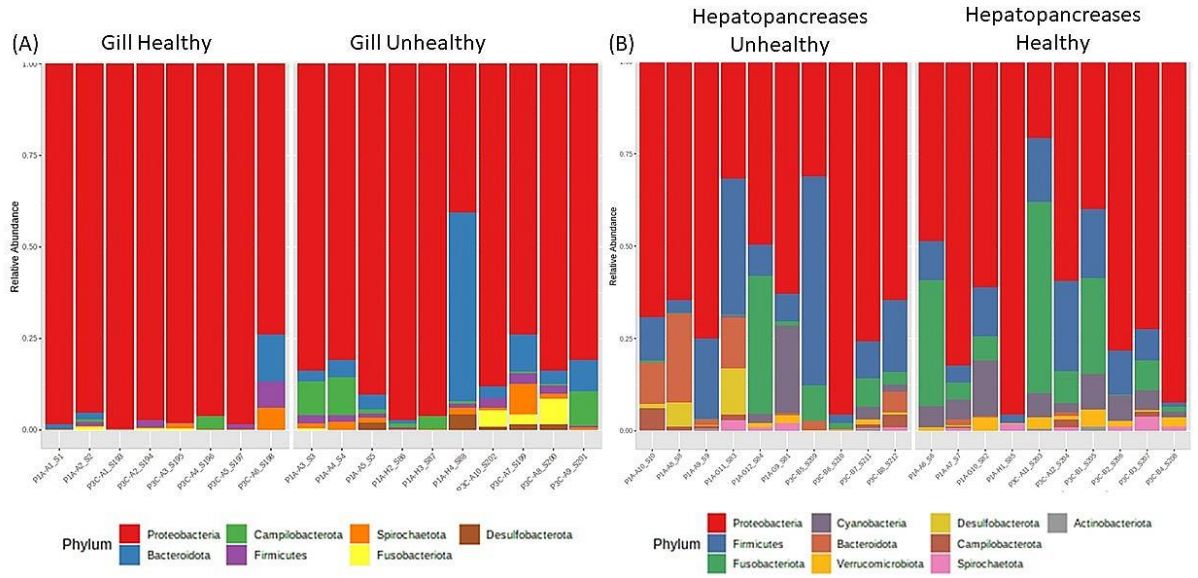


Figure 20. Phylogenetic classification of the bacterial communities of gill tissue samples: (A) at the phylum level, and (B) at the genus level.

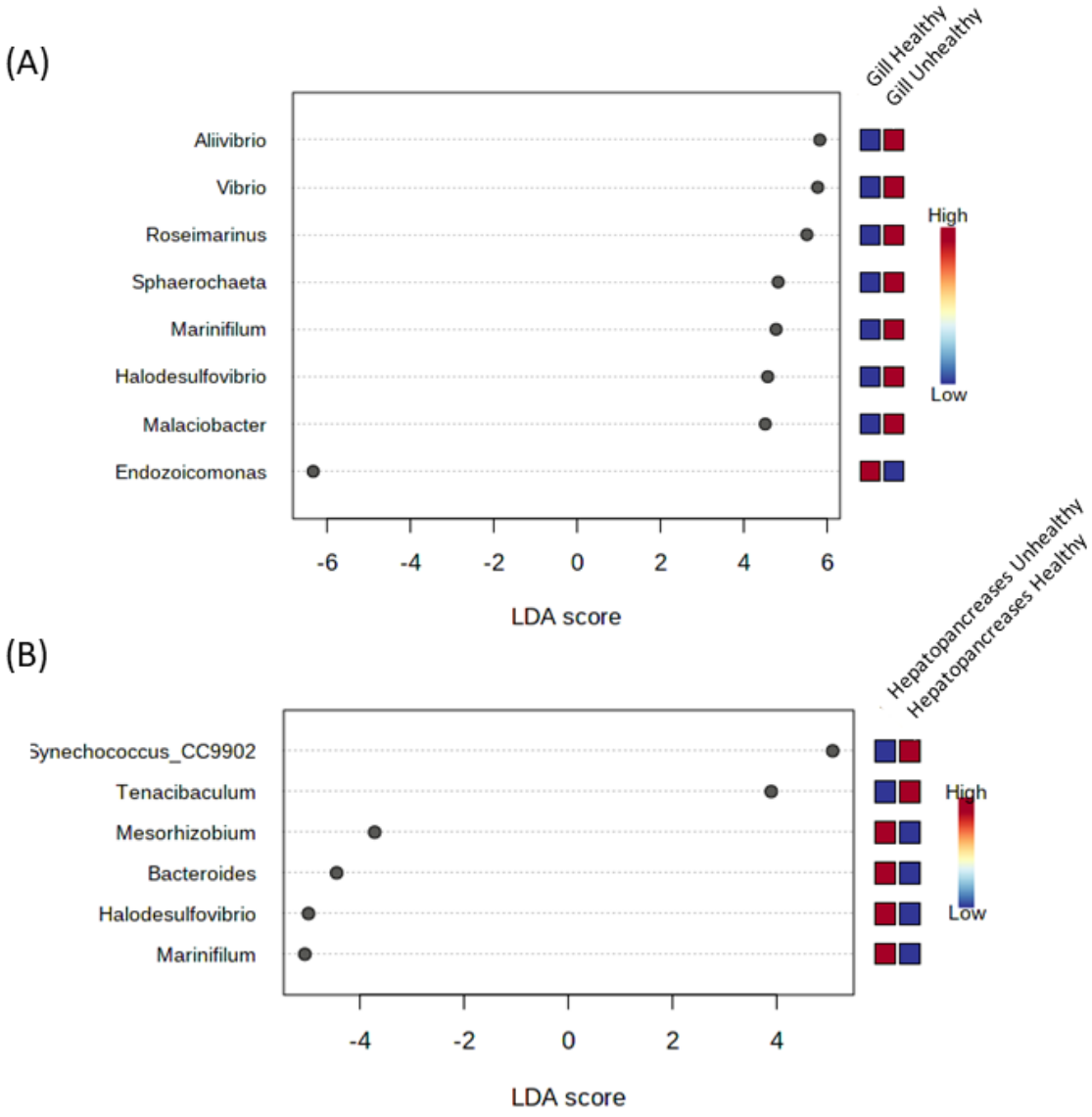


Figure 21. LEfSe analysis of taxonomic biomarkers of (A) gill tissue samples microbiota and (B) hepatopancreases tissues. a LEfSe analysis identified the most differentially abundant taxons at the genus level. LDA score ±2 is shown.

Phylum-level community profile of the hepatopancreas tissues, on the other hand, showed insignificant differences between healthy and unhealthy groups at the phylum level (Figure 22A). However, ANCOM-BC analysis revealed the existence of increases in relative abundances Firmicutes (healthy group = 12%, unhealthy group = 18%; ANCOM-BC p-value = 2.9e-4; q value = 4e-3), Bacteroidota (healthy group = < 0.1%, unhealthy group = 4%;

ANCOM-BC p-value = 1.8e-5; q value = 2.9e-4) and a decrease in Cyanobacteria (healthy group = 6 %, unhealthy group = 3%; ANCOM-BC p-value = 1.3e-23; q value = 2.5e-22). Additionally, healthy and unhealthy hepatopancreases tissue were also dominated by Phylum Gammaproteobacteria with 65% and 62%, respectively (ANCOM-BC p-value = 2.3e-19, q value = 4.3e-18). Further examination of the hepatopancreas tissue community structure at the genus level revealed alterations of numerous genera, including increases of *Mycoplasma* (healthy group = 6 %, unhealthy group = 13%; ANCOM-BC p-value = 4.8e-21; q value = 6.8e-19) and decreases of *Synechococcus_CC9902* (healthy group = 5 %, unhealthy group = 2.3%; ANCOM-BC p-value = 1.9e-26; q value = 2.7e-24) and *Endozoicomonas* (healthy group = 20 %, unhealthy group = 5%; ANCOM-BC p-value =8.6e-11; q value = 1.2e-8) (Figure 22B). LEfSe analysis of the hepatopancreas tissues at an LDA score of ±2 showed the taxa contributing most to the dissimilarity (effect size) of the hepatopancreas tissue to be Synechococcus_CC9902, *Tenacibaculum*, *Halodesulfovibrio*, and *Marinifilum* (Figure 21B).

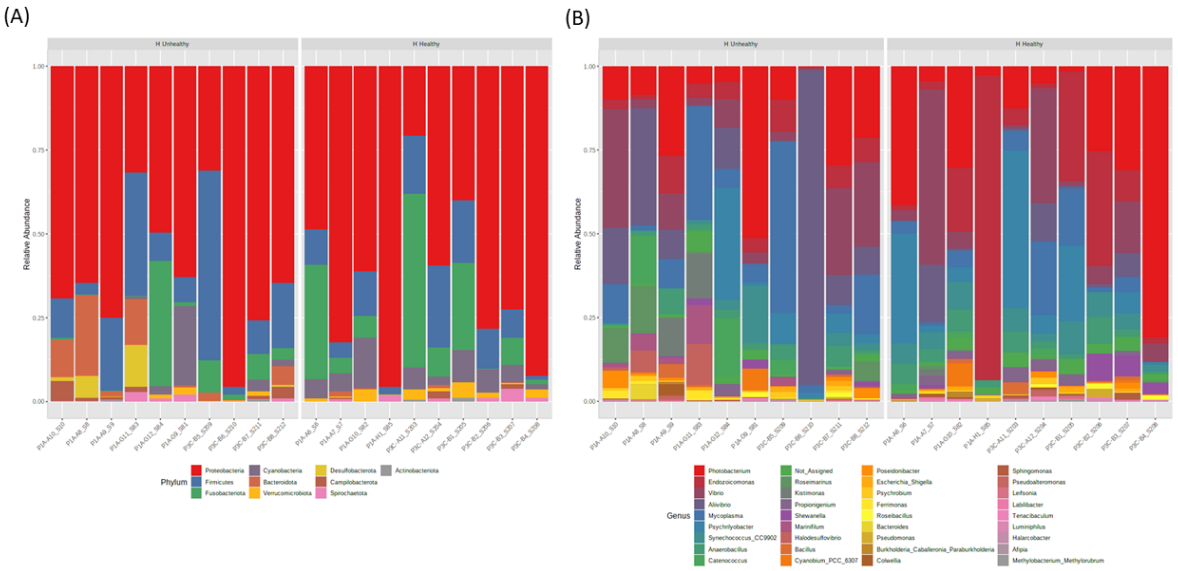


Figure 22. Phylogenetic classification of the bacterial communities of hepatopancreas tissue samples: (A) at the phylum level, and (B) at the genus level.

Finally, the SparCC network analysis returned a complex structure depicting the interactive associations of the prevalent gill tissue genera with others (Figure 23), as the gill tissues showed larger microbiome dissimilarity compared to hepatopancreas. It was noted that the changes of the most relatively abundant bacteria *Vibrio*, *Aliivibrio* and *Roseimarinus* (as indicated by the size of the nodes) were related to unhealthy gill tissues (r-values > 0.5 and p-values < 0.01). These notable (hub) bacterial genera were also correlated to multiple genera, highlighting significant co-occurrence.

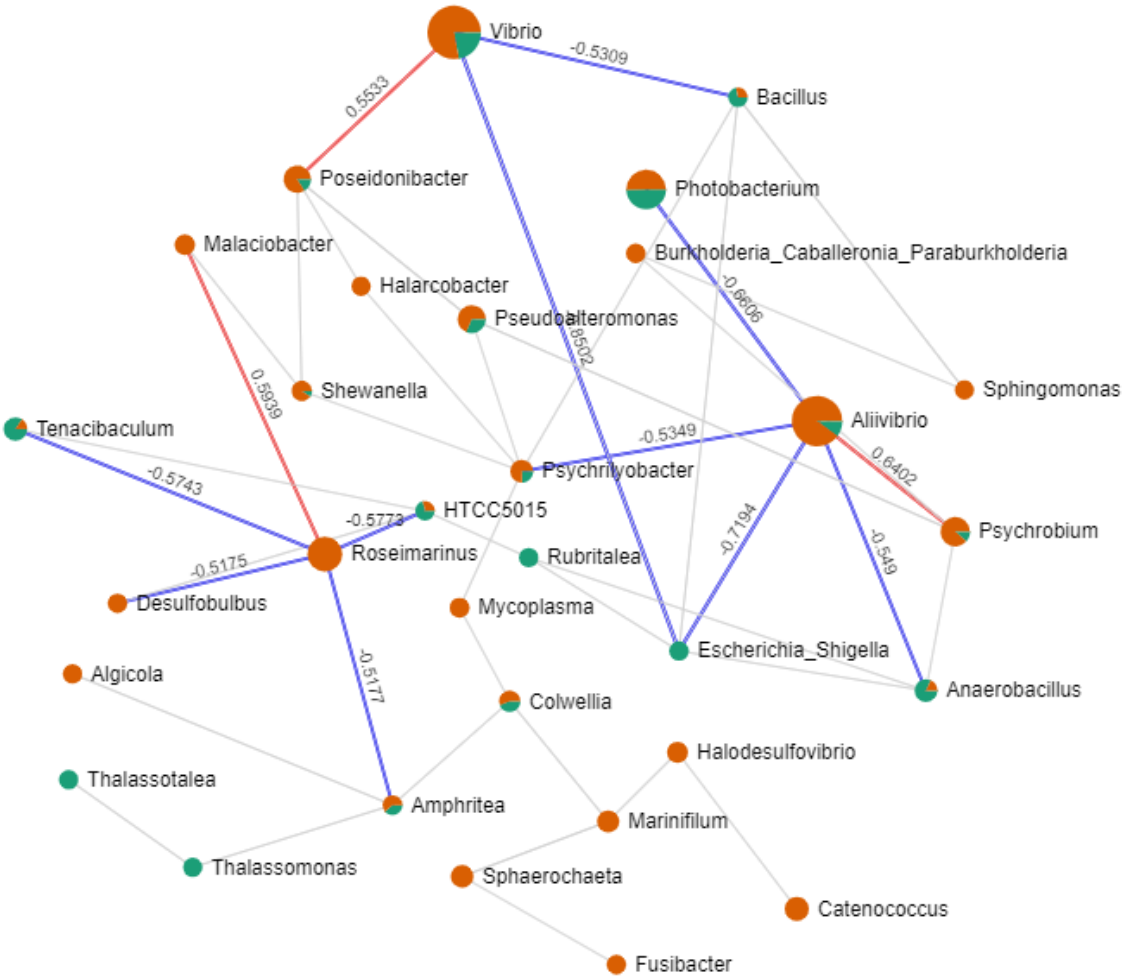


Figure 23. Co-occurrence network analysis of significant correlations (SparCC; absolute r-values > 0.5; p-value < 0.05) between genera. Each node represents a bacterial genus and the edges represent the correlation coefficients among genera. Vibrio, Aliivibrio and Roseimarinus were selected as significant hub genera and the blue and red edges from these nodes represents positive and negative correlations between these selected genera and other genera. The values on the edges signify the correlation coefficients. Nodes are coloured according to the gill tissue sample groups (healthy & unhealthy gill tissues).

5.4. Discussion

The New Zealand mussel (*Perna canaliculus*) aquaculture industry suffers from mortalities during summer months and potential pathogenic outbreaks, which threatens this commercial production. Mussel microbiome interactions, responses to potential pathogen infections and

changes in environment need to be understood to develop mussel health assessment approaches and mitigation strategies. This exploratory study provides the first microbiome analysis for *P. canaliculus* affected by summer mortality effects. The study also provides detailed information on key bacterial perturbations for mussel gill tissues and hepatopancreas tissue, including changes in bacterial diversity and profile, microbiome structural alterations, potential bacterial indicators and functional microbiome information that may have contributed to the occurrence of unhealthy mussels during the summer mortality event.

Warm climtate temperatures can lead to diversification of bacterial community composition (Erwin et al. 2012). During colder winter months, decreases in bacterial counts, as well as species diversity is expected to vary considerably. These trends hold true, regardless of bivalve host species or tissue type (Pierce and Ward 2018). Results from the present study show that bacterial richness and diversity within gill tissues of *Perna canaliculus* were significantly higher in unhealthy mussels compared to healthy mussels. Conversely, there was no significant change in bacterial richness and diversity observed in the hepatopancreas tissues. Similar result of high bacterial species diversity and richness in gill tissues have been observed in multiple previous study, such as higher microbial species richness in summer versus winter seen in the gills of *Chama pacifica* (Zurel et al. 2011). Similarly, gill-associated microbial communities of *Spondylus* species had significantly higher diversity indices in months with warmer water temperatures than gill communities sampled during times of low water temperatures (Roterman et al. 2015). Finally, a positive correlation between seawater temperature and the alpha diversity of the hemolymph microbiome of *Crassostrea gigas* from the Wadden Sea was also observed (Lokmer et al. 2016b). The gill microbiome of bivalves is a good indicator of the interactions between the animal and its surrounding environment, since gills and the hemolymph within, are constantly in direct contact with the aqueous environment and as they

performs respiratory, excretory, and feeding functions (Brito et al. 2018; Li et al. 2022). Therefore, they better reflect the effect of the environment on the microbiome community. Our results, and those of previous studies, indicate that increased microbial diversity and compositional shifts in the gill tissues of unhealthy mussels affected by higher summer temperatures may lead to the mortality levels observed in the mussel farm. However, it remains to be established whether the shift in the bacterial community was due to a change in the mussel's physiology caused by the direct increase in water temperature or a response of pathogen overload as a result of temperature elevation. Hepatopancreas tissues, on the hand, did not have different bacterial diversity or richness values between the healthy and unhealthy groups. There are few studies on the effect of mussel hepatopancreas microbiome changes with regard to temperature or pathogen loads. However, multiple studies on marine bivalve gut/digestive gland microbiome contradict our finding, as they show that a rise in temperature causes either increase or decreases in bacterial diversity and richness (Pierce 2016; Li et al. 2018, 2019b). More studies need to be conducted to verify the microbial response in terms of diversity and richness within hepatopancreas samples as a result of increases in temperature and/or pathogen loads. These studies are particularly important since the hepatopancreas is a vital tissue in mussels, which serves as a site for storage of metabolic reserves and provides energy sources for gametogenesis and physiological stress (Walne 1977; Yasmeen 2013).

