Dynamics of Gut Microbiota in Bivalves: Exploring the Impact of Species, Habitat, Season and Feed Composition

By

Shirin Akter
B.Sc., M.Sc.

Thesis
Submitted to Flinders University
for the degree of

Doctor of Philosophy

College of Science and Engineering

8 August 2022

TABLE OF CONTENTS

CHAPTER 4

Seasonal pattern of bacterial community composition in Pacific oyster *Crassostrea gigas* gut and the environment

CHAPTER 5

Impact of feed composition on the gut bacterial composition in Pacific oyster *Crassostrea gigas* and Mediterranean mussel *Mytilus galloprovincialis*

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF FIGURES

CHAPTER 1

ABSTRACT

Gut microbiota research in marine invertebrates is an emerging field driven by the interest to understand the microbial association and their impact on animal health. Many microbes in the gut of aquatic animals are either sourced from the environment or food intake. Therefore, some gut microbes remain stable over time, but others are transient and established in the host gut. The bacteria community is mainly species-specific, but members in the gut of marine invertebrates are similar to those of teleost and mammals. Habitat differences, dietary intervention, host age, physiological conditions and environmental disturbances can modulate bacterial community and biomass. Gut microbiota plays a crucial role in food transformation into nutrients and energy and in disease resistance. Potential pathogenic bacteria and resident bacteria may be present in a healthy animal, but the opportunistic pathogens may outcompete endosymbionts during extreme environmental, seasonal and physiological stress to cause epidemic diseases. Furthermore, low food availability can alter intestinal homeostasis leading to a shift of the bacterial species composition. Therefore, sustainable bivalve aquaculture requires a better understanding of gut bacteria. The dynamics of gut bacterial composition due to host phylogeny, seasonal, environmental, and dietary variations is of particular interest to better understand bacterial symbiosis and health management in oysters and mussels. Yet, despite the commercial importance of these bivalves and susceptibility to diseases, little is known regarding the host and environmental pressures that drive gut microbiota composition between inter-genic populations. The research aims to understand bacterial community composition and assess if the gut microbial change is related to species, habitat, season, or feed composition. To achieve these goals, I carried out four experiments with four specific objectives: (1) to compare gut bacteria between oysters

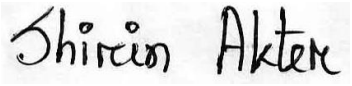
and mussels, (2) to compare gut bacterial community in oysters between two different habitats, (3) to investigate the seasonal pattern of gut bacterial community composition in oysters, and (4) to identify the impact of feed composition on gut bacteria in oysters and mussels.

Experiment 1 demonstrates both inter-generic and intra-specific host specificity in colonising bacterial composition in the gut of oysters and mussels from same sampling site. The seasonal change of gut bacteria in oysters differed from mussels. Experiment 2 defines the colonization of host-specific bacteria in oyster gut. The bacterial abundance and diversity were both season and habitat-dependent. The gut bacteria of oysters in the oceanic nutrient based Coffin Bay had higher diversity than those in local nutrient based habitat Franklin Harbor. This trend was more obvious in winter than in summer. Experiment 3 shows a significant effect of seasonal temperature on the oyster gut microbial community and its ambient environment whole year-round. Experiment 4 shows that diet type strongly affected the gut microbial composition in oysters and mussels in a laboratory condition, and the response of bacteria to diet change varied with feed composition and bivalve species.

In summary, Tenericutes was the most prevalent bacterial class in oyster guts. The low microbial diversity, especially in summer, provides evidence to partially explain the vulnerability of oysters to bacterial infection and mass mortality in summer. The high bacterial diversity of oysters in winter and oceanic nutrient based Coffin Bay indicates the resistance of oysters to environmental stress. In contrast, the low bacterial diversity of oysters in local nutrient based Franklin Harbor may be less resilient to environmental stress, triggering oyster disease or mass mortality in summer. Modulation of gut microbes could be used to predict the growth of beneficial bacteria in marine bivalves in aquaculture.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference is made in the text.