Unhealthy mussel gill tissues had a distinct microbial profile and structure that was indicative of the effect summer mortality, confirmed via NMDs and hierarchical clustering. Perturbations in gill tissue microbiome community were largely caused by the lower dominance of Proteobacteria (gamma) and Bacteroidetes, and increases in proliferation of Campilobacterota and Desulfobacterota at the phylum level. The Phylum Proteobacteria dominated in the gill microbiome of *Perna canaliculus* in both mussel groups. This dominance has previously been

demonstrated in marine bivalve gill microbiota (Roterman et al. 2015; Pierce and Ward 2018; Musella et al. 2020; Li et al. 2022). Additionally, Proteobacteria are thought to play crucial roles in supporting the mucosa's microbial barrier of fish gill, with many being opportunistic pathogens. These microbes may even contribute to the development and maintenance of the host immune system through stimulatory mechanisms (Gomez et al. 2013; Wu et al. 2021). Interestingly, a study by Pathirana *et al.* (2021) examined the effect of seawater temperature on *Crassostrea gigas* microbiome susceptibility to Ostreid herpesvirus-I (OsHV-I), and found that Proteobacteria and Bacteroidetes did not change in absolute abundance after acclimating at different temperatures. However, compared with the negative control oysters, both phyla increased in abundance after they were challenged with the OsHV-1 virus (Pathirana et al. 2022). Campilobacterota has previously been identified in *P. canaliculus* (Li et al. 2022), and includes sulfur-oxidizing bacteria that are commensals of livestock, but also includes the most common foodborne pathogenic bacteria, *Campylobacter* species. The significant change in Campilobacterota dominance between healthy and unhealthy mussel gills needs to be examined furthuras the mussel farm from where the mussels were collected are surrounded by dairy farming and there are no literature available on the role of this phylum in mussel health. Regarding Desulfobacterota (sulfate-reducing bacteria), not many studies have identified alterations of this particular phylum in bivalve gill tissues. However, its increase in relative dominance is associated with gill microbiome dysbiosis of American bullfrogs (*Lithobates catesbeianus*) (Jiang et al. 2022).

For hepatopancreas community structure at the phylum level, decreases in dominance of Cyanobacteria and increases in dominance of Firmicutes and Bacteroidota were revealed in unhealthy mussels. The lower C*yanobacteria* abundance in the present study were expected. Cyanobacteria within the hepatopancreases are most likely derived from the environment

(ingested food and seawater), as they form an important part of the phytoplankton in the water column (Moore et al. 2019). Cyanobacteria contribute significantly to the diet of most bivalve filter feeders as they are rich in proteins and contain carotenoids, vitamins, minerals, and essential fatty acids (Wells et al. 2017). Some bivalve species, such as the swan mussel (*Anodonta cygnea)* have been shown to preferentially select Cyanobacteria from the water column to boost their nutritional state and contribute to the accumulation of energy reserves during host gametogenesis (Lopes-Lima et al. 2014). Therefore, a decrease in Cyanobacteria in the unhealthy hepatopancreas tissues of *P. canaliculus* can potentially be reflective of the mussel's energy deprivation and the effect of temperature and/or pathogen load on the mussels during summer months. Microbes within the Phylum Firmicutes can produce short-chain fatty acids from complex polysaccharides, which provide nutrition for the intestinal mucosal cells (Muegge et al. 2011; Koh et al. 2016). High levels of *Firmicutes* may also contribute to the maintenance of the normal function of the intestinal mucosa and the regulation of the intestinal microbial environment (Koh et al. 2016; Hao et al. 2017). Bacteroidetes participate in carbohydrate transport and protein metabolism, which are involved in digestive processes (Karlsson et al. 2011). Interestingly, Bacteroidota/Firmicutes ratios have been extensively researched as markers for gut health and dysbiosis in humans and mice (Mariat et al. 2009; Jami et al. 2014; Nguyen et al. 2015; Magne et al. 2020). Based on previous findings and the results from the present study, differences in key hepatopancreases phyla abundance indicates a disruption in gut health leading to energy deprivation and digestive capabilities.

Interestingly, at the genus level, there were remarkable changes in the relative abundances of bacteria, including *Endozoicomonas*, *Aliivibrio*, *Vibrio* and *Roseimarinus*. Particularly interesting is the dominance of *Endozoicomonas* and its increase in gill tissues, but decrease in the hepatopancreas tissues. The association of *Endozoicomonas* with molluscs, and other

marine invertebrates has been widely reported and often is one of the more dominate members of the host's microbiome (Kurahashi and Yokota 2007; Pike et al. 2013; Vezzulli et al. 2013; Ransome et al. 2014). Previous studies have described that the functionality of *Endozoicomonas* species is most likely to participate in nutritional symbiosis, transport and secretion processes between symbiont and host (Neave et al. 2017a), production of antimicrobial substances to deter potential invading microbes from host (Bourne et al. 2008). Yet, trends of these associations of *Endozoicomonas* species with their hosts are not clear. For example, in New Zealand green-lipped mussels, clams and scallops affected by mortalities there is an increased *Endozoicomonas* abundance in the gill and digestive epithelium tissues (Howells et al. 2021). There is also an observed increase of *Endozoicomonas* presence in the gill tissues of king scallop suffering from summer mortalities (Hooper et al. 2019). Based on these studies it is suspected that the increase in relative abundance of *Endozoicomonas* in gill tissues of marine shellfish is associated with pathogenic and environmental stresses. However, in the gut microbiome, a lower abundance of *Endozoicomonas* was demonstrated in the fish gut microbiomes of butterflyfish (*chaetodon capistratus*) in response to severely degraded Caribbean reefs causing stress in the host (Clever et al. 2022). Furthermore, *Endozoicomonas* dominated the gut of Mediterranean mussel (*Mytilus galloprovincialis*) in response to thermal stress (27 °C) (Li et al. 2019b). Previous environmental effect studies on *Endozoicomonas* gut communities show inconsistent results with those obtained in this study. However, *Endozoicomonas* has chemical defence capabilities, high dominance in multiple tissues and significant relative abundance alterations in response to stress (as mentioned previously). Therefore, Endozoicomonas is an important target for future microbial-host interaction research in *Perna canaliculus* to develop potential host health biomarkers.

The abundance of *Vibrio* spp. in the gill tissues of *Perna canaliculus* is to be expected as they are ubiquitous in marine environments, and aggregate on surfaces of both gill and digestive tissues of marine animals (Thompson et al. 2004a). Although many *Vibrio* species are harmless, several can be highly pathogenic for humans and/or marine animals (2010; Petton et al. 2015; Froelich and Noble 2016; Castinel et al. 2019b). Indeed, warm temperature favours the proliferation of *Vibrio* species and have contributed to mass mortalities in shellfish farms (Eiston et al. 2008; Le Roux et al. 2016), which support the findings of the present study. Interestingly, we also revealed an elevation in dominance of *Aliivibrio*, (a newly reclassified species from the genus *Vibrio* via ribosomal RNA comparison) in the gill tissues of *Perna canaliculus*. *Aliivibrio* (also known as *Vibrio fischeri*) can often be found in temperate and subtropical marine environments, and are associated with eukaryotic host, forming beneficial relationships with marine animals, sediment and decaying matter (McFall-Ngai 2014). For example, one study hypothesized that *Aliivibrio* can utilize host-produced carbon sources to secret pyruvate, which is the utilized by host's light organ cells in squids in the genus *Euprymna* and *Sepiola* (McFall-Ngai 2014). However, the response of *Aliivibrio* in *Perna canaliculus* to temperature and pathogenic stresses is poorly understood. In the present study, our network analysis results further highlights *Vibrio*, *Aliivibrio* and *Roseimarinus* as key indicator genera of *P. canaliculus* summer morality, as they are highly significant and mostly correlated with numerous genera with highly increased dominance in the summer months. Although in-depth functional insights of these genera are currently lacking, our network association analysis highlights opportunities to further study of the metabolic implications for these correlated genera and their roles in host–microbe interactions during summer mortalities.

In the hepatopancreas tissues, alterations of numerous genera, including increases in relative dominance of *Mycoplasma* and decreases in relative dominance of *Synechococcus (CC9902 strain)* were uncovered in unhealthy mussels. Reports have shown that *Mycoplasma* naturally dominates the microbiome of oysters in warmer seawater environments (King et al. 2012). In an another study, *Mycoplasma* was found to be increased in the gill tissue microbiome of oysters after subjecting the animals to temperature disturbance treatments (2–26°C) (Wegner et al. 2013). The authors suggest that *Mycoplasma* species represent a temperature-sensitive component of oyster microbiota, and may selectively proliferate at higher temperatures (Wegner et al. 2013). In humans, *Mycoplasma* is considered a pathogen which causes diseases of the bronchial and urogenital tract (Römhild 2016). *Mycoplasma* are also considered parasites of plants, utilizing the plant's vascular system to move throughout the host causing disease (Tattar 1978). In addition, *Mycoplasma* have been associated with disease in shellfish (Paillard et al. 2004) and other aquatic invertebrates (Krol et al. 1991; Azevedo 1993), as well as in fish (Kirchhoff et al. 1987). Based on these findings, increases of *Mycoplasma* in the gill tissues of *P. canaliculus* during the summer months further indicates its pathogenic role. Finally, *Synechococcus* cyanobacteria are one of the most important components of photosynthetic picoplankton (Partensky et al. 1999; Flombaum et al. 2013; Sohm et al. 2016), and their lower abundance in the hepatopancreas is reflective of a decreased digestive capability due to stress induced via temperature and/or pathogen load increases. These findings highlight the importance of monitoring the microbiome signatures within the hepatopancreas tissues as they have potential effect on the digestive capability and susceptibility to pathogen loads, particularly in the face of climate change and increasing temperature.

The alterations found in the hepatopancreas tissues of unhealthy mussels reveal a potential pathogenic role of *Mycoplasma* in shellfish, and its selective profliferation in higher

temperatures. Futhurmore, the decreased adundance of *Synechococcus* in the same tissues indicates a reduced digestive capability due to stress indiced by temperature and/or pathogen load increase.

5.5. Conclusions

This study presents the first microbiomics investigation of microbiota responses of *P. canaliculus* mussels during a period of high mussel farm mortality in summer. The microbiome profile of the gill and hepatopancreas tissues revealed strong evidence of alterations in gill tissue bacterial diversity and community structure in unhealthy mussels. Perturbations of dominant phyla in the gill tissues potentially represent disturbance of gill tissue microbial barrier, potentially affecting the development and maintenance of the host immune system. Alterations of phyla in the hepatopancreases may also indicate signs of dysbiosis of the unhealthy mussels. Our results also highlight multiple genera that are potential indicators of temperature stress and pathogenic overloads, which provide novel information and a base for further functional analysis of environmental effects that underpin host-microbiome interactions, as well as a potential assessment tool for the pathophysiological state of mussels exposed to summer mortality events.

Chapter 6

Seasonal Variation on the Gill Tissue Microbiome of Green-Lipped Mussels, *Perna canaliculus*

6.1. Introduction

Modern human activities are driving increases in the concentration of atmospheric greenhouse gases at an alarming rate and magnitude (Solomon et al. 2009). These climate change conditions can have a detrimental effect on marine environments, and can lead to perturbations in ocean temperature, pH, salinity, sea level, wind and current patterns, and intensity of extreme climate evens, which are likely to affect marine biodiversity and resources (Brander 2007; Brierley and Kingsford 2009). Ultimately, these factors will have an impact on the global aquaculture industry. Aquaculture is one of the fastest growing primary industrial sectors, producing significant global social and economic benefits (Bostock et al. 2010). However, the changes in climate are predicted to critically impact many aquaculture systems around the world through effects on species' physiology (e.g. changes in growth rate, reproductive output and disease susceptibility) and farming practises (e.g. changes to farm locations, infrastructure and husbandry) (Brander 2007; De Silva and Soto 2009; FAO 2009).

The green-lipped mussel, *Perna canaliculus* (Gmelin 1791), is an economically and ecologically important native species of New Zealand, commonly located within intertidal and subtidal coastal habitats. Economically, the highly valued *Perna canaliculus* supports a major export sector leading to a fast growing New Zealand aquaculture industry, which proves a net

worth of over $300 million in export revenues (Aquaculture New Zealand 2017). Ecologically, mussel beds provide vital environmental functions, such as removal of suspended sediment and particulate organic material, resulting in improved water quality. Due to their importance, monitoring and maintenance of both wild mussels and cultivated stocks is of vital importance. Despite the fact that the New Zealand mussel industry has experienced relatively few health issues compared to other cultured shellfish, mussels such as the *P. canaliculus* are vulnerable to changes in their environment (Stewart-Sinclair et al. 2020; Srisunont et al. 2022a, b). This can be due to storm events, shifting sediments, and increase in sea temperatures resulting in heat stress. Opportunistic pathogens, such as *Vibrio* spp. have been implicated in mussel mortality events, often taking advantage of variations in water temperature, salinity, pH, and algal bloom (chlorophyll *a*), weakening the host's immune system (Riisgård et al. 2013; Romero et al. 2014; Kapsenberg et al. 2018; Karlson et al. 2021). For example, there have been multiple reports of mass mortalities on mussel farms and beaches in the North Island of New Zealand during summer months (Nguyen and Alfaro 2020).

Direct impacts of diseases and marine heatwaves on *Perna canaliculus* have been monitored and researched greatly (Nguyen et al. 2019a; Nguyen and Alfaro 2020). However, given the impending changes in climate in the coming years, and the importance of *Perna canaliculus* to the New Zealand aquaculture industry, it is vital to comprehensively explore the effect that environmental factors can have on the physiology of this species. Such studies on marine shellfish experiencing dynamic and variable aquatic environment are limited for *Perna canaliculus*. Host-microbe interactions are thought to provide a key role in maintaining mussel health and organ-level functioning. Alterations in this interaction may reflect changes in host microbiome and the environment and may lead to detrimental outcomes. For example the gill tissues of marine mollusc function as an important physical barrier to the environment and is

generally thought to be colonized with a unique microbiome and reflective of environmental influences (Gomez et al. 2013).

Currently, there is a gap in knowledge regarding seasonal variation and their effect on the microbiome of *Perna canaliculus.* Previous work investigating seasonal or temporal microbiome changes of marine shellfish in seawater environments span less than a year and mostly focused on the gut and hemolymph microbiome (Lokmer et al. 2016a; Pierce et al. 2016; Pierce and Ward 2019). For example, a study on the he eastern oyster, *Crassostrea virginica,* revealed that oysters maintain similar microbial communities over time and space, which are largely influenced by seawater temperature. The same study also observed a decrease in genetic and functional diversity of the oyster-associated microbial communities in winter, as did environmental microbial communities (Pierce et al. 2016). The authors also argued, in another follow up study, that Blue Mussel (*Mytilus edulis*) maintained gut microbiomes which did not experience a decrease in functional diversity in winter months. This functional stability is likely to be due to the higher feeding activity of mussels in the winter compared to oysters (Pierce and Ward 2019). Although these studies provided interesting discoveries and vital information for future research, temporal microbiome data sets spanning multiple years are scarce. Multi-year temporal microbiome data would increase result accuracy, lower biases and provide critical insights into structure and function microbial communities that can be used to further build predictive models and diagnostics tools.

The purpose of this study was to utilize 16S sequencing targeting the V3-V4 variable region to explore, for the first time, the effects of multiple marine environmental drivers, including temperature, pH, salinity, and chlorophyll *a* on the gill tissue microbiome structure in *Perna canaliculus* in a mussel farm over a 3-year period. Specifically, this study attempts to discover

significant patterns in microbiome diversity, structure and signatures that reflect multiple seasonal fluctuation via comparative analysis.

6.2. Methods

6.2.1. Mussel collections

From July 2018 to July 2021 farmed *Perna canaliculus* mussels were sampled monthly from Kaiaua Greenshell™ mussel Farm, Firth of Thames, New Zealand (GPS coordinate: -37.0610, 175.3002). Approximately, 50 healthy adult mussels (length = 93.5 mm ± 5.1; weight = 62.3 g ± 7.9) were collected and measured per sampling event and immediately transported while submerged in a container with seawater to the Aquaculture Laboratory at the Auckland University of Technology (AUT), Auckland, New Zealand (approximately 1-hour travel time). Once at the lab, 10 mussels were cleaned by washing the mussels externally with fresh filtered seawater to remove biofouling. Then, the mussels were dissected to obtain the appropriate samples. Gill tissues were collected by cutting the posterior and anterior adductor muscles using a sterile dissection knife. The mantle was peeled off with sterile forceps revealing the inner cavity and gill tissues. A clean cut was then made dorsally of the mantel along the median plane to isolate and separate the gill tissues. A 20–30 mg gill section was carefully removed using sterile forceps and transferred to sterile 2 mL cryovials (BioStor™) containing 20 µL RNA stabiliser (Qiagen, Germany), and immediately snap-frozen in liquid nitrogen and stored at –80°C until further analyses. To select samples for the microbiome analysis, 10 samples (representing summer) were selected from February 2019, 2020 and 2021; 10 samples (presenting autumn) were selected from May 2019, 2020 and 2021; 10 samples (representing winter) were selected from July 2018, 2019 and 2021; 10 samples (representing spring) were selected from November 2018, 2019 and 2020.

Environmental parameters

Seawater temperature, salinity and chlorophyll *a* were previously recorded using SeaFET sensors deployed in conjunction with a SeaBird SBE37SMP-ODO-RS232 (MicroCAT) (SeaBird, USA), which has a high accuracy active pumping system and measures conductivity, temperature, pressure and includes an Optical Dissolved Oxygen (DO) sensor. Pre-deployment, deployment, maintenance during deployment and data management were accomplished and conducted by Frost (2019). Sensors were deployed in the northern Firth of Thames, New Zealand in association with the Barstrom Greenshell^{TM} mussel farm in two deployments 150 m apart (deployment one: -36.996667, 175.294722; deployment 2: -36.997222, 175.296389) (Figure 24). The mussel farm consists of a block of nine farms, located less than 1km from the shore and has a maximum depth at high tide of 12.5m and has a bottom substrate mainly consisting of fine sediment (Frost 2019).

Figure 24. Map of the Firth of Thames including location of the mussel farm base, mussel lines, buoy 1 and buoy 2.