Shirin Akter

08 August 2022

Acknowledgements

All praises and thanks to our Creator, who enable me to successful completion of my PhD. First of all, I would like to express my heartfelt gratefulness to my principal supervisor Professor Jian G. Qin, for his scholastic guidance and supervision to accomplish my PhD. I sincerely acknowledge his inspiration and all supports from very beginning to overcome my critical situations during this journey. I also sincerely thank my associate supervisor Professor Xiaoxu Li for giving me opportunity to work on this fascinating project. Special thanks to my co-supervisor Dr. Andrew Oxley for his invaluable direction to successful ending of this project and teach me basics of microbiological aspects. I would like to acknowledge another co-supervisor Dr. Sarah Catalano for teaching me each laboratory fundamentals and helping me in library preparation and data analysis. I also would like to say special thanks to Dr. Melissa Oxley for teaching me basics of data analysis with its interpretations.

I acknowledge Dr. Md. Mahbubul Hassan for helping me in field sampling. Special thanks to Dr. Maziidah Ab Rahman and Shaun Henderson for helping me to run the feeding trial experiment. I sincerely acknowledge Dr. Md. Motiur Rahman and his family for supporting me in my critical moments of this PhD journey.

Special thanks to my lovely family. I am blessed to have a caring, innocent, friendly, and lovely soulmate. This PhD would never be possible without his overall support, help and leadership. I acknowledge my husband's each particular encouragement, endless support and contribution towards this journey. Special thanks to my two princes- Farhan Zahin and Raiyan Rafi for keeping me motivated to overcome all struggles positively. I sincerely acknowledge my beloved parents for their continuous support and encouragement from my birth to date, and my lovely sisters for their positive, caring words. I also acknowledge my elder brother Dr. Mostafa Hossain for his continuous inspiration from my childhood to pursue a PhD.

Lastly, I would like to acknowledge Fisheries Research and Development Corporation (FRDC) for funding the project (Project No. 2014/027), South Australian Research and Development Project (SARDI) for laboratory facilities, and Flinders University for providing Flinders University Research Scholarship (FURS) to pursue my PhD. I acknowledge the comment and help by Prof Jim Mitchel in the early stage of my research project.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1. General Introduction

Microbial symbiosis is a feature of most biotic systems, and it is the base for growth and evolution of eukaryotic organisms. The mutualism that evolved between bacteria and metazoan hosts dates back hundreds of millions of years (Ley *et al*., 2008). Anton Von Leewenhoek was the first to observe microbes in 1674 (DoBell, 1932), and isolation of *Escherichia* from human gut has laid the foundation to study our "second genome". Microbes regulate majority of the aspects of animal life including nutrient metabolism, immune response, pathogen resistance and animal development. An important concept in microbial ecology 'the hologenome theory of evolution' recognizes that a metazoan with its symbiotic microbiome evolves as a single unit 'holobiont' (Rosenberg *et al*., 2007). Since microbial symbiosis is integral for functioning of every ecosystem on earth, our understanding of the underlying mechanisms of host-microbe symbiosis is crucial for science and society.

Vertebrates represent only 4% of the total extant species but received the most attention in microbial studies. Vertebrates have complex body plans, and their microbial communities are highly diverse, whereas invertebrates have a more straightforward body plan and harbour less diverse microbial communities. As a result, vertebrates are difficult experimental subjects in many cases to tease apart specific molecular and cellular interactions between host tissue and associated microbes. Furthermore, aquatic invertebrates are under constant selective pressure from the physical condition of habitat that delineates many interesting microbial mutualisms. For example, the chemoautotrophic bacteria and marine invertebrates have developed a mutualistic relation, thereby accessing nutrition for each other in the deep-sea hydrothermal vents (Cavanaugh, 1983; Nakagawa & Takai, 2008). Sea sponge, an aquatic invertebrate, is the oldest species in plant originated 760 million

years back. In addition, some aquatic invertebrates, for example, red sea urchin, ocean quahog and the immortal jellyfish, can live for hundreds of years. The antiquity and diversity of aquatic invertebrates highlight the need to study their microbiome as we attempt to understand microbial symbiosis across different life forms.