6.2.4. Microbial DNA extraction, PCR amplicon and sequencing

Frozen samples of gill tissue were thawed and then homogenised using a FastPrep 24 system (MP Biomedicals; Irvine, California) at six movements per second for one minute prior to sub-sampling of uniform 250 µL volumes for DNA extraction. The total microbial DNA was extracted from the gill tissue samples using the DNeasy PowerSoil kit (Qiagen, Germany) following the manufacturer's instructions with the elution step repeated twice with 50 µL Tris elution buffer. Extracted DNA samples were stored at −20°C before subsequent processing. Purified DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen; USA). MiSeq (Illumina, USA) libraries were prepared as per manufacturer's protocol (16S Metagenomic Sequencing Library Preparation; Part # 15044223; Rev. B [Illumina; San Diego, CA, USA]) and as previously described (Archer et al. 2020). PCR analyses were conducted with primer sets targeting the V3-V4 regions of the bacterial 16S rRNA gene: PCR1 forward (5' CCTACGGGNGGCWGCAG 3') and PCR1 reverse (5' GACTACHVGGGTATCTAATCC 3').

6.2.5. Bioinformatics and statistical analysis

Data were pre-processed using our established workflow (Archer et al. 2020). Briefly, 16S rRNA genes were processed using the R package DADA2 v1.8 (Callahan et al. 2016) and cutadapt v3.4 (Martin 2011) to remove forward (CCTACGGGNGGCWGCAG) and reverse (GACTACHVGGGTATCTAATCC) primer sequences for 16S rRNA genes. Bacterial reads were uniformly trimmed to 280 bp (forward) and 250 bp (reverse) and then filtered by removing reads exceeding maximum expected errors of 2 for forward reads and 5 for reverse

reads or reads containing ambiguity N. High quality bacterial reads were then clustered into amplicon sequence variants (ASVs), The resulting taxa were subsequently process using R v3.5.2 (R Core Team 2020). A total of 1386 amplicon sequence variants (ASVs) were inferred from high quality bacterial reads. which were assigned taxonomic ranks using R package DADA2 v1.8 (Callahan et al. 2016) and SILVA nr v132 database (Quast et al. 2013). Excel and R software were used to plot environmental variables pH temperature, salinity and chlorophyll a.

Statistical analyses of microbiome data were performed using marker-gene data profiling in MicrobiomeAnalyst (Xia Lab, McGill University, Quebec, Canada) (Dhariwal et al. 2017). A low count filter was used to filter all features with <4 counts in at least 20% of values. Features with <10% variance, based on the inter-quartile rank, between all sampling months were filtered using a low variance filter. All samples were rarefied to even sequencing depth using the minimum library size (total sum scaling was applied). Diversity measures to calculate alpha diversity were Chao1 (richness of a group) as well as Shannon, and Simpson (richness and evenness of a group) using a Kruskal-Wallis. In order to calculate beta diversity, distance methods used were Bray-Curtis Index using PERMANOVA. R packages phyloseq (McMurdie and Holmes 2013) and ggplot2 (Wickham 2011) were used to compare and visualise relative bacterial abundances differentials, differences in group means/median (via Kruskal-Wallis). To further substantiate our findings, we also applied the pattern search feature of MicrobiomeAnalyst. Sparse Correlations for Compositional data (SparCC) (Friedman and Alm 2012) was used as distance measure of taxa between the sampling months. As SparCC assumes a sparse network and uses log-ratio transformed data and performs iterations to identify correlations that are distinct from correlations resulting from network changes within a compositional dataset, the results of this pattern search approach take the compositional

character of our data into account. Finally, Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to determine the taxa contributing to the effect size between gill tissue microbial communities across the different seasons. This analysis incorporated the non-parametric Kruskal-Wallis sum-rank test for significant differential abundance set at a significance of $p = 0.05$, followed by LDA to estimate effect size at log (10) values.

6.3. Results

Oceanographic data

Results of the environmental variable measurements obtained from the sensors are shown in Figure 25. As expected, temperature and salinity follow a seasonal trend, with higher temperatures and lower salinities during summer months compared to winter months (p value < 0.05). Chlorophyll a measurements revealed the occurrence of algal blooms, particularly in the months of November 2019 and February 2021. The overall seasonal trend for pH is less clear due to missing and limited data.

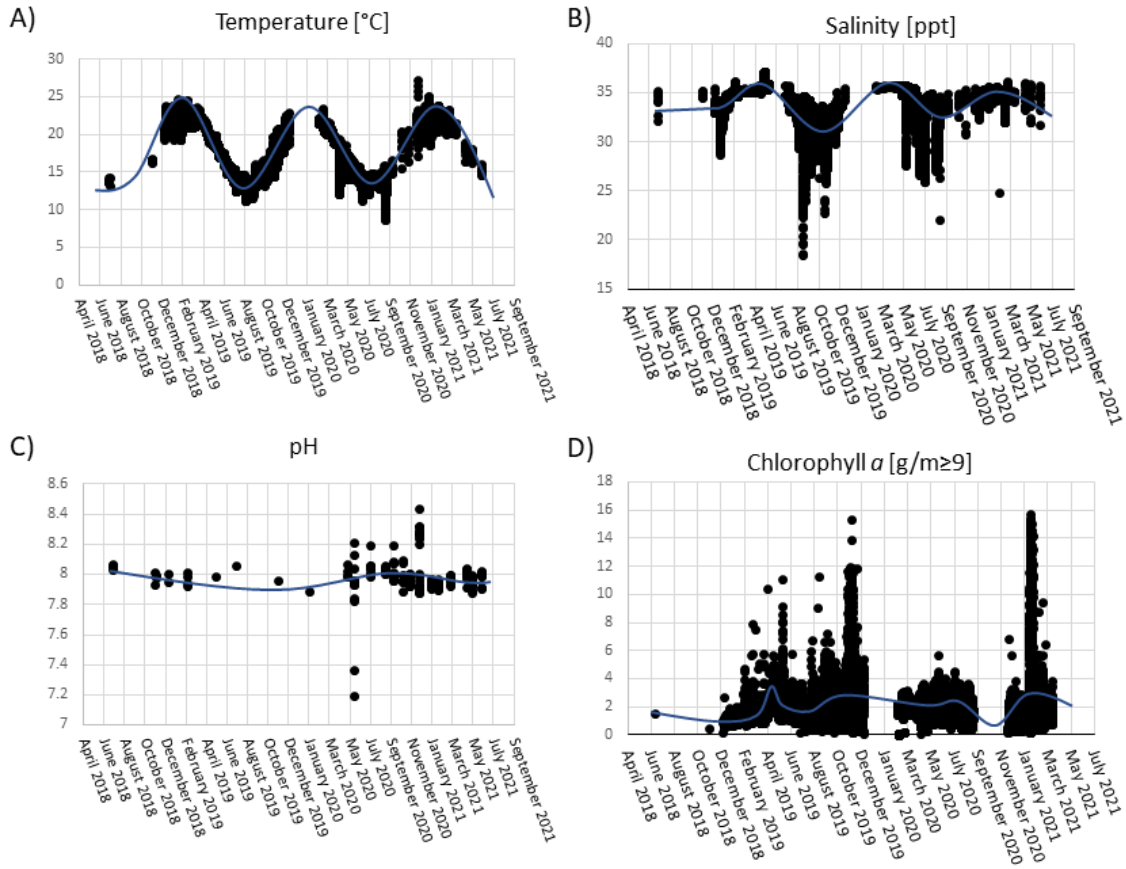


Figure 25. Environmental data from the Firth of Thames collected from the 2 buoys between April 2018 to September 2021 overlaid. Seawater measurements of A) temperature, B) salinity, C) pH, and D) chlorophyll *a* were recorded and collected from the SeaFET and MicroCAT sensors every 15 minutes daily.

Mussel Data:

Amplicon sequencing of gill tissues from all mussel samples generated a total sequence library size of 8,380,831 before filtering with 5575920 read pair sequences passing quality filtering. High quality reads were clustered into 4116 bacterial ASVs. Alpha-diversity indices reflected diversity and richness of symbiotic microbiota in each season, incorporated Chao1 and Shannon indices. There were significant differences in Shannon (Kruskal-Wallis p-value 0.003) and Chao1 (Kruskal-Wallis p-value 0.002) alpha-diversity indices. Chao1 index showed a

significant reduced alpha diversity in summer (December to April) which coincided with increased temperature and lowered salinity. Chao1 index also showed a significantly higher richness in Winter (June to October) coinciding with higher temperature and increased salinity due to increases in rainfall (Figure 26A). On the other hand, Shannon index revealed a decrease in evenness in Winter and increased evenness in Autumn (Figure 26B).

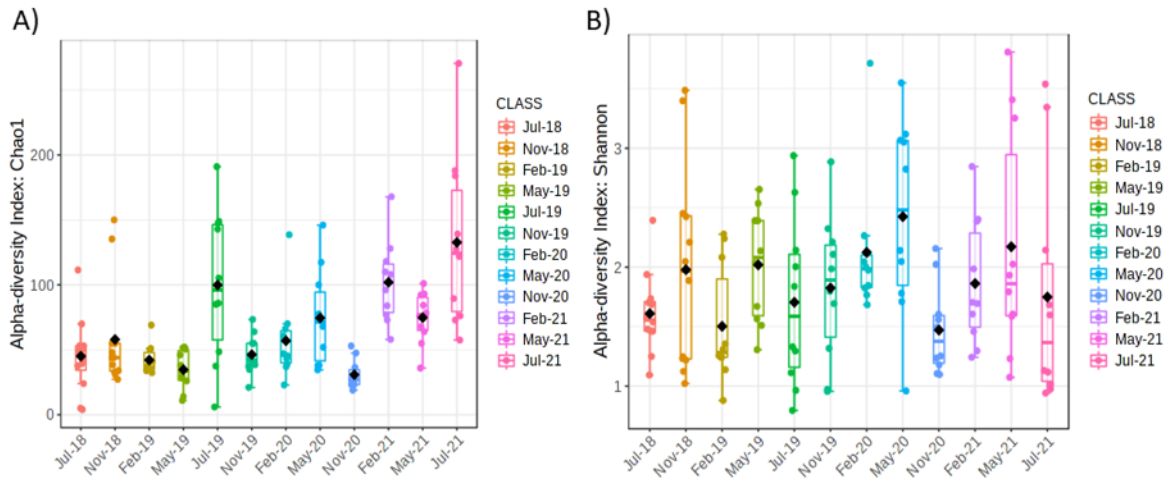


Figure 26. Richness and diversity indices for A) Chao1 and B) Shannon at ASV level for microbiome in Green-lipped mussel gill tissues via box and whiskers plot. Black diamonds represent the mean of the data.

The relative abundance of major taxonomic groups of the gill microbiota at the phylum level was found to be significantly affected by the sampling month (Figure 27A). In particular, relative abundance of Proteobacteria and Firmicutes was found to be significantly dependant on seasonally (Kruskal-Wallis test, $p < 0.05$). Proteobacteria was elevated in the seasons of spring and autumn and less abundant in winter and summer (Figure 28A). On the other hand, Firmicutes was most abundant in autumn and least abundant in winter (Figure 28B). Further examination of the gill tissue community structure revealed alterations of numerous genera in relation to sampling month. Noticeably, genera *Endozoicomonas*, *Photobacterium* and

Anaerobacillus were significantly elevated in Spring and Autumn. Furthermore, *Vibrio* was shown to be significantly more abundant in summer and autumn (Figure 27B).

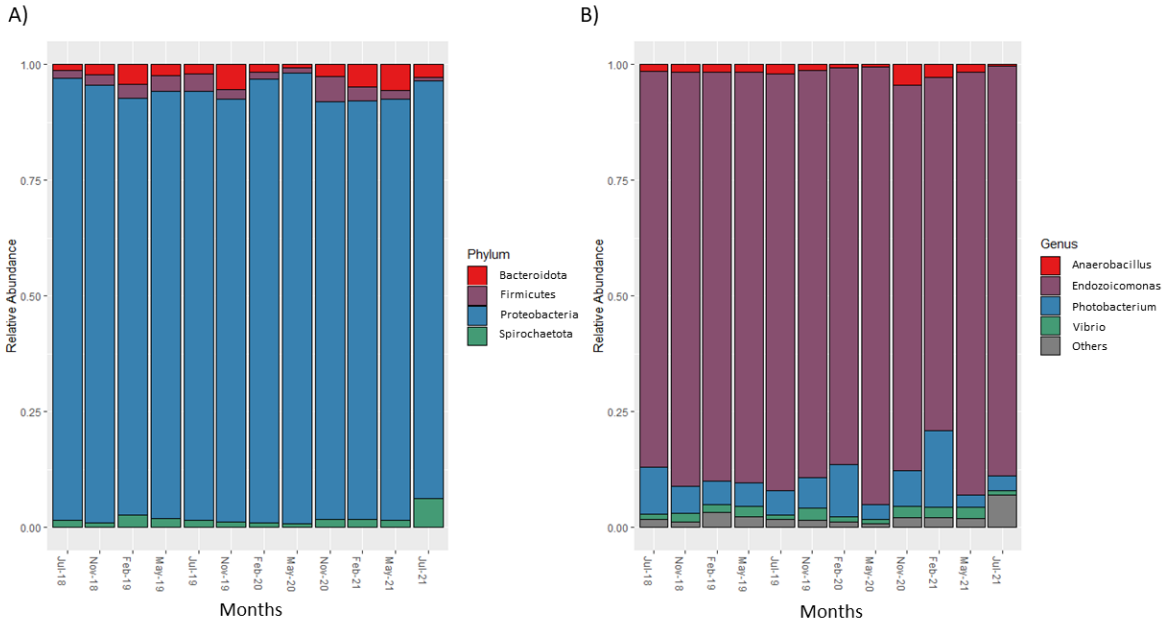


Figure 27. Phylogenetic classification of the bacterial communities of gill tissue samples: A) at the phylum level, and B) at the genus level.

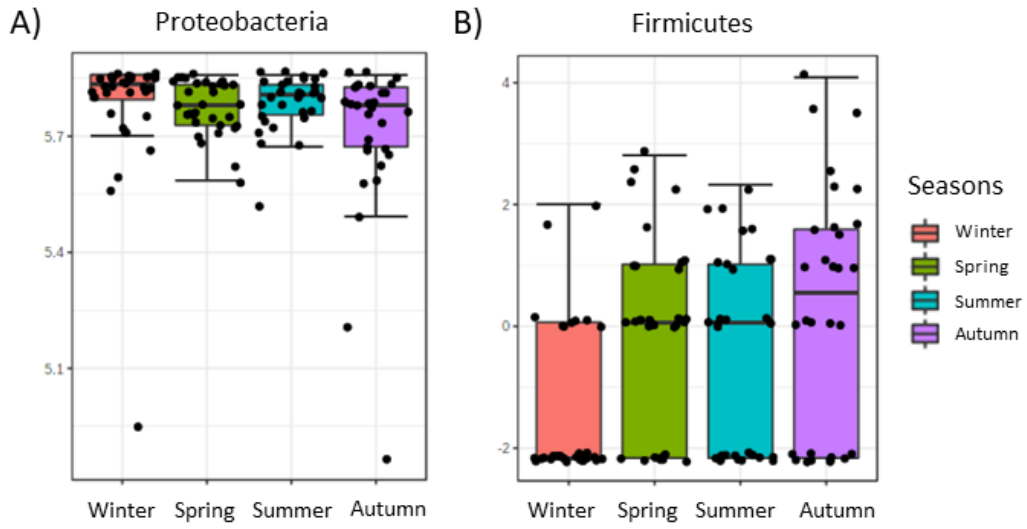


Figure 28. Relative abundance of major gill microbiota phyla in Green-lipped mussels, by Seasons of sampling A) Relative abundance of Proteobacteria B) Firmicutes. In each box-and-whisker plot, the box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line inside the box represents the median. The whiskers above and below the box show the locations of the minimum and maximum values. All relative abundance values were additionally log-transformed.

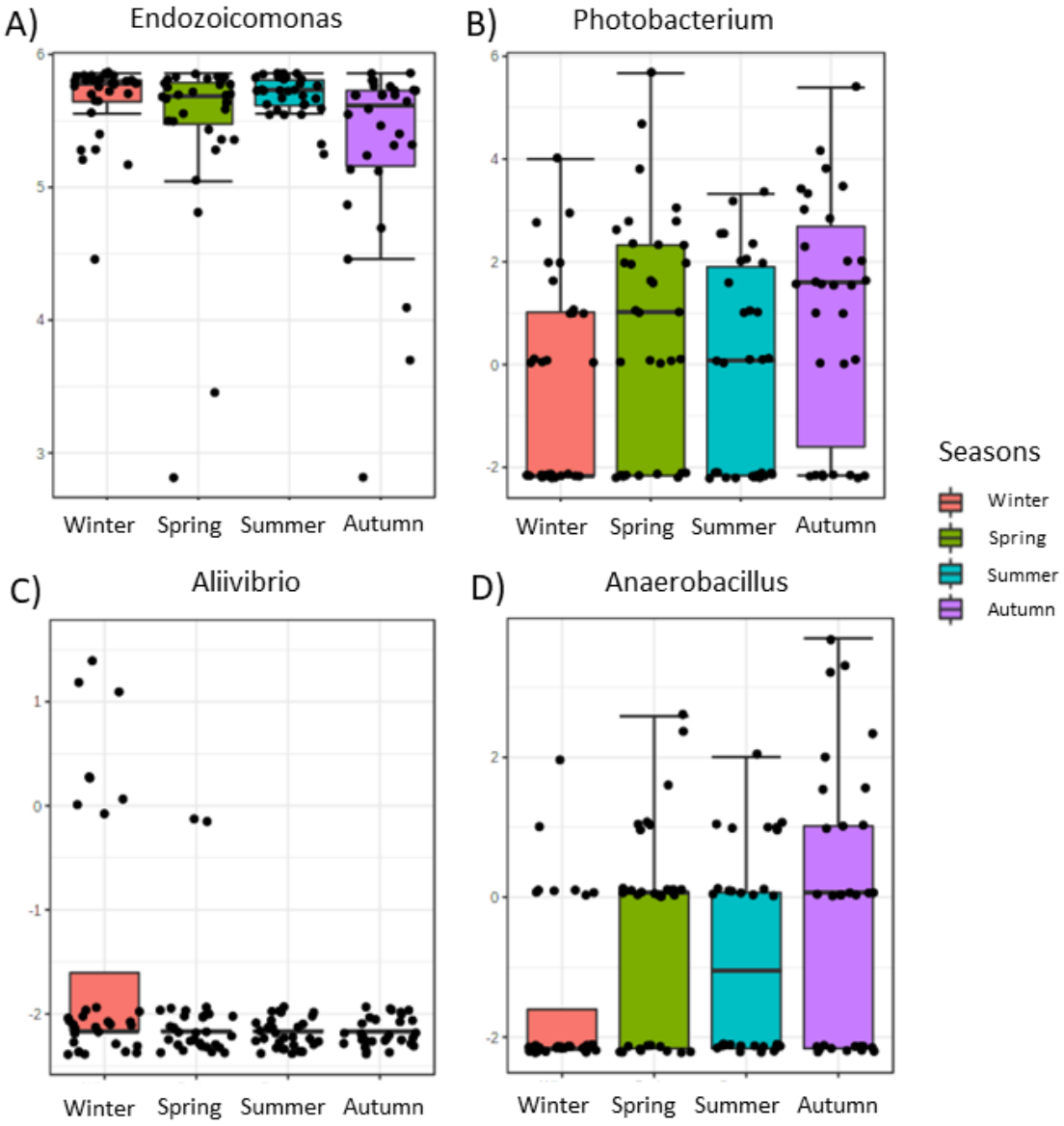


Figure 29. Relative abundance of major gill microbiota phyla in Green-lipped mussels, by Seasons of sampling A) Relative abundance of Endozoicomonas B) Photobacterium C) Aliivibrio D) Anaerobacillus. In each box-and-whisker plot, the box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line inside the box represents the median. The whiskers above and below the box show the locations of the minimum and maximum values. All relative abundance values were additionally log-transformed.