1.1. Invertebrate Microbial Community: Healthy Ecosystem

Aquatic invertebrates are important aquaculture candidates globally, and beneficial microbes can improve current health management practices. In aquaculture, animals are exposed to a variety of stressors that incline the host to potential pathogens. Since most invertebrates have non-adaptive innate immunity (Rinkevich, 1996; Frank *et al.*, 1997), microbial mutualism is important for protecting from potential invaders. The use of antibiotics is no longer a preferred option for health management in aquaculture species due to environmental unsustainability and appearance of antibiotic-resistant bacteria (Holmström *et al*., 2003). The use of vaccines is expensive and have limited use in invertebrate aquaculture. Therefore, the application of beneficial microbes such as probiotics and prebiotics is increasing as a health management tool in aquaculture (Ninawe and Selvin, 2009; Mohapatra *et al*., 2013). Characterization of gut bacteria will contribute to understand host-microbe relations and the possibility of identifying potentially probiotic bacteria for aquaculture. The gut bacteria community structure and their dynamics due to phylogenetic, seasonal, environmental, and dietary change will improve gut microbial interaction leading to the efficient application of probiotics and prebiotics. A better understanding of gut microbiota can improve marine invertebrate health management.

1.2. Invertebrate Microbial Community: Diseased Ecosystem

Mass mortality and infectious diseases are a global concern in marine invertebrate aquaculture. Generally, pathogenic diseases are manifested by a synergistic impact of pathogens, environmental stress, and physiological imbalance of host. Therefore, it is intrinsically difficult to treat an infected population of marine invertebrates because (1) they cannot be treated with antigen as a preventive measure, (2) their enormous number and relatively smaller size do not allow mass treatment to be applied even if only a small number of individuals are infected, (3) some species, sedentary bivalves, for example, are cultured in open sea which is not suitable for chemotherapeutic applications and (4) the microbial association and susceptibility to disease depends on life stages. Given these limitations, understanding microbial dynamics is important for health management of marine invertebrates. In a healthy ecosystem, animals maintain microbial equilibrium and protect themselves from pathogenic infection. The microbes contribute to holobiont fitness, but their modulations impact the chance of survival. Therefore, microbial diversity in a holobiont has become increasingly essential to understanding microbial symbiosis.

1.3. The Transient and Resident Gut Microbe

Gut bacteria either originate with host or from environment, and some bacteria remain stable over time, but others establish for a limited time. Some gut microbes invade with incoming water and food and form transient bacteria in host, but the others permanently exist and form resident bacteria. The resident bacteria generally do not cause any harm to the host despite its continuous contact with host tissue, and they contribute to host fitness. The nature of microbial association also depends on how the microbes are acquired, the lateral transmission from the environment to animal tissues are generally transient, but the vertical transmission from the parents to offspring is

long-lasting. Similar life history and selective pressure in the vertical transmission result in a stronger association between microbes and the host, and thus the resident microbes maintain a life-long association with the host (Dubilier *et al*., 2008).

1.4. Factors Affecting Microbial Association

Multiple factors are involved in the colonization of gut bacteria in an animal, but primarily, a host gut comprises the resident microbes and evolves as a selection unit. Past research on microbial colonization has been placed within the framework and theories of microbial mutualism, co-evolution, and microbes' ecology. The inheritance of symbiotic microbes has propelled the concept 'the hologenome theory of evolution' (Rosenberg *et al*., 2007), which different animal models have supported, including shrimp (Oxley *et al*., 2002; Rungrassamee *et al*., 2014), zebrafish (Roeselers *et al*., 2011), hydra (Fraune and Bosch, 2007), drosophila (Cox and Gilmore, 2007) and mice (Wilson *et al*., 2006). In addition to species specificity, the microbes have a selective association with host tissues, organs, geographic locations (Sweet *et al*., 2011; Weiland-Bräuer *et al*., 2015) and environmental conditions (Schöttner *et al*., 2009). The coral and sponge models have demonstrated that the selective microbiota association in invertebrates are distinct from the seawater and sediment microbial communities (Hentschel *et al*., 2012; Morrow *et al*., 2012).