Using pattern search in MicrobiomeAnalyst with Pearson r as distance measure, we observed that the Phylum *Gammaproteobacteria* (r = -0.203; p - value = 0.02) was positively correlated with seasons and *Firmicutes* (r = 0.27, 0.003) was negatively correlated with seasons (Figure 30A). At the genus level, *Pseudoalteromonas* (*r* = 0.3179; *p* - value = 3.2077E-4), *Anaerobacillus* (*r* = 0.266, *p* - *value* = 0.002), and Vibrio (*r* = 0.23, *p* = 0.01) were positively correlated with seasons, whereas *Endozoicomonas* (r = -0.22; p - value = 0.01) and *Aliivibrio* (*r* = -0.2937, *p* - *value* = 9.304E-4) showed negative correlations with seasons (Figure 30B). Finally, Linear discriminant analysis Effect Size (LEfSe) of the gill tissue at a logarithmic discriminant analysis (LDA) score of ±2 showed relevance or effect size of differential phylum in response to season of the gill tissue to be *Proteobacteria, Firmicutes, Bacteroidota*, and *Campilobacterota* (Figure 31A). At the genus level, LEfSe analysis of the gill tissue at an LDA score ± 2 taxa contributing most to the dissimilarity (effect size) of the gill tissue to be *Endozoicomonas, Photobacterium, Anaerobacillus, Pseudoalteromonas, Tenacibaculum, Vibrio, Escherichia Shigella, Bacillus* and *Aliivibrio* (Figure 31B).

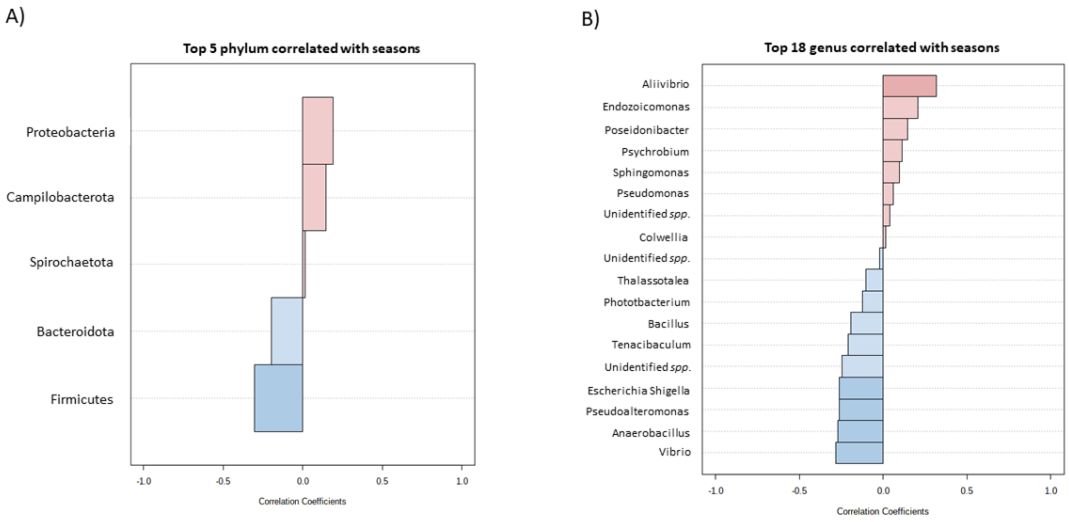


Figure 30. To further strengthen the described alterations via a compositionally aware method, we used pattern search in Microbiomeanalyst with SparCC as distance measure to identify correlations of individual taxa with groups. Bars indicate the value for the correlation coefficient of a significantly correlated taxon with seasons on **(A)** phylum level and **(B)** genus level. Correlation coefficients are depicted as positive (red) or negative correlations (blue).

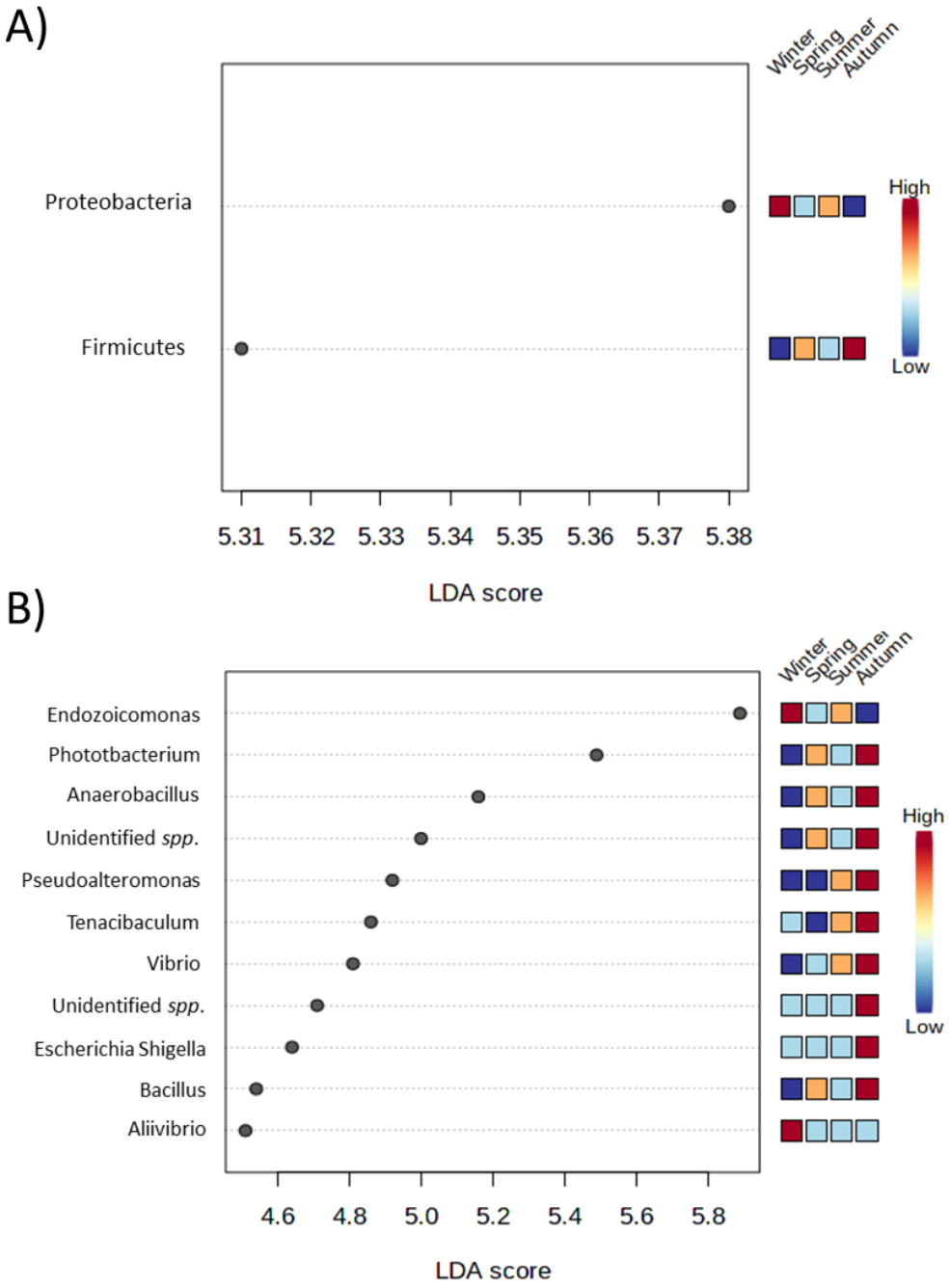


Figure 31**.** LEfSe analysis of taxonomic biomarkers of gill tissue samples microbiota. LEfSe analysis identified the most differentially abundant taxons at the **A**) phylum and **B**) genus level. LDA score ±2 and p values of <0.05 are shown. On the right side of the figure is a mini heatmap. Colours of the min-heatmap indicate high (red spectrum) or low abundances (blue spectrum) of the taxon in the corresponding seasons.

6.4. Discussion

Exploration of the seasonal effects of multiple marine environmental drivers on the microbiota of *Perna canaliculus* is important, as changes in climate may have devasting effect on the mussel industry in NZ aquaculture. Currently, there are no multi-seasonal studies on the microbiome composition and structure in the *Perna canaliculus.* This study was designed to comparatively analyse and explore differences in the gill microbiota composition of Green-lipped mussels across multiple seasons while attempting to observe associations of the microbiota with seawater parameters, such as temperature, pH, salinity, and chlorophyll *a*. Our findings suggest that gill tissue microbiome changes that overlaps with seasonal fluctuations in temperature and salinity. In particular, there are significant alterations in multiple bacterial phylum and genus abundances, as well as variations in diversity among seasons in the gill tissue microbiome of *Perna canaliculus*.

According to the results from our diversity analysis, the richness in bacterial species was lowest in spring and gradually increased for summer, before reaching its peak richness in winter. This richness is possibly associated with the diversity at lower taxonomic levels, since the relative abundance of bacterial phyla in the two seasons remained relatively constant. The observed microbiome changes from our results seems to be conflicting with previous findings of bacterial diversity with respect to seasonal variations. For example it has been suggested that warm sea water temperatures can lead to diversification of bacterial community composition (Erwin et al. 2012). Indeed, higher water temperature ranges provide optimal environmental conditions for the growth of many bacteria. During colder winter months, decreases in bacterial counts as well as diversity can be expected with some variations, and these trends hold true regardless of bivalve host species or tissue type (Pierce and Ward 2018). However, a study by

Li *et al*. (2018) revealed that gut microbiome diversity in Korean mussels (*Mytilus coruscus)* exposed to increased water temperatures showed lower diversity indexes. It is worth noting that the temperature increase in this study is more acute, suggesting heat stress and not gradual increase in temperature during the summer season. Another, study on the effect of Amazonia seasons on Mangrove oysters (*Crassostrea gasar*) discovered that alpha-diversity indexes of the oyster bacterial microbiota are richer during the rainy season. Rainy season coincides with winter in New Zealand as shown by our environmental variable. Therefore, high bacterial diversity from winter season could potentially be a result of increased rainfall, as well as lowered salinity. One explanation for this occurrence could potentially be that The Firth of Thames (where the samples were collected) is surrounded by agricultural farms. During the colder rainy climate of winter, rainfall carries of organic matter from the surround farms to the ocean, leading to an increase bacteria diversity.

From our analysis of the mussel gill tissue samples, we identified that Proteobacteria maintained high dominance across all seasons and does not go through major fluctuations in relative abundance across the seasons and years. This may be due to Proteobacteria being the largest bacterial community in aquaculture systems, as well as the being the most abundant phylum in ocean sediments and seawater (Herlambang et al. 2021). It is also closely associated with the gill tissues of many marine animals (Li et al. 2022). For example, Proteobacteria have been found to dominate fish gill tissues where they are thought to play crucial roles in supporting the mucosa's microbial barrier, and, with many being opportunistic pathogens, they may even contribute to the development and maintenance of the host immune system through stimulatory mechanisms (Gomez et al. 2013; Wu et al. 2021). Proteobacteria also play a major role in nitrogen and carbon cycles in aquaculture environments as highlighted by study on sea urchin (Hakim et al. 2016) and sulfur-related processes demonstrated by rice-fish farming

(Yamamoto and Takai 2011). On the other hand, Firmicutes were found to have significantly lower abundance in gill tissues of *Perna canaliculus* during winter season. Microbes within the Phylum Firmicutes produce short-chain fatty acids from complex polysaccharides food sources, which in turn supply nutrition for the host mucosal cells (Muegge et al. 2011; Koh et al. 2016). Based on this information, it is possible that a decrease in food sources during winter potentially resulted in less Firmicutes. Indeed, phytoplankton (main diet of mussels) experience optimal growth during spring and summer conditions when there are high levels of light and water temperature.

Particularly interestingly is the dominance of Endozoicomonas at the species level in the gill tissues of the mussels, which revealed an elevated dominance in autumn and spring compared to winter and summer, with highest relative abundance in autumn and lowest in winter. The relationship between Endozoicomonas and molluscs, as well as other marine invertebrates are well reported, and in most cases Endozoicomonas is one of most dominate members of the host's microbiome (Kurahashi and Yokota 2007; Pike et al. 2013; Vezzulli et al. 2013; Ransome et al. 2014). Functionally, Endozoicomonas species are likely to participate in nutritional symbiosis between symbiont and host (Neave et al. 2017a), production of antimicrobial substances to deter potential invading microbes (Bourne et al. 2008) and upcycling of carbohydrates or supply of proteins to host (Neave et al. 2017). A study on the gill tissues of Mediterranean oysters (*Spondylus spinosus*) revealed that Endozoicomonas was a major determinant for the differences between oysters held under summer and winter conditions, with members of the Endozoicomonas genus shown to be more abundant in the gills of *Spondylus spinosus* during winter than during summer (Rina-Dor et al. 2020). Although this finding contradicts the result of this study, it indicates that substantial changes take place in the Endozoicomonas community of *Spondylus spinosus* in response to increased

temperatures. Furthermore, multiple studies on marine corals have highlighted shifting abundance of Endozoicomonas in response to temperature (Neave et al. 2017b; Shiu et al. 2017; Rina-Dor et al. 2020). Therefore, it is reasonable to hypothesize that Endozoicomonas abundance is potentially linked with temperature stress (hot or cold). It is likely, however, that other factors could play a role in influencing Endozoicomonas dominance within in the host (e.g., algal toxins during bloom events), but these were not identified from the results of this study. Thus, Endozoicomonas presents an opportunity for future research involving microbial-host interactions in response to environmental changes and a potential biomarker reflective of physiological status of the animal.

Based on our results, *Vibrio* spp. in the gill tissues of *Perna canaliculus* mussels appeared to be elevated in dominance during summer when the temperature is higher and autumn when the salinity was higher. The positive correlation of *Vibrio* spp. with temperature and salinity is highly supported and well reported (Huehn et al. 2014; Paranjpye et al. 2015; Di et al. 2017; Trinanes and Martinez-Urtaza 2021). *Vibrio* species can be highly pathogenic for humans and/or marine animals (2010; Petton et al. 2015; Froelich and Noble 2016; Castinel et al. 2019b). Rising seawater temperature in seasons like summer promotes the development of *Vibrio*. and has contributed to mass mortalities in shellfish farms (Eiston et al. 2008; Le Roux et al. 2016). We also observed a significantly higher abundance of *Anaerobacillus* during summer and autumn. *Anaerobacillus* are facultative or strict anaerobes and accordingly they change with changes in dissolved oxygen levels (Krotman et al. 2020). In New Zealand, algal blooms occur during summer and early autumn, and this overgrowth consumes oxygen and lowers the dissolved oxygen within the seawater, hence providing optimal condition for anaerobic bacterium such as *Anaerobacillus* to thrive. The genus Photobacterium includes a large family of Gram-negative, facultatively, aerobic, motile bacteria that is also a close relative

of *Aliivibrio* and *Vibrio* from the Vibrionaceae family. Temperature and salinity are the main abiotic factors that shape their abundance and distribution (Thompson et al. 2004b; Takemura et al. 2014; Baker-Austin et al. 2018). Additionally, *Vibrio-Photobacterium* species appear to cause disease under changing environmental conditions, such as ocean temperature and salinity by taking advantage of host's stressed condition (Matanza and Osorio 2018). In the gill tissues of *Perna canaliculus*, our analysis showed that there was an increase in relative abundance of Photobacterium during autumn and spring compared to winter and summer, coinciding with months where an increase in salinity is observed. These results underscore the potential impact of environmental factors on multiple bacteria genus abundances. This enables us to further study and understand the potential for pathogenic bacteria to take advantage of stressed hosts under changing environmental conditions.

6.5. Conclusions

This study presents a multi-seasonal analysis of *P. canaliculus* microbiomes to explore trends and potential environmental drivers of microbiota variability. The findings suggest that there is little of temporal variation in the composition and structure of the gill tissue microbiome depending on season. However, the results reveals that changes in salinity and temperature overlaps with gill tissue microbiome variability in mussels and indicates alterations of multiple phylum and genus signatures that overlaps with said changing seawater parameters. These bacterial signatures serve to better understand host-microbiome interactions, and physiological wellbeing of the animals as a response to seasonal and climate changes. This study also lays the ground work for much needed future research into multi-seasonal microbiome variations for farmed animals in the aquaculture industry.

Chapter 7

General Discussion

7.1 Preface

Aquaculture is the fastest growing food-producing sector, with a current production of 80.03 million tonnes and proposed the goal of reaching 1 billion dollars in export target by 2025 (FAO 2005-2018). Among the species produced by aquaculture, bivalves are an important contributes to 90% of global molluscan production and 14% of all aquaculture production in 2017 (FAO 2021). However, the rapid development of bivalve aquaculture during the last decades has been accompanied by disease outbreaks and mortality events in most global regions (Chapter 1). Yet, there exists a gap in knowledge about molecular response of bivalves to these mortality evens, which present a challenge for disease management, environmental monitoring and overall aquaculture production. Hence, there is an urgent need for the application of new integrated, innovative approaches and platforms to unravel the complexities of bivalve molecular mechanisms that mediate biological pathways and influence of environment on the microbiota to decipher the pathogen-host-microbiome-environment interactions.

The research presented in this thesis has made significant contribution towards our understanding of the underlying metabolic and protein correlations as well microbiome responses of the ecologically and culturally important endemic mussel species in New Zealand, *P. canaliculus*, when it is exposed to different levels of stress variables, seasonal and environmental effects. The results obtained from 5 data chapters, involving different

experimental condition and omics platforms allowed for better understanding of the metabolite and protein perturbations and interactions underpinning the effects of summer mortality on adult *P. canaliculus*. In addition, the data chapters also explore the microbiome makeup of different tissue types of *P. canaliculus* and how mussels respond to changes in nutritional state, as well as potential overlaps in temperature, pH, salinity, and chlorophyll *a* in the Firth of Thames. This chapter summarises the overall findings of the thesis and highlights the research challenges, implications, and the potential for future applications of omics tools to study the health of *P. canaliculus* and other shellfish species.

7.2 Metabolomics and Proteomics Integration

GC-MS-based metabolomics and proteomics were utilized to understand the underlying pathophysiological mechanism of *P. canaliculus* when under stress from increased temperature and pathogenic overloads. While metabolomics has been extensively used to investigate *P. canaliculus* metabolic processes, this is the first application of proteomics in this species. Furthermore, this thesis attempted to apply statistical integrative method to the metabolomics and proteomics data in order to gain a more comprehensive and holistic understanding on the interaction mechanism between metabolites and proteins.

In this part of the thesis, changes in metabolite profiles of *P. canaliculus* revealed distinct differences in metabolite signatures (i.e., succinic, malic, citric, cis-aconitic and itaconic acids) and energy-related pathways between healthy and unhealthy mussels suffering from summer mortality (Chapter 2). Indeed, accumulation of TCA cycle metabolites indicates interruption to this central metabolic pathway (Jha et al. 2015). Hence, the results indicate disruptions of the tricarboxylic acid (TCA) cycle in unhealthy mussels. Upregulation of glutathione (GSH) alongside a relative high coverage of metabolites within the glutathione pathway (i.e., cysteine,

glutamate, 5-oxoproline, and ornithine) was also revealed within this thesis (chapter 2). Reactive oxygen species (ROS) is well known to be regulated by glutathione (GSH) metabolism in invertebrates (Espinosa-Diez et al. 2015; Sies et al. 2017). The result suggests the involvement of GSH metabolism when mussels were under oxidative stress from summer morality. Finally, a reduction in free fatty acids (FFA), such as the essential long-chain polyunsaturated fatty acids docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and gamma-linolenic acid were identified in the gills of unhealthy mussels, pointing to decreased energy metabolism and decreased resistance to stress.

Through the application of proteomics, for the first time, this thesis identified 17 significantly under-expressed proteins in the unhealthy mussels, of which six were cytoskeletal proteins (Chapter 2). Among these cytoskeletal proteins, three are related to the structure complex of the cilia and flagella. The first one is a radial spoke head protein 9, a multi-unit protein that plays a role in the mechanical movement of the flagellum/cilium (Pigino and Ishikawa 2012). The other two structural proteins are tektin-3 isoforms (tektin-3 X1 and tektin-3 X2). Tektins are integral microtubule proteins in cilia and flagella found within the gill tissues (Pirner and Linck 1994; Amos 2008). Based on these results this thesis, it is hypothesised that stresses caused by heat or pathogenesis in unhealthy mussels led to the changes in cytoskeletal protein abundance and gill structural changes. Two other decreased cytoskeletal proteins in the unhealthy mussels, namely coil-coil domain-containing protein 151 (CCDC151) and kinesin-2 (KIF17), present evidence of altered regulation, assembly and motile mediation of the gill tissue cilia/flagella. CCDC151 is an evolutionary conserved protein responsible for controlling motile intra-flagellar transport (IFT) dependent dynein arm (inner and outer rows of arms associated with the doublet microtubules of motile cilia) assembly (Jerber et al. 2014), and is critical for flagellar beat frequency and cilia waveform (King 2012).

Protein identification is an integral part of proteomics. It enables us to identify potential protein markers which are currently lacking in *P. canaliculu*s. Since the identification of protein depends on reference genome of the species in question, as well as comparable sequence content, application of proteomics in *P. canaliculus* is usually limited to the species that are adequately represented in the database. This limitation can be detrimental for researching response to external factors such as *P. canaliculus*. As a result, metabolomics has been utilised by the NZ aquaculture industry as it is a non-invasive and less expensive approach due to its close link to the phenotype. Therefore, proteomic results from this thesis are valuable and add a layer of molecular understanding that did not exist for the farmed *P. canaliculus* prior.

In order to perform omics data integration, a decision was made on the integrative method and how it could be implemented. The challenge was that many strategies (component-based, message-passing, Bayesian methods, network analysis, classification schemes) exist for integration of multiple omics to answer differing questions, combining experimental data and curated data from omics databases (Bersanelli et al. 2016; Meng et al. 2016; Huang et al. 2017; Rohart et al. 2017; Zeng and Lumley 2018). These include data-driven methods for identifying novel phenotypic clusters (Kirk et al. 2012; Wang et al. 2014), methods for extracting common sources of variation (Li et al. 2012; Zhang et al. 2012; Lock et al. 2013), and methods that incorporate curated data with experimental data in order to reconstruct biological networks (Zhu et al. 2012; Glass et al. 2013). Most of these methods are examples of unsupervised multi-omics data integration, that is, without the need of sample labels that categorize samples based on a certain phenotype or trait. In this thesis, we were interested in multi-omics biomarkers that could be predictive of mussel diseases, i.e. supervised methods in which molecular patterns that span across biological domains explain or characterize a known phenotype, such as

mortality of the mussels during summer.

However, there are limitations with employing supervised data integration approaches for the classification of multiple phenotypes (e.g. Healthy vs. Unhealthy). For instance, biases can occur with supervised data integration when certain omics data types do no account for interactions between omics layers (Aben et al. 2016; Ma et al. 2016). Considering the discussion above, we applied 'data integration analysis for biomarker discovery using latent variable approaches for omics studies' (DIABLO) to examine underlying structure, Pearson's correlation and data variability. DIABLO a multi-omics method that simultaneously identifies key omics variables during the integration process and discriminates phenotypic groups. DIABLO maximizes the common or correlated information between multiple omics datasets via the implementation of dimension reduction techniques that extends both sparse PLS-DA (sPLS-DA) and sparse generalized canonical correlation by projecting the data into a smaller subspace while capturing the correlation structure and highlighting the largest sources of variation from the data, resulting in reliable sample classification and powerful explanatory capacity (Singh et al. 2016) (Chapter 2).

Integrative analysis in this thesis work revealed a distinctive correlation between the metabolic and proteomic profiles of the mussels. Particularly, positive relationship between the calcium binding protein Annexin A4 and structural protein CCDC151 (both significantly under-expressed in the unhealthy mussels) and three down-regulated saturated fatty acids (pentadecanoic acid, 10,13 dimethyltetradecanoic acid and myristic acid) in unhealthy mussels. Another finding from our integrative analysis was the negative relationship between 2 aminoadipic acid (significantly upregulated in unhealthy mussels) and lowered expression of cytoskeletal proteins tektin-3 (both isoforms), CCDC170 like, radial spoke head protein 9, EF-

hand domain containing-protein, and annexin A4. These results suggested that temperature stress from the summer may result in degradation in gill tissues cytoskeleton structure and cilia/flagellum regulation of the unhealthy mussels and potentially contributes to undesired changes in gill membrane fluidity, permeability, and lipid composition, impairing its function.

7.3 Microbiomimcs

Microbiomics was employed to explore the microbiome diversity and its perturbations as well as shifts in composition structure in *P. canaliculus* when it is subjected to various stress factors, disease state and seasonal influence as discussed in Chapter 3, 4, 5 & 6. At the beginning of this work, various *P. canaliculus* tissues samples, such the gill tissue, haemolymph, and stomach and digestive tissue were compared in order to select the target tissue/organ for the rest of the experiments (Chapter 3). This pilot study also provided information, for the first time, on the microbiome and fungal composition on the different tissue/organ of *P. canaliculus* to provide a baseline for the subsequent microbiomics study on the physiological and health of this important species.

Microbiome profiling of different tissue types revealed distinct compositional patterns of microbes, which were identified at various taxonomic levels (Chapter 3). Bacterial communities were distinct by tissue type except for stomach and digestive gland tissues which were highly similar to each other, but distinct from the gill tissues and haemolymph. Seawater, gills, and haemolymph contained proteobacterial groups, while digestive gland and stomach tissues were dominated by common anaerobic gut microbes involved in fatty acid synthesis, carbohydrate digestion and gut maintenance. These results highlight the open association between the circulatory physiology (gills and haemolymph) of mussels and surrounding seawater, and the high selectivity of microbiomes in the digestive system (digestive gland and

gut). This information established the gill tissue as the most optimal tissue type to examine host-microbiome interactions (Chapter 5&6), as the gills of bivalves perform respiratory, excretory, and feeding functions, which require them to interact directly with seawater. Close contact between gills and haemolymph allows waterborne microbiota from the external environment to be transferred to the haemolymph via the gills as has been shown previously (Brito et al. 2018).

Interestingly, this thesis identified patterns of bacterial signatures at the phylum level and species level from multiple data chapters for the *Perna canaliculus*. For instance, all microbiome data from this thesis have shown that gill tissues, haemolymph and digestive tissues are largely dominated by the Phylum Proteobacteria (Chapter 3,4,5 & 6). The dominance of Proteobacteria, specifically gamma, in the gill tissues and haemolymph is consistent with multiple prior findings in other mussel species (Li et al. 2018, 2019a), oysters (Lokmer and Mathias Wegner 2015), and abalone (Mizutani et al. 2020). Proteobacteria are thought to play crucial roles in supporting the mucosa's microbial barrier of fish gill, with many being opportunistic pathogens, they may even contribute to the development and maintenance of the host immune system through stimulatory mechanisms (Gomez et al. 2013; Wu et al. 2021). Additionally, proteobacteria appears to significantly change its dominance in response to various stress and environmental factor even in the gut of the mussels in response to starvation (Chapter 4). Thus, the results highlight proteobacteria as an important member of the *P. canaliculus* community structure.

Alongside proteobacteria, the studies have also revealed the appearance of Campilobacterota in the gill tissues (Chapter 3&5). In particular, unhealthy *P. canaliculus* suffering from summer mortality appeared to have a higher proliferation of Campilobacterota. The significant change

in dominance of Campilobacterota between healthy and unhealthy gills tissue needs to be examined via an environmental forensics approach (Baily et al. 2015) as the mussel farm from where the mussels were collected are surrounded by dairy farming. Chemicals and degraded biomass produced by these farms and animals, leaches into the Firth of Thames where the mussels were collected. This should be considered when question is brought up regarding optimal location of the mussel farms for the healthy growth and development of *P. canaliculus.*

Others dominating phyla includes Firmicutes, Bacteroidota and Cyanobacteria (Chapter 3,4,5 & 6). It's important to note here that Bacteroidota/Firmicutes ratios have been extensively researched as markers for gut health and dysbiosis in humans and mice (Mariat et al. 2009; Jami et al. 2014; Nguyen et al. 2015; Magne et al. 2020). Based on previous findings and the results from this thesis (Chapter 4), alteration in this ratio is potentially a good indicator of disruption in gut health leading to energy deprivation and digestive capabilities.

The identification of bacterial markers provides the industry with valuable knowledge and information that can be applied to develop diagnostics tools. Indeed, if we can comprehensively understand the relationship between the microbiome and the biological process within the mussels, we can utilize microbiome markers as a robust, low-cost diagnostic tool to rapidly identify biological dysfunctions and created intervention methods at an earlier stage. This would not only reduce cost for the industry, but also save stock from succumbing to disease leading to increase in production yields. For example, a rapid and accessible handled laser-based NMR could be developed to access the metabolome of a particular animal. The data could be uploaded and analysed via cloud. Using this result the farm would be able to decide on appreciate mitigation, preventative and recovery actions. In addition to diagnostic methods, we can use probiotics and nutrition to supplement the microbiome. For example, in scenarios

where a particular microbiome is well understood, we may be able to alter the microbiome structure to achieve a desired physiological state within the mussel hosts via changes in diet and/or supplementation via probiotics.

One of the most interestingly microbiome signature presented in this thesis is the species is *Endozoicomonas.* Not only is this species the most dominate member of the proteobacterial phylum in tissue types, such as gill tissue and haemolymph (Chapter 3), but it also varied significantly in all experimental conditions (Chapter 5 & 6). Particularly, remarkable changes in the relative abundances of bacteria of *Endozoicomonas* was detected in the gill tissues of unhealthy mussels suffering from summer mortality (Chapter 5). More detail in Chapter 6 revealed that *Endozoicomonas* proliferation alters in response to seasonal effects, potentially linked to temperature and salinity. The association of *Endozoicomonas* with molluscs, and other marine invertebrates is widely reported and often one of the more dominate members of the host's microbiome (Kurahashi and Yokota 2007; Pike et al. 2013; Vezzulli et al. 2013; Ransome et al. 2014). Functionally, studies have described that *Endozoicomonas* species are likely to participate in nutritional symbiosis, transport and secretion processes between symbiont and host (Neave et al. 2017a), production of antimicrobial substances to deter potential invading microbes from hosts (Bourne et al. 2008). There is also evidence to suggest that *Endozoicomonas* increase in gill and digestive epithelium *Endozoicomonas* presence has been identified in New Zealand green-lipped mussels, clams and scallops mortalities (Howells et al. 2021), as well as infecting the gill tissues of king scallop (Hooper et al. 2019). Due to its ability to produce chemical defences in animals, such as marine molluscs and bivalve, and its community dominance and polarised change in relative abundance in response to stress, as well as in different tissue types, marks *Endozoicomonas* an important target for future microbial-host interaction research in *P. canaliculus* to develop potential host health biomarkers.

7.4 Challenges and Future Research

Recent advances in modern analytical tools and bioinformatics have led to the rapid growth of omics, such as metabolomics, proteomics and microbiomics platforms in the biological research. However, many challenges still exist for its application, especially in aquaculture.

In this thesis, there were many challenges, the first of which was the utilization of bioinformatic tools to analyse metabolomics and proteomics datasets. Regarding metabolomics, the first challenge is metabolite identification. Since metabolite databases are not completely available, unknown metabolites are often encountered, which makes their analysis limited, and presents an obstacle for pathway analysis and biological interpretation. Proteomic datasets present the most difficulty, as there was no reference genome available for the animal in question, *P. canaliculus*. To be able to quantify candidate protein genes, reference genomes of the species or close relative must be used, or there is danger to produce inaccurate amino acid predictions. For the future advancement in aquaculture research, it is important to be able to access these vital gaps of information. At the completion of this thesis, the complete genome of the *P. canaliculus* is nearing complete, bringing a huge stride forward into future omics (such as genomics and transcriptomics) studies of the species.

Another major challenge was the application of bioinformatics. Analysis of large multivariate dataset, such as metabolomics and proteomics presents a hurdle, as strong computational skill is required to analyse these datasets using tools, such as R. However, many researchers have limited knowledge and access to programming and software, rendering analysis of metabolomics and proteomics data difficult. Omics research is coupled with bioinformatics

and must be presented as a whole package. It is important to not only understand omics from the biological aspect, but also from the bioinformatics aspect. Greater emphasis should be placed into developing more refined, polished and user-friendly bioinformatics tools.

Multi-omics research is the future, and can be more powerful, especially when large datasets are available. This approach allows multi-faceted insights into complex biological processes. Hence, marine scientists may take advantage of this emerging approach to greatly advance our understanding of complex host–pathogen–environment interactions, such as during mass mortality outbreaks of wild and cultivated stocks. This integrated approach is also more likely to lead to the identification of relevant biomarkers. However, multi-omics research is still at the developmental stage and is facing big challenges due to lack of easy-to-use workflows to integrate big data obtained from different omics platforms. To this end, future advances in bioinformatics, such as deep learning algorithms are expected to bring forth new wealth of information, and place multi-omics at the forefront biological research.

The microbiomics work in this thesis is novel in a sense that it was the first time to be applied to *P. canaliculus.* Despite great stride being made in this exploratory project, there is room for more in-depth analysis. A sensible follow-up research is to further understand causality by investigating biological roles of microbes driving the community abnormalities prior to and during onset of mortality. This can be achieved by overlaying microbial community shift data with microbial activity data. However, this would require a more refined and holistic approach. A metagenomic shotgun sequencing of microbial communities would be the appropriate step. Metagenomics is the study of a collection of over thousands of bacterial genomes from one environmental sample whereas shotgun sequencing is the technique of DNA shearing and

sequencing. Instead of using single gene marker (e.g. V3 to V4 regions of the 16S rRNA gene as employed in this thesis), the output of bacterial genomes will allow species and potentially genotypic level delineation, therefore greatly facilitating high resolution characterisation of bacterial communities (Segata et al. 2012).

A major limitation faced in this thesis was the capability to produce sufficient samples for analyses. Indeed, 16S profiling of the microbiome introduces a lot of variability and biases among samples. The costs of preforming Next-Generation Sequencing of the microbiome proved to be too high, and careful consideration was taken in this thesis with regards to sample selection. This particularly limited the quality of the results and the types of analysis that could be conducted in chapter 6. Indeed, chapter 6, where the effects of seasonal variation was investigated on the gill tissue microbiome of the *P. canaliculus*, only 10 samples for each season where possible to analyse. Seasonal microbiome analysis in aquaculture species is extremely vital to our understanding and characterization of how the animal responds to the surrounding environment. If we combine advanced modelling techniques, such as deep learning (Michel-Mata et al. 2022) and Microbial Temporal Variability Linear Mixed Mode (MTV-LMM) (Shenhav et al. 2019) with a large number of microbiome samples, there is a potential to produce novel findings, and also predict changes in the microbiota that benefits the industry in terms of developing mitigation strategies, probiotics and manage the health of the wild stock.