Along with the species-specific nature of the gut microbial evolution, the ecosystem also contributes to the selection of resident gut microbe of marine invertebrates. However, the resident microbial composition is not simply altered by changing the food or environment. The presence of filamentous bacteria in hydrothermal shrimp, irrespective of habitat and food source, suggests that they are resident microbes (Durand *et al*., 2010). In addition, the stability of the microbial community in sponges and oysters irrespective of seasonal and temperature shifts

supports the identification of host specificity resident microbes (Erwin *et al*., 2012; Pierce *et al*., 2016). The characteristic resident microbes are developed to meet the nutritional demand of the host in different ecosystems. The resident gut microbes of the animals living in the freshwater and seawater are likely to be different. The animals living in the complex local ecosystems on the deep-sea floor, such as wood falls, whale falls, hydrothermal vents, and cold seeps, have the characteristic of gut microbial communities. For example, the echinoids, gastropod and galatheid crabs living in wood falls have the characteristic of sulfur-oxidizing gut microbes to digest the wood-based diets (Becker *et al*., 2009; Hoyoux *et al*., 2009; Zbinden *et al*., 2010). The deep-sea invertebrates have a unique mechanism to survive in the nutrient-poor environment by developing mutual relations with sulfur-oxidizing bacteria that provide them access to oxygen and reduce inorganic compounds (Dubilier *et al*., 2008). The sulphur-oxidizing bacteria use sulphide and inorganically reduced sulphur to produce energy by oxidative phosphorylation, thus providing synthesized organic compounds for the host and contributing to host nutrition (Yamamoto and Takai, 2011). The inverse gradient of oxygen and sulphide or methane has prompted chemosynthetic symbioses (Stewart *et al*., 2005), and thus characteristic gut bacteria have evolved in the animals living in such an extreme ecosystem.

1.5. Variation in Microbial Community Dynamics

1.5.1. Host Age and Its Developmental Stages

The host age and physiological conditions also influence selection of gut bacterial community composition. The gut bacterial community was different in post-larvae, juvenile, and adult stages in *Crassostrea gigas* and *C. corteziensis* (Trabal *et al*., 2012), and a relatively diverse community was present in post-larvae than adults (Fernández *et al*., 2014). A unique gut bacteria community develops in different

developmental stages partly because the digestive enzymes produced are diverse at different stages (Zhao *et al.*, 2012). In addition, different microbial communities from polyp, strobila, ephyra and juvenile medusa of jellyfish suggest the life stage-specific microbial community (Weiland-Bräuer *et al*., 2015). The bacteria in gut and seawater were similar in juvenile abalone, but the adults have a different microbial community than seawater, suggesting that resident gut bacteria do not develop until the gut tissues start functioning efficiently (Tanaka *et al*., 2003). Most of the gut bacteria in juvenile abalone were replaced by algal polysaccharide-degrading bacteria from four months of age (Tanaka *et al*., 2003). Furthermore, the gut bacterial community was also found influenced by sexes in octopus (Iehata *et al*., 2013) and gonad development stage in shrimp (Cheung *et al*., 2015). Many of the transient bacteria eventually become resident as larvae develop into adults, but the dynamics of such microbial changes is not well understood.

1.5.2. Habitat

The immediate environment has a more significant impact on aquatic animals' life history than on terrestrial animals, and composition of gut bacteria in live animals generally depends on habitat environment. The differences in gut bacterial community composition of juvenile shrimp reared in oligotrophic water and eutrophic ponds indicate role of habitat in the selection of gut bacteria (Moss *et al*., 2000). In addition, the cultured and wild Chinese mitten crab have different gut bacteria communities (Li *et al*., 2007a). Furthermore, the deposit-feeder holothurians have specialized gut bacterial community only when living in nutrient-poor sediments, but such characteristic bacterial communities disappear when living in nutrient-rich sediments (Amaro *et al.*, 2009). The digestive tracts of aquatic species are in continual contact with surrounding water, and environmental microbes influence the gut bacteria.

However, the gut bacteria composition does not reflect the mirror image of environmental bacteria. For example, there was no significant difference in gut bacteria community between the wild and domesticated shrimp (Oxley *et al*., 2002; Rungrassamee *et al*., 2014).