Despite these limitations, this thesis presents comprehensive information tackling some areas of science that were not previously attempted in the *Perna canaliculus* species. The exploratory analyses and results will be a stepping stone for others to build upon and expand. The contributions made in this study to the fields of health, nutrition and fitness of mussels are

significant towards this growing aquaculture industry, and provide new insight on the pathophysiological mechanisms, molecular and bacteria biomarkers, host-microbiome interactions involved in mussels exposed to summer mortality, pathogen loads, nutritional stress, and other factors. With this thesis laying the groundwork and providing this novel information, there is hope for improved management strategies via probiotics and diet changes within the mussel industry. This will contribute to increased survival rates, fitness and quality, especially, during the summer months. Additionally, the identified biomarkers can be used to monitor the health status of the animals and develop targeted interventions such as diagnostic and nutritional tools to improve their health and productivity. Overall, this thesis emphasizes the need for continued application of advanced techniques and platforms to increase the health of animals and productivity of the aquaculture industry in New Zealand and overseas. By embracing the power of technology, the industry can tackle the impending challenges associated with animal health and increase its competitiveness in the global market.

Supplementary information

Supplementary table 1

Name	G1	G5	G9	G11	G13	G15	G10	G12	G14	G16	G6	G8
1-Aminocyclopropane-1-carboxylic acid	0.000196	0.000299	0.000233	0.000209	0.000229	0.000526	0.000166	0.000604	0.00052	0.000235	0.000283	0.000727
10,13-dimethyltetradecanoic acid	0.371288	0.542813	0.24087	0.44962	0.574334	0.540281	0.229688	0.340389	0.448945	0.273384	0.188063	0.30987
11,14-Eicosadienoic	0.03173	0.040969	0.021421	0.042807	0.071786	0.045876	0.042315	0.05165	0.046165	0.022786	0.023692	0.035395
11,14,17-Eicosatrienoic acid	0.001353	0.001409	0.000729	0.002902	0.002592	0.002327	0.000754	0.001105	0.001072	0.001133	0.000454	0.000676
13,16-Docosadienoic acid	0.007668	0.012053	0.003245	0.007351	0.009004	0.011275	0.008129	0.008797	0.009712	0.003521	0.00458	0.009479
Strombine	0.854827	0.520553	0.623431	0.515773	0.682196	0.192649	0.425304	0.109987	0.573536	0.196864	0.346923	0.267775
2-Aminoadipic acid	0.016771	0.013356	0.051182	0.017787	0.022562	0.036815	0.089318	0.118543	0.075249	0.065392	0.099744	0.106577
2-Aminobutyric acid	0.031457	0.040882	0.032627	0.026803	0.033093	0.0504	0.016903	0.10571	0.034674	0.039212	0.031207	0.088287
2-Oxobutyric acid	0.001389	0.002145	0.000263	0.002248	0.000955	0.002558		0.00166	0.000938	0.001496	0.001533	0.001597
2,3-Butanediol	0.001748	0.000304	0.001481	0.000871	0.000637	0.002655	0.00041	0.000444	0.000125	0.00018	0.000239	0.000307
2,4-Diaminobutyric acid	0.000883	0.001439	0.000924	0.00127	0.000821	0.001388	0.000305	0.002043	0.00129	0.000922	0.000425	0.002613
4-Aminobutyric acid (GABA)	0.018195	0.039212	0.021415	0.022872	0.034631	0.044573	0.010154	0.017469	0.017276	0.035049	0.016368	0.037909
4-Hydroxyphenylacetic acid	0.000694	0.000844	0.00182	0.002159	0.001726	0.001585	0.000804	0.003868	0.012606	0.004057	0.005212	0.008738
5-Hydroxy-L-lysine	0.000469	0.000387	0.000402	0.000335	0.00047	0.000787	0.000423	0.003481	0.0007	0.000519	0.001143	0.000531
9-Heptadecenoic acid	0.001073	0.000912	0.000615	0.001385	0.001718	0.00132	0.00103	0.001137	0.001446	0.000563	0.000495	0.001003
Alanine	2.418104	3.886538	1.686775	2.103213	3.141727	4.321555	1.421056	3.079039	2.825733	2.26316	2.024557	2.752338
Arachidonic acid	0.039468	0.052172	0.029267	0.052366	0.090799	0.053459	0.049986	0.064484	0.054222	0.030064	0.029229	0.045703
Asparagine	0.1261	0.101634	0.033772	0.100996	0.130918	0.085129	0.018551	0.020081	0.063187	0.027545	0.034033	0.026873
Aspartic acid	1.243051	2.116866	0.923074	1.146051	1.419313	2.397525	0.496896	1.45826	1.858808	1.863316	1.321653	1.291348
beta-Alanine	0.026683	0.046976	0.02483	0.018849	0.092337	0.075113	0.009337	0.034738	0.019384	0.046057	0.013066	0.033336
beta-Methylamino-alanine (BMAA)	0.001002	0.001167	0.001477	0.000848	0.001076	0.001133	0.000612	0.005661	0.003003	0.001643	0.001032	0.002986
Caprylic acid			0.00017		8.58E-05	2.57E-05	5.02E-05	0.000144	0.000623			3.14E-05
cis-4-Hydroxyproline	0.003524	0.014812	0.00415	0.006227	0.00268	0.023856	0.011297	0.023317	0.007381	0.02139	0.004406	0.026725

cis-Aconitic acid	0.000342	0.000317	0.0003	0.000209	0.000942	0.000607	0.000973	0.001427	0.002141	0.001463	0.001667	0.00069
Citraconic acid	0.012018	0.009205	0.007688	0.010797	0.018396	0.019658	0.014766	0.012855	0.018695	0.010356	0.00996	0.017211
Citric acid	0.008923	0.008193	0.006802	0.008526	0.019345	0.01138	0.023713	0.032124	0.052969	0.038475	0.042366	0.017222
Creatinine	0.008899	0.005825	0.007651	0.009065	0.015166	0.007842	0.008832	0.013754	0.01874	0.013051	0.01157	0.012915
Cystathionine	0.003362	0.008348	0.002652	0.002098	0.004093	0.01327	0.004358	0.016228	0.005204	0.004677	0.00454	0.011282
Cysteine	0.011525	0.008429	0.007243	0.011974	0.034128	0.012228	0.009191	0.007985	0.014281	0.008365	0.006795	0.008773
DHA	0.296256	0.482434	0.217663	0.394306	0.497689	0.475148	0.225384	0.399396	0.340985	0.20558	0.169056	0.285607
Dimethyl aminomalonic acid	0.31326	0.327155	0.251627	0.283082	0.320704	0.462141	0.155288	0.393573	0.294534	0.303905	0.174581	0.33357
DL-3-Aminoisobutyric acid	0.010061	0.009339	0.0087	0.005951	0.018122	0.017418	0.008238	0.02538	0.013885	0.01595	0.005923	0.008696
Dodecanoic acid (Lauric)	0.002572	0.001356	0.001088	0.002381	0.003201	0.001387	0.000997	0.001328	0.00164	0.001241	0.000783	0.001641
DPA	0.021138	0.031992	0.010095	0.017951	0.025427	0.024342	0.009791	0.019863	0.017351	0.01041	0.007057	0.014768
EPA	0.138308	0.167317	0.074146	0.142244	0.200043	0.146593	0.08488	0.141283	0.105364	0.109351	0.04738	0.077679
gamma-Linolenic acid	0.011232	0.018614	0.008362	0.022385	0.03347	0.019025	0.006863	0.011219	0.014276	0.011292	0.005088	0.007193
Glutamic acid	0.57026	0.335672	0.392703	0.531143	0.640657	0.419655	0.277142	0.346357	0.570843	0.459761	0.367152	0.262517
Glutamine	0.005769	0.005274	0.002174	0.004486	0.002449	0.007535	0.000775	0.022601	0.003145	0.002043	0.001864	0.00293
Glutaric acid	0.000467	0.001856	0.0005	0.001261	0.000841	0.00402	0.004054	0.002086	0.001494	0.001415	0.000513	0.000979
Glutathione	0.139826	0.088012	0.074324	0.091566	0.089592	0.115898	0.047602	0.062054	0.102311	0.0837	0.068201	0.05962
Glycine	4.238162	5.635458	3.536154	4.159922	4.780147	5.977166	3.598643	5.016059	4.550113	4.378197	3.148514	4.732973
Histidine	0.034768	0.035003	0.033015	0.022172	0.035224	0.052926	0.054283	0.078623	0.104565	0.038962	0.058051	0.095611
Homocysteine	0.001321	0.000694	0.001821	0.001019	0.001063	0.001227	0.001847	0.001272	0.001788	0.001413	0.000761	0.001633
Hydroxybenzoic acid	0.000106	0.000107	0.000167	0.000174	0.00021	0.000307	0.000117	0.000411	0.000364	0.000201	0.000206	0.000159
Isoleucine	0.049307	0.028824	0.033526	0.042991	0.075825	0.043644	0.046088	0.069489	0.090337	0.070135	0.066557	0.069126
Itaconic acid	0.001402	0.001073	0.000954	0.001109	0.002554	0.001491	0.015463	0.010792	0.003384	0.003988	0.001626	0.003662
Lactic acid	0.013763	0.00249	0.008069	0.008652	0.0071	0.006581	0.055425	0.012291	0.015433	0.010842	0.007385	0.010375
Leucine	0.146257	0.083601	0.11218	0.142154	0.26275	0.136983	0.136587	0.199451	0.346825	0.251771	0.210514	0.18683
Linoleic acid	0.014754	0.026111	0.010952	0.024941	0.036056	0.021403	0.014919	0.017392	0.027813	0.011051	0.010279	0.015776
Lysine	0.110496	0.084614	0.084045	0.112706	0.236188	0.140825	0.10847	0.394329	0.329286	0.20022	0.152202	0.145365
Maleic acid	0.02337	0.010093	0.018401	0.015042	0.020263	0.015113	0.031104	0.035597	0.096908	0.073997	0.06995	0.020115
Malic acid	0.016254	0.016155	0.01333	0.015576	0.031823	0.016198	0.029838	0.022251	0.065903	0.047935	0.062108	0.021488

Malonic acid	0.000286	0.000247	0.000454	0.000425	0.000958	0.000445	0.000482	0.000517	0.000609	0.00052	0.000387	0.00041
Margaric acid	0.063168	0.105845	0.027734	0.060716	0.08974	0.09673	0.033054	0.061493	0.068032	0.031488	0.027012	0.049675
Methionine	0.020019	0.018477	0.011415	0.017665	0.024443	0.028245	0.011504	0.119219	0.039037	0.033911	0.018595	0.036816
Myristic acid	0.139098	0.172045	0.095505	0.205049	0.250051	0.209184	0.076733	0.098634	0.152032	0.108302	0.07028	0.090728
Nicotinic acid	0.006966	0.006217	0.005693	0.010176	0.016899	0.01256	0.004059	0.006134	0.008055	0.005995	0.004456	0.008549
Oleic acid	0.020221	0.02773	0.011793	0.027653	0.039914	0.027578	0.016165	0.017597	0.030016	0.013821	0.01136	0.018013
Ornithine	0.066647	0.037595	0.082221	0.04631	0.014975	0.054753	0.051121	0.99215	0.230203	0.207073	0.13643	0.109228
Oxalic acid	0.002319	0.001684	0.002344	0.002001	0.003012	0.002539	0.001173	0.003051	0.001734	0.002278	0.001141	0.002263
Palmitelaidic acid	0.01572	0.016346	0.008286	0.025157	0.031705	0.016601	0.013667	0.012577	0.023828	0.011667	0.009432	0.012524
Palmitic acid	0.002006	0.003322	0.001525	0.003087	0.004175	0.003809	0.001618	0.002834	0.002651	0.00184	0.001415	0.001992
Pentadecanoic acid	0.070211	0.113662	0.03875	0.077885	0.088467	0.103897	0.036105	0.065894	0.076984	0.047034	0.03018	0.055506
Phenylalanine	0.038459	0.020402	0.024952	0.040063	0.057349	0.037007	0.035755	0.063675	0.083541	0.064031	0.055975	0.028281
Proline	0.275633	0.377726	0.195544	0.254978	0.456066	0.511087	0.192691	0.341464	0.355612	0.288557	0.175939	0.209494
Putrescine	0.000506	0.000698	0.000521	0.000669	0.000764	0.000952	0.000438	0.001256	0.008228	0.001049	0.000945	0.000749
Pyroglutamic acid	0.121281	0.068874	0.079346	0.111449	0.144019	0.096796	0.05265	0.063907	0.110578	0.08724	0.066791	0.052668
S-Adenosylmethionine	0.001161	0.003837	0.00214	0.005087	0.003087	0.006116	0.000359	0.00072	0.00063	0.002028	0.001052	0.003602
Serine	0.08976	0.07923	0.052128	0.048737	0.057602	0.148967	0.092819	0.09029	0.177579	0.067026	0.077893	0.226862
Stearic acid	0.380795	0.406644	0.289898	0.376139	0.491053	0.476581	0.408485	0.396311	0.431018	0.348772	0.253203	0.350404
Succinic acid	1.811242	0.018498	1.278762	1.592985	1.111788	0.044578	0.379209	0.048532	0.208581	0.113758	0.153519	0.070241
Threonine	0.065416	0.06683	0.034253	0.051103	0.078488	0.131678	0.098151	0.050129	0.082659	0.049256	0.068343	0.102867
trans-4-Hydroxyproline	0.002833	0.000712	0.001449	0.002463	0.004393	0.00171	0.013396	0.003872	0.009593	0.004045	0.008527	0.005356
trans-Vaccenic acid	0.007262	0.007966	0.003639	0.009608	0.016478	0.0074	0.009052	0.006695	0.011977	0.004638	0.004939	0.007194
Tridecanoic acid	0.049446	0.082279	0.036559	0.046263	0.055231	0.092823	0.019342	0.057717	0.076146	0.072976	0.053584	0.051979
Tryptophan	0.027605	0.024281	0.017198	0.025538	0.032731	0.034656	0.034156	0.029112	0.01863	0.023211	0.027185	0.026144
Tyrosine	0.078023	0.102457	0.047651	0.064092	0.114549	0.137261	0.056701	0.06699	0.075209	0.063881	0.056929	0.048475
Undecanoic acid	0.000397	0.000587	0.000443	0.000458	0.001017	0.000479	0.000384	0.000633	0.000594	0.000273	0.000375	0.000402
Valine	0.159082	0.126744	0.11584	0.140267	0.258429	0.21747	0.166352	0.347279	0.308314	0.252689	0.216657	0.353352

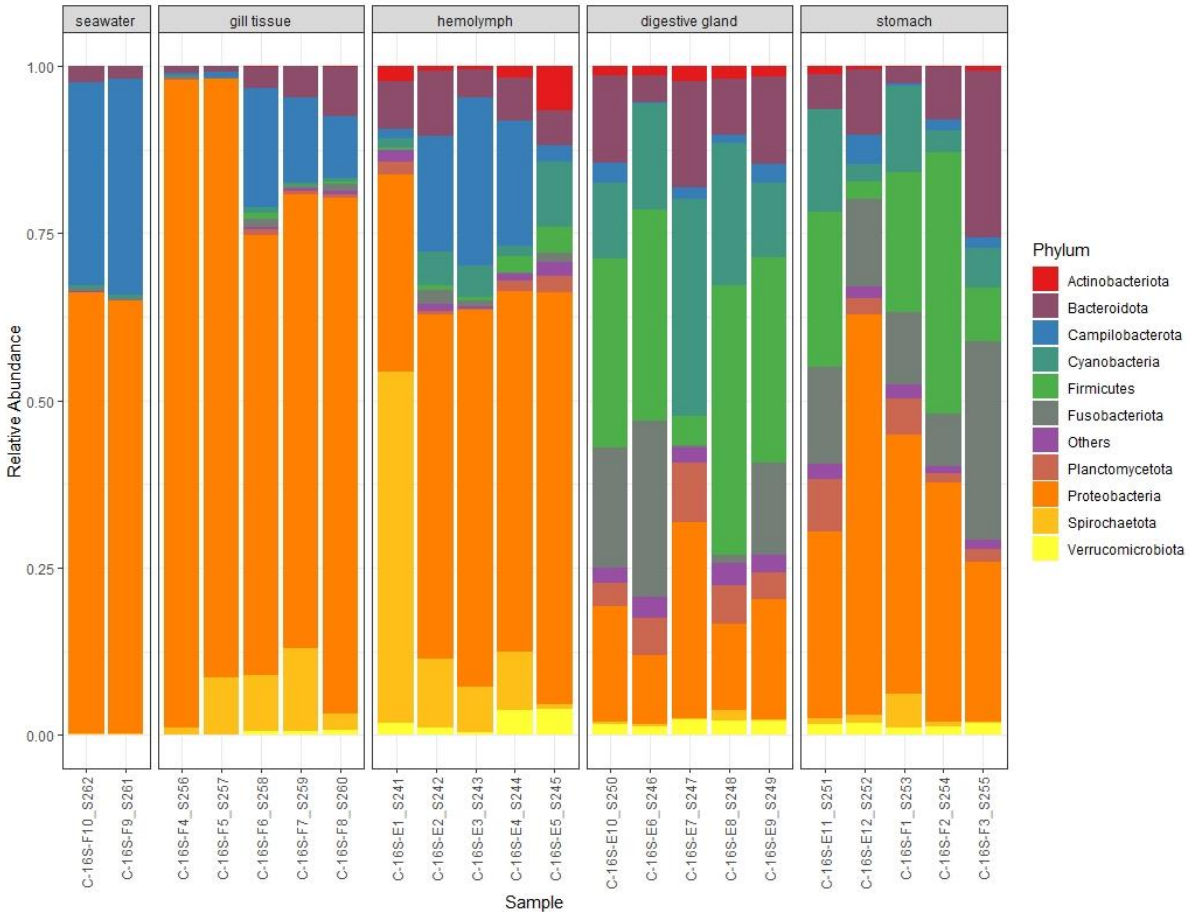
Supplementary table 2

	d.value	stdev	rawp	q.value	Regulation
2-Aminoadipic acid	6.5559	0.26307	0.000127	0.000654	Up
Citric acid	4.2702	0.36037	0.001519	0.002618	Up
Asparagine	-4.0882	0.37048	0.001899	0.002618	Down
cis-Aconitic acid	3.966	0.37751	0.002025	0.002618	Up
Histidine	3.2349	0.42329	0.007089	0.005972	Up
trans-4-Hydroxyproline	3.2247	0.42397	0.007089	0.005972	Up
Myristic acid	-3.0973	0.43259	0.008101	0.005972	Down
Maleic acid	3.0295	0.43726	0.009241	0.005972	Up
2,3-Butanediol	-2.8194	0.45197	0.015063	0.007417	Down
11,14,17-Eicosatrienoic acid	-2.805	0.453	0.015696	0.007417	Down
Malic acid	2.7304	0.45833	0.017215	0.007417	Up
Valine	2.7262	0.45863	0.017215	0.007417	Up
Isoleucine	2.5903	0.46844	0.022532	0.008115	Up
4-Hydroxyphenylacetic acid	2.5649	0.47028	0.023291	0.008115	Up
Succinic acid	-2.5547	0.47103	0.023544	0.008115	Down
gamma-Linolenic acid	-2.4464	0.47894	0.027342	0.008289	Down
S-Adenosylmethionine	-2.4074	0.4818	0.029873	0.008289	Down
10,13-dimethyltetradecanoic acid	-2.4063	0.48188	0.029873	0.008289	Down
DPA	-2.369	0.48463	0.031266	0.008289	Down
EPA	-2.3587	0.48538	0.032278	0.008289	Down
Glutathione	-2.3334	0.48725	0.033671	0.008289	Down
Pentadecanoic acid	-2.3084	0.48909	0.035823	0.008418	Down
Itaconic acid	2.2735	0.49165	0.037468	0.008422	Up
Creatinine	2.1847	0.4982	0.04519	0.009581	Up
Strombine	-2.169	0.49936	0.046329	0.009581	Down
Pyroglutamic acid	-2.1429	0.50128	0.048861	0.009716	Down

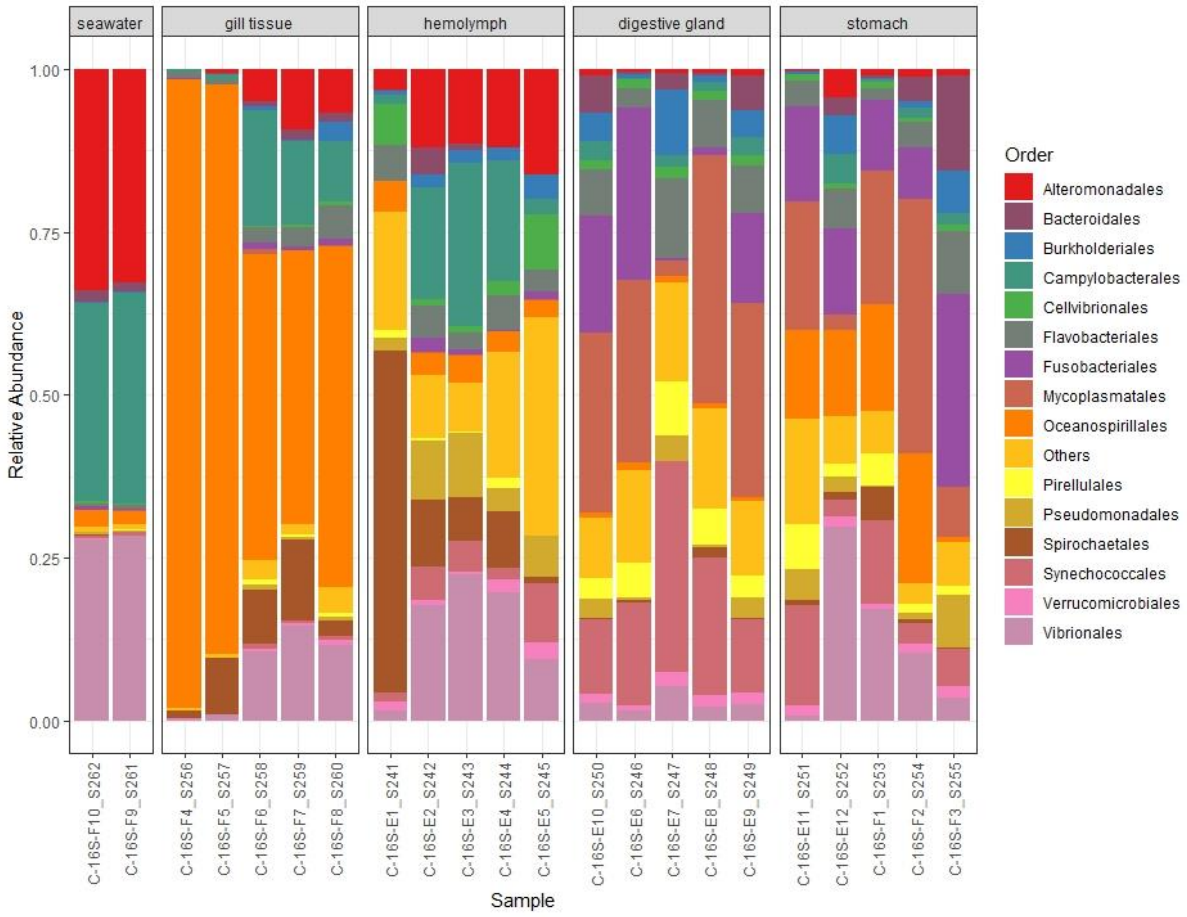
Supplementary table 3

UOA Identified Proteins	NCBI Blast RefSeq ID	Gene ID	Mean Healthy ± SD	Mean Unhealthy ± SD	FC (Unhealthy/Healthy)	d.value	stdev	rawp	q.value	Regulation direction
tektin-3-like isoform X1 0.9958|88.983|0.0	XM_022484351.1	111134864	3234421 ± 319911	1517387 ± 560107	2.13	-1.7983	0.096628	8.82E-05	0.063248	Down
LOW QUALITY PROTEIN: annexin A4-like 0.9948|63.472|0.0	XM_022462400.1	111121229	399418 ± 51525	216406 ± 54858	1.85	-1.5801	0.091617	0.00044101	0.10014	Down
testis, prostate and placenta-expressed protein-like 0.9903|81.068|1.83e-130	XM_022150236.1	110453417	112894 ± 28497	25178 ± 30896	4.48	-1.4999	0.10537	0.00070562	0.10014	Down
radial spoke head protein 9 homolog 0.9928|77.899|3.05e-159	XM_022150766.2	110456738	725910 ± 114898	419799 ± 127355	1.73	-1.447	0.12554	0.00084895	0.10014	Down
MACPF domain-containing protein 5 0.79|41.922|3.46e-119	KP125925.1		966901 ± 214029	408359 ± 199032	2.4	-1.4385	0.11256	0.00093716	0.10014	Down
transmembrane 9 superfamily member 2-like 0.9555|77.623|0.0	XM_022152109.1	110465344	192764 ± 35134	59719 ± 69000	3.23	-1.4232	0.12963	0.00104 74	0.10014	Down
coiled-coil domain-containing protein 170-like 0.997|56.437|0.0	XM_022148368.2	110440545	652550 ± 87164	357468 ± 140188	1.83	-1.4099	0.12051	0.00121 28	0.10014	Down
cilia- and flagella-associated protein 47-like isoform X2 0.9981|73.813|0.0	XM_022436971.1	111103594	44548 ± 20653	11840 ± 10414	3.76	-1.4076	0.1737	0.00125 69	0.10014	Down
tektin-3-like isoform X2 0.9961|85.799|0.0	XM_022460731.1	111120071	4093389 ± 575759	2182582 ± 782507	1.88	-1.3579	0.09942	0.00183 02	0.1265	Down
coiled-coil domain-containing protein 151-like 0.9709|78.839|0.0	XM_022464489.1	111122642	144587 ± 62478	37649 ± 19640	3.84	-1.3333	0.12688	0.00211 69	0.1265	Down
uncharacterized protein LOC110460860 0.9968|75.836|0.0	XM_022514054.1	110460860	366360 ± 56149	220071 ± 63364	1.67	-1.3175	0.11469	0.00244 76	0.12699	Down
uncharacterized protein LOC110460435 isoform X8 0.9984|49.573|0.0	XM_022513323.1	110460435	99514 ± 37812	26686 ± 18538	3.72	-1.3167	0.11444	0.00248 07	0.12699	Down
kinesin-like protein KIF17 isoform X8 0.9839|48.540|0.0	XM_011417113.2	105319529	106987 ± 40741	44721 ± 15507	2.4	-1.3039	0.15074	0.00272 33	0.12699	Down

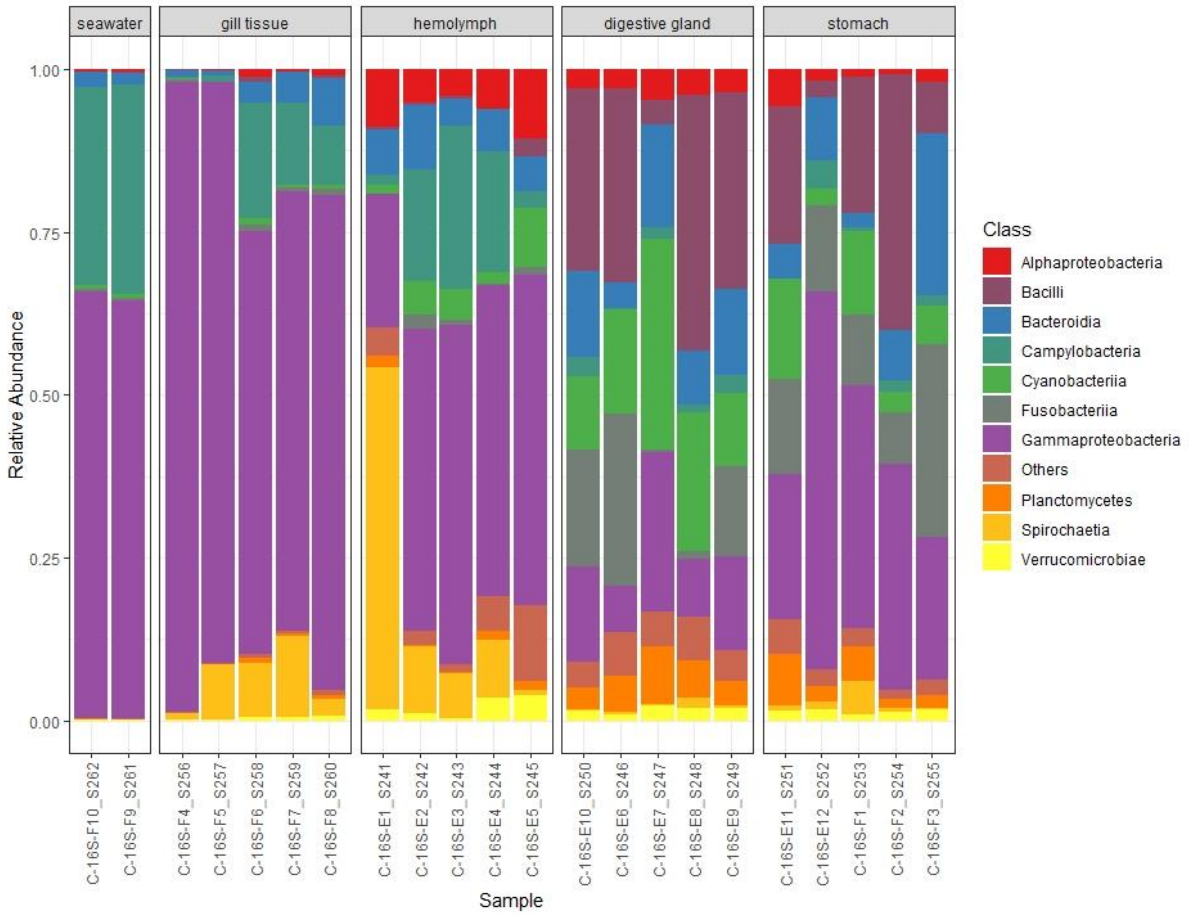
DDX6 0.9955|94.395|0.0	KT694372.1		540785 ± 128162	259222 ± 120154	2.1	-1.2963	0.12509	0.0028335	0.12699	Down
aldehyde dehydrogenase, dimeric NADP-preferring-like isoform X2 0.9895|62.712|0.0	XM_021486918.1	110442994	56810 ± 20073	18936 ± 17846	3	-1.2688	0.14732	0.0032415	0.13133	Down
annulin-like 0.8235|42.760|0.0	XM_021524730.1	110467514	197177 ± 42149	71523 ± 76842	2.76	-1.2677	0.14333	0.0032966	0.13133	Down
glutathione S-transferase sigma 2 0.9901|46.377|7.55e-58	JX485637.1		643911 ± 166569	218252 ± 192923	2.95	-1.2526	0.11199	0.0036714	0.13856	Down



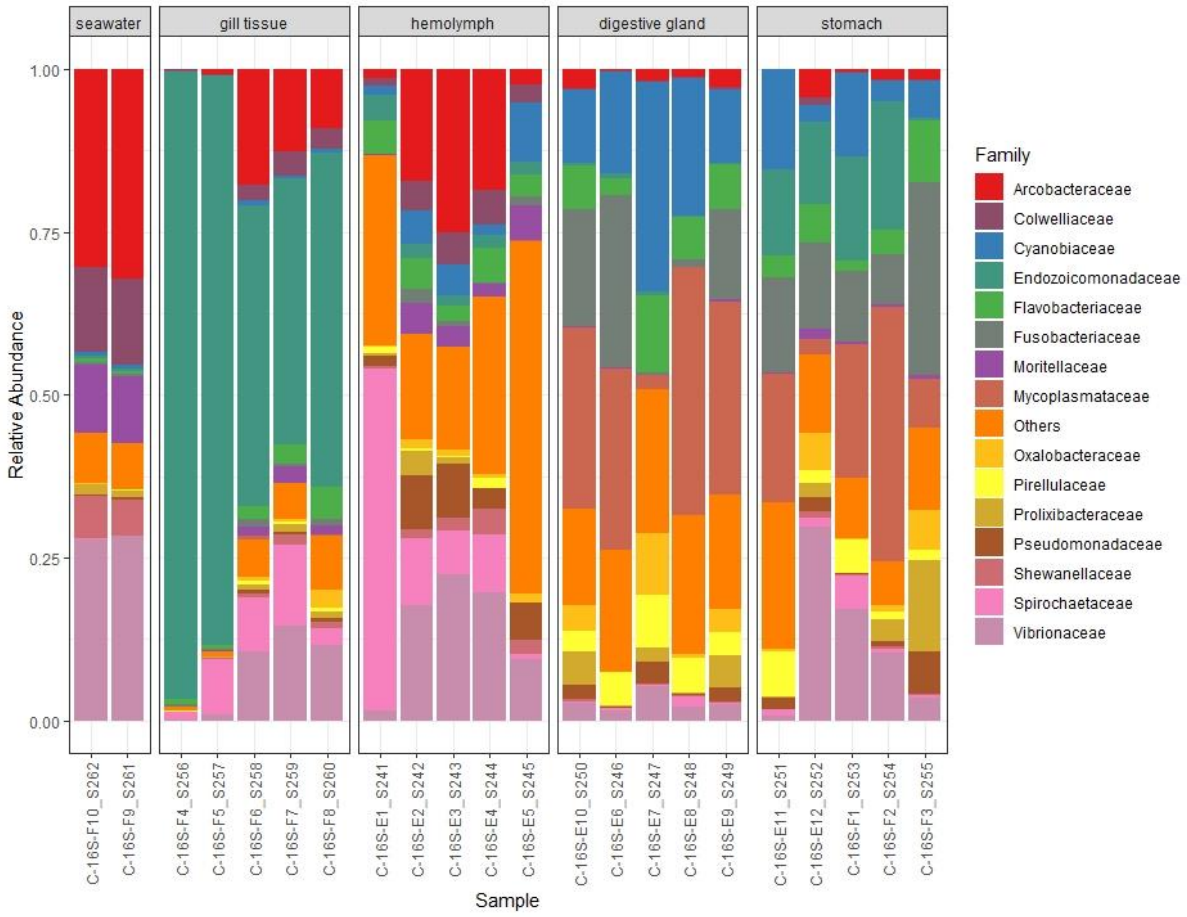
S1 Stacked barplot of relative abundance of bacteria at the phylum level across all tissue groups.