1.5.3. Environmental Parameters

The effects of environmental factors on gut microflora are of special interest due to their consequences on animal physiology and the ecosystem. Environmental disturbances such as pollution, elevated temperature, food depletion, toxicity cause microbial community shift and dynamics of this shift depend on the magnitude of host stress and initial community structure. Such modulations of microbial community lead to disease susceptibility and mass mortality in marine invertebrates. Global warming and environmental stress have imbalanced microbial association leading to coral bleaching, which has imposed threats to the destruction of coral reefs worldwide (Rosenberg *et al*., 2009). In addition, heat stress destabilizes the sponge symbionts, leading to stress response, new microbes, and low functional redundancy, thus increasing the host's vulnerability to disease and mortality (Fan *et al*., 2013). The bacterial community of gut tissue and pallial fluid was found changed from winter to summer in eastern oyster *Crassostrea virginica* (Pierce *et al*., 2016). The gut bacteria composition was modulated by experimental infection compared to healthy Sydney rock oysters. Rickettsiale like prokaryotes dominated the gut flora, but effect of such microbial alteration on host health remained uncertain (Green and Barnes, 2010).

1.5.4. Sampling Location in The Gut

The location in gut has a characteristic gastrointestinal micro-environmental factors. Therefore, the resident microbe may develop for that particular location in the gut. The different compartments in gut reflect specific micro-environmental factors, thus imposing selective pressure on bacteria community. The pH, oxygen level and concentration of digestive enzymes vary along gradients in the digestive tract. The chitinous lining acts as a substrate for colonization of certain bacteria in the hindgut of crustaceans (Harris *et al*., 1991). The differences in total numbers, community composition and diversity of colonized bacteria exist in gut compartments. In holothurians, a dense and diverse community was found foregut than hindgut, and these changes reflect differences in microbial environment along the gut (Roberts *et al*., 2001; Plotieau *et al*., 2013; Gao *et al*., 2014). Three phyla represented the bacteria community in stomach in Eastern oyster, while the gut bacterial community was represented by twelve phyla (King *et al*., 2012). In addition, the density of microbes increases from anterior to posterior regions in shrimp gut (Gomez-Gil *et al*., 1998; Oxley *et al*., 2002). The microbial biomass and composition along the gut were markedly different in irregular sea urchin as a strategy to better exploit nutrients from organic matter through microbial degradation (Thorsen, 1999).

1.5.5. Diet

The dietary shift from low-fat to high-fat diet revealed an alteration of microbial metabolic pathway in germ-free mice (Turnbaugh *et al*., 2009). The community composition of gut bacteria can be modulated by dietary intervention. Besides, gut pH varies according to the animal feeding status, such as starved or fed, and the type of food intake (Tang *et al*., 2011). The gut bacterial composition was changed fed with different sources of food in lobster (Meziti *et al*., 2012) and abalone (Zhao *et al*., 2012. In another study, the gut bacteria community shifted when abalone diet

switched from microalgae to algal pellets (Tanaka *et al*., 2003). In addition, the dietary lipid sources affected microbial community composition in shrimp (Zhang *et al.*, 2014). On the contrary, gut bacteria community of copepod did not change after feeding different algae (Tang *et al*., 2009). The succession of bacterial community in different developmental stages in the abalone gut is associated with live food, and introduction of an artificial diet could alter this microbial succession (Zhao *et al*., 2012). The symbiotic bacteria in midgut of coastal isopods rely on either leaf litter or phenolic compounds as food sources, demonstrating that they have evolved specific endosymbionts to support their nutritional demand (Zimmer *et al*., 2001). The herbivorous gastropods have polymer-degrading and denitrifying gut bacteria to support their nutritional requirement (Zbinden *et al*., 2010).

1.6. Role of Gut Bacteria in Marine Invertebrate Wellbeing and Survival

1.6.1. Provision of Nutrients

Transplantation of obese human microbiome to germ-free mouse regulated nutrient metabolism towards obesity (Ridaura *et al*., 2013). The digestive tract is a dynamic and complex ecological niche where gut bacteria are source of various functions, including synthesis of digestive enzymes, nutrient absorption, and energy homeostasis. Gut bacteria influence host nutrient acquisition, but bacteria community composition also depends on the ingested food. For example, diet-induced obesity prompted growth of Mollicutes bacteria, and after transplantation of the bacteria to a germ-free recipient, they reduced fat deposition in mice (Turnbaugh *et al*., 2008). The proper functioning of gut microbes in nutrient metabolism has been a key factor in human health (Musso *et al*., 2011; Tremaroli and Bäckhed, 2012). In teleosts, gut bacteria are source of a range of digestive enzymes (Bairagi *et al*., 2002; Ray *et al*., 2012) and vitamins (Sugita *et al*., 1991), and also contributes to metabolism of

proteins (MacDonald *et al*., 1986), complex polysaccharides (Sugita and Ito, 2006), lipids (Rawls *et al*., 2004) and fatty acids (Semova *et al*., 2012).

Even though gut microbes are expected to play a similar role in providing host nutrition, it has not been explored extensively in marine invertebrates because of the paucity of empirical studies. The gut bacteria in marine invertebrates assist the production of different digestive enzymes to catalyse complex substrates (Erasmus *et al.* 1997; Sakai *et al*., 2003; Liu *et al*., 2013). Ingestion of flagellates and ciliates bacteria contributed to nutrient acquisition, and these bacteria are considered a food complement for shrimp larvae (Thompson *et al*., 1999). The alginolytic gut microbes in abalone and sea urchin convert algal polysaccharides into acetic acid, becoming an essential source of energy and precursors of anabolism (Sawabe *et al*., 1995; Sawabe et al., 2003). Sea cucumber has polysaccharides degrading gut bacteria to digest organic matter (Zhang *et al*., 2013). The gut bacteria of xylophagous marine invertebrates have chemosynthetic properties to metabolize sunken woods (Becker *et al*., 2009; Hoyoux *et al*., 2009). The microbes in anterior and intestinal caeca contribute to carbohydrate fermentation and sulphide oxidation, which is important for energy acquisition of a sea urchin (Thorsen, 1998). Enzyme activity of gut bacteria contributes to digestion among male and female octopus leading to the distinct provision of nutrients (Iehata *et al*., 2013). Interestingly, Amaro *et al*. (2009) suggest that deep-sea holothurians do not require bacteria symbiosis for nutrient acquisition in organic matter rich substances, but such relations are crucial in nutrient-poor sediments.

1.6.2. Immunity

Despite lacking adaptive immunity, marine invertebrates have strong resilience to pathogenic microbes and biotic and abiotic stressors. Different invertebrate species have similarities in immune defence, but because of diversified evolutionary trajectories among members of different phyla, they also have different approaches to innate immune system (Loker *et al*., 2004). The invertebrate immune system defends pathogens by activating cellular and humoral responses that recognize a broad spectrum of pathogens. The cellular response includes haemocyte mobilization and phagocytosis, whereas releasing cytokines, enzymes and immune effectors to infection site is considered a humoral response. In addition, molecular mechanisms such as antimicrobial peptides and proteins have also evolved to defend against infections and stressful environmental conditions.

Human and mouse models have shown that microbial mutualism contributes to innate and adaptive immunity. In addition, a healthy microbiome plays a vital role by providing critical signals for the regulation of immune system. The development of innate immunity in marine invertebrates with associated microbes has not been extensively explored like that of mammals, but a similar microbial consortium for development of immunity is expected. Gut bacteria act as a physical barrier inhibiting the invasion of pathogens into host gut, thus improving host immunity. Colonization of beneficial bacteria in gut mucosal surface competitively excludes pathogens by limiting nutrients and space in binding sites. In addition, certain microbes show antagonistic activity by preventing colonization of pathogens in gut. Bacteria-bacteria interaction is a vital structuring force of the microbiome such as 35% of surface bacteria from marine invertebrate and seaweed produced antimicrobial compounds (Burgess *et al*., 1999). In a healthy coral holobiont, complex antagonistic interactions of resident bacteria with potentially pathogenic bacteria structured coral microbiome