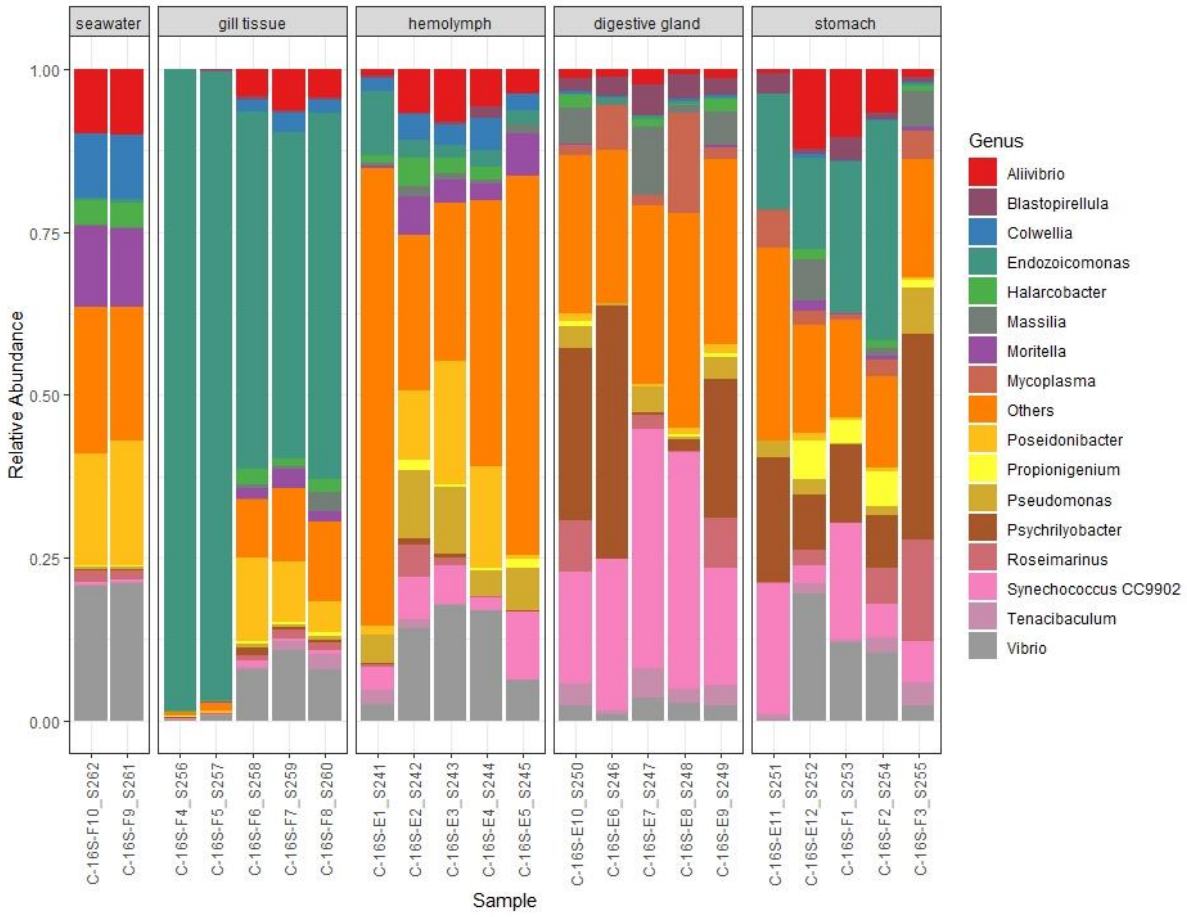
S2 Stacked barplot of relative abundance of bacteria at the order level across all tissue groups.



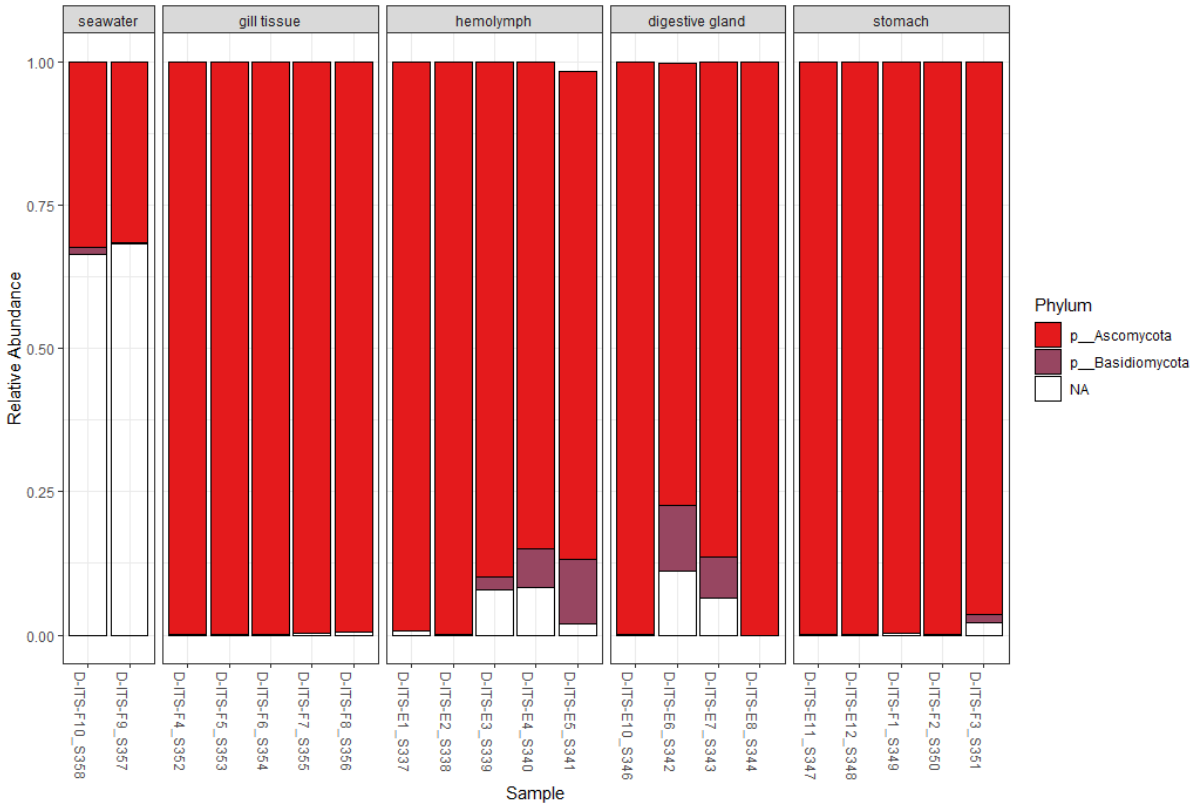
S3 Stacked barplot of relative abundance of bacteria at the class level across all tissue groups.



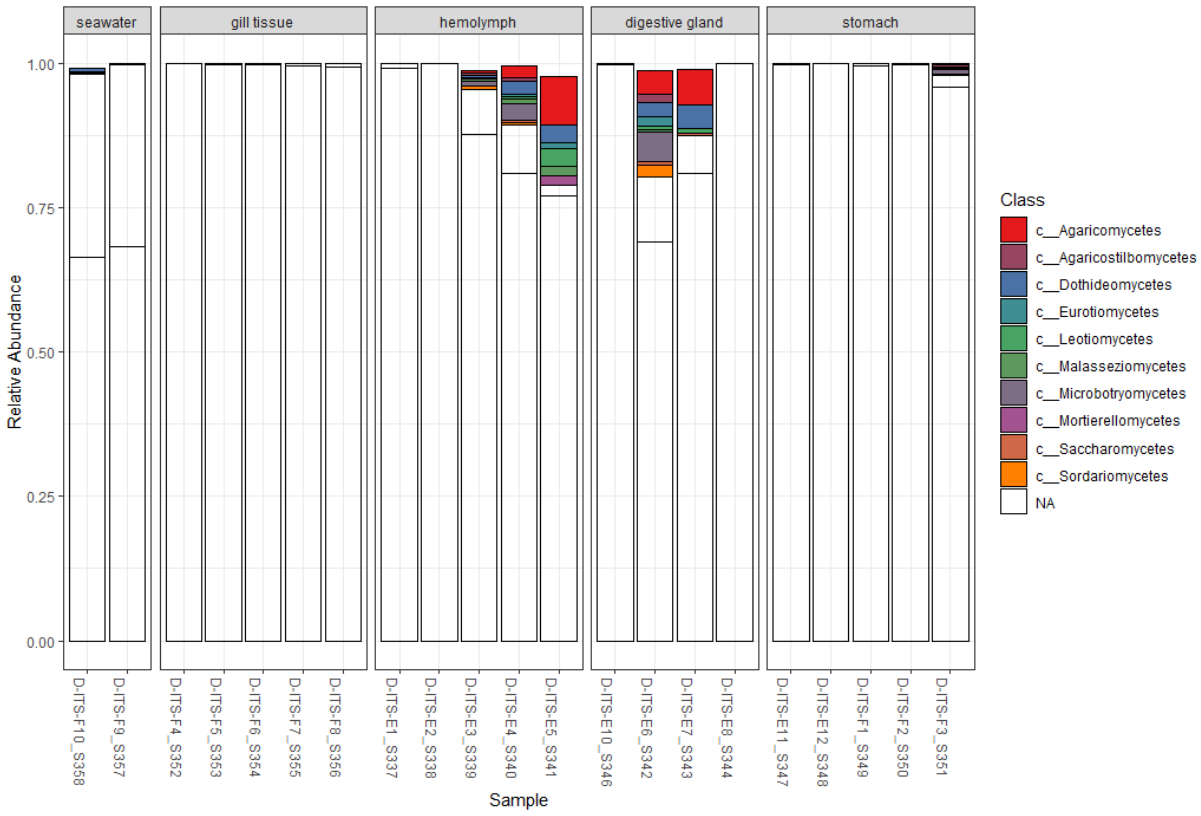
S4 Stacked barplot of relative abundance of bacteria at the family level across all tissue groups



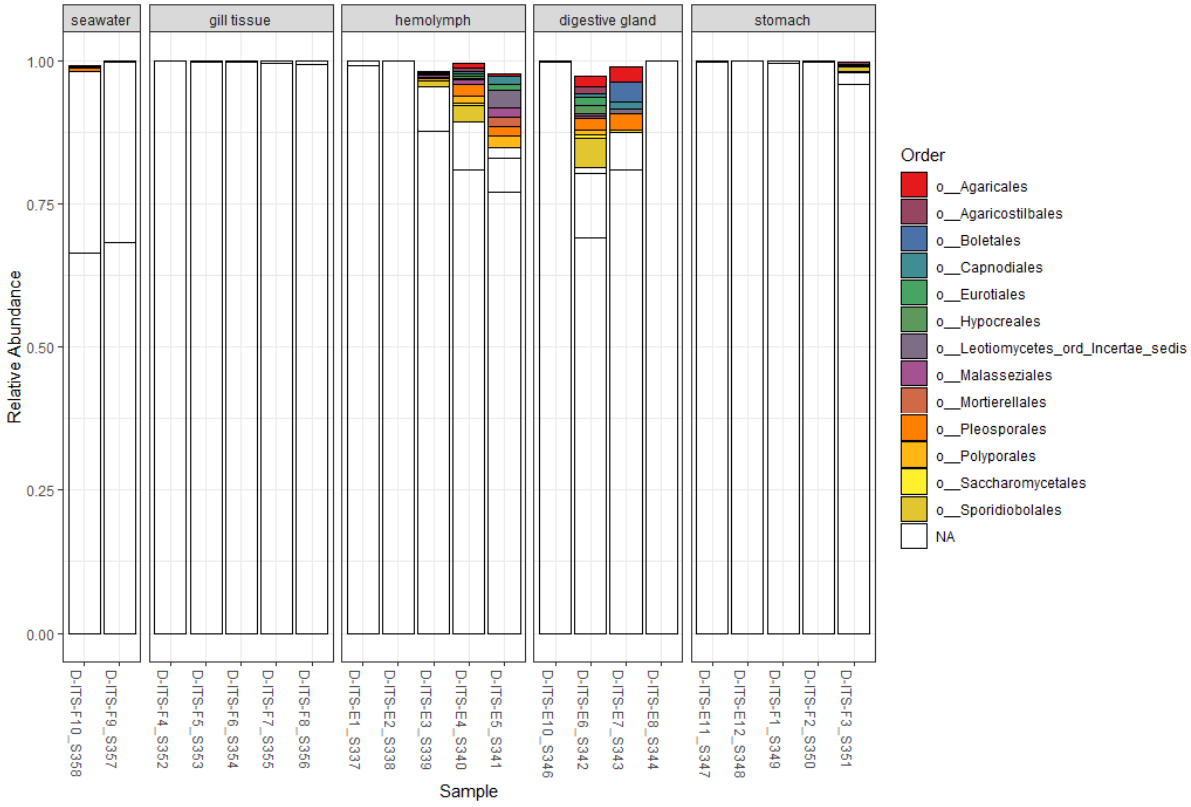
S5 Stacked barplot of relative abundance of bacteria at the genus level across all tissue groups.



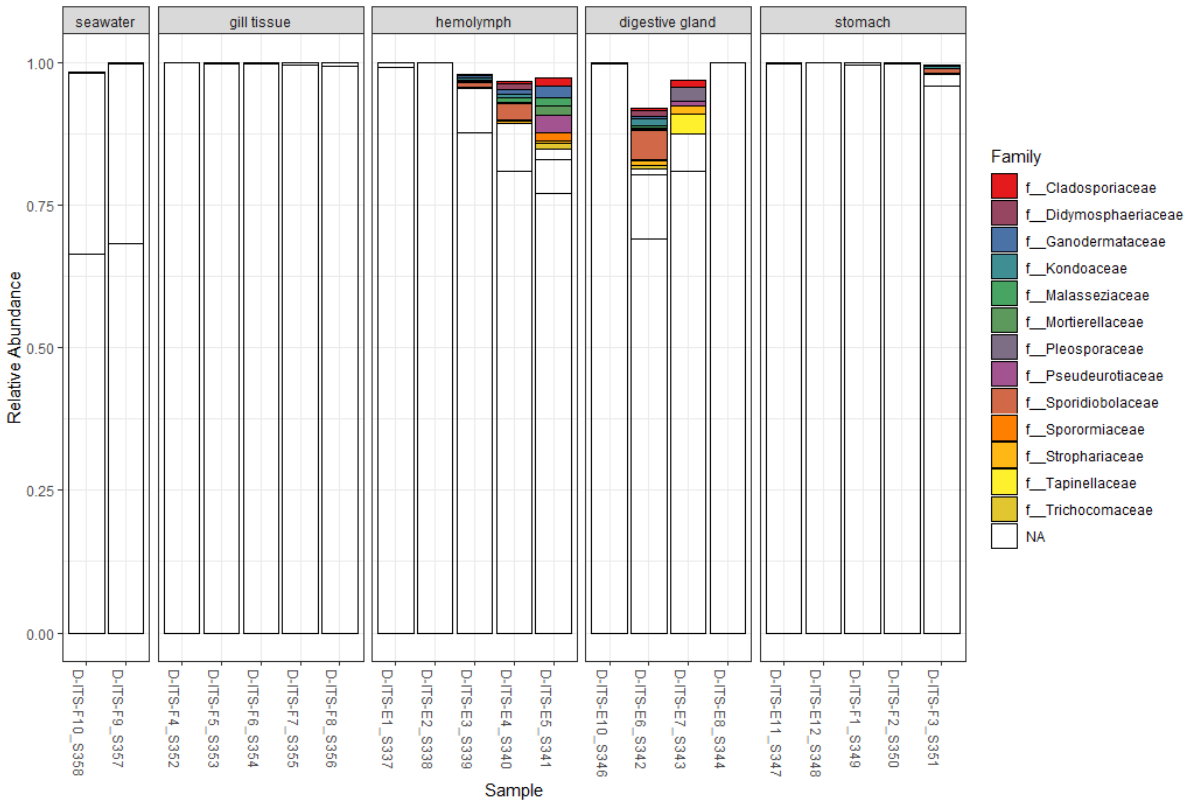
S6 Stacked barplot of relative abundance of fungus at the Phylum level across all tissue groups. NA represents unclassified fungus ASVs.



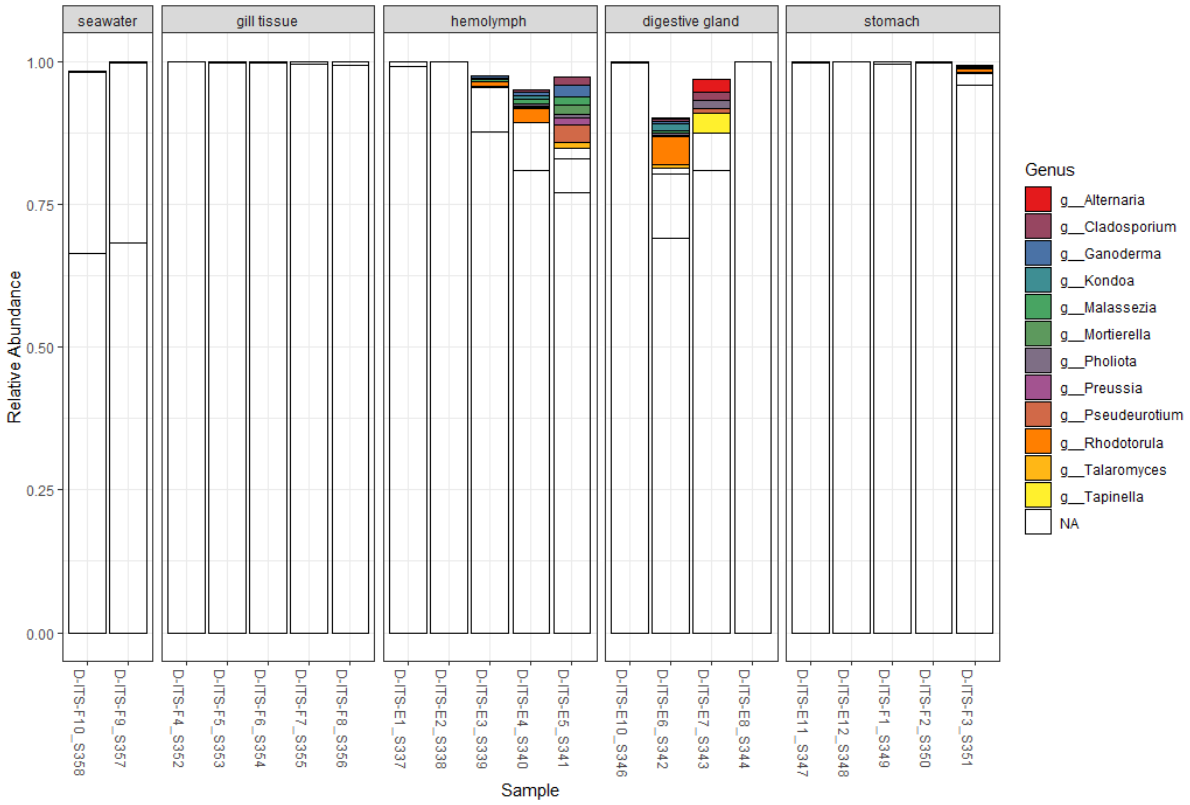
S7 Stacked barplot of relative abundance of fungus at the class level across all tissue groups. NA represents unclassified fungus ASVs.



S8 Stacked barplot of relative abundance of fungus at the order level across all tissue groups. NA represents unclassified fungus ASVs.



S9 Stacked barplot of relative abundance of fungus at the family level across all tissue groups. NA represents unclassified fungus ASVs.



S10 Stacked barplot of relative abundance of fungus at the genus level across all tissue groups. NA represents unclassified fungus ASVs.

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