to maintain coral health (Rypien *et al*., 2010). Some bacteria act as an immune modifier by reinforcing antibody level and macrophage activity. The production of antimicrobial compounds improves ability to compete with pathogenic bacteria, and thus they can be a powerful weapon to protect marine invertebrates from epizootics (Desriac *et al*., 2010). The production of bacteriocin, an antimicrobial peptide of major lineages of bacteria and archaea, forms a shield in host gut that limits establishment of pathogens by inhibiting pathogen introduction and stimulating immune function (Dobson *et al*., 2012). A bacterial strain *Aeromonas media*, capable of producing bacteriocin like substances, effectively controlled infections in Pacific oyster larvae (Gibson *et al*., 1998). The expression of immune-related genes during an experimental immune challenge by pathogenic bacteria revealed immune function in shrimp gut (Soonthornchai *et al*., 2010).

1.7. Major Challenges in Sustainable Invertebrate Aquaculture

Bacterial diseases are major limiting factors for sustainable aquaculture. The combined effects of animal physiological status, environmental conditions, and pathogenic bacteria have caused mass mortality, becoming a global concern for marine invertebrate aquaculture. Aquatic animals are exposed to stressful conditions in intensive aquaculture, which has led to occurrence of infectious diseases. Poor water quality, high stocking density, inappropriate feeding causes physiological imbalance and may lead to loss of gut bacterial homeostasis. Disease susceptibility is related to the loss of gut microbial homeostasis leading to lack of mucosal barrier to pathogens, occupancy of space and nutrients by opportunist pathogens, and lack of release of substances that inhibit pathogens. Environmental stress such as elevated temperature minimizes the antagonistic interactions and impedes antibiotic

production, thus leading to a proliferation of pathogens (Long *et al*., 2005; Rypien *et al.*, 2010).

Marine invertebrate gut harbours a rich bacterial community, including opportunistic bacteria like *Vibrio*, *Pseudomonas, Aeromonas,* and *Flavobacterium*. *Vibrio* spp, for example, constitute majority of bacteria biomass present in invertebrate gut. The *Vibrio* has a diverse existence in marine ecosystem and host tissues, including free-living, symbiotic, opportunistic, and pathogenic forms, and these lifestyles are modulated by environmental variability (Thompson *et al*., 2004). High seawater temperature weakens host immune defence and increases pathogenicity of *Vibrio* by up-regulating virulence factors, including motility, host degradation, secretion, and antimicrobial resistance (Kimes *et al*., 2012), thereby causing the disease and mortality. Mass mortalities in marine invertebrates cause severe economic impacts on aquaculture industry, and *Vibrio* spp. have been implicated as one of the major causative agents of diseases (Gómez-León *et al*., 2005; Saulnier *et al*., 2010). *Vibrio* strains are also associated with mortality episodes, which have been demonstrated by experimental infections in Pacific oysters (Gay *et al*., 2004; Garnier *et al*., 2007).

1.8. The Necessity to Study Bivalve Gut Microbial Dynamics

Sustainable bivalve aquaculture requires a better understanding of gut bacteria because they influence energy transfer, metabolism, absorption, and immune function. During extreme seasonal and environmental variability, the host becomes stressed, and opportunistic pathogens exploit advantage of host stress. Low food availability can alter intestinal homeostasis leading to a shift of bacterial species composition. Under such a condition, when host physiology is imbalanced, pathogenic bacteria multiply further and cause host mortality (Lacoste *et al.*, 2002). Bivalves can be used

as a model research organism. The factors that influence gut bacteria community composition and their physiological consequences in marine invertebrates have been explained in Figure 1.1.

1.9. Main Research Hypotheses

Although research has unveiled the indigenous bacterial flora in oyster gut, the composition tends to alter by seasonal, environmental, and dietary variations. The dynamics of bacterial composition in oyster gut due to seasonal, environmental, and dietary variation is of particular interest to better understand bacterial symbiosis and oyster health management. Considering the economic importance of aquaculture activities worldwide and consumer demand, I have chosen the Pacific oyster and Mediterranean mussel as a model for bivalve research. Pacific oyster is a critical marine bivalve globally that significantly impacts the economy of many countries, including China, Japan, Korea, France, USA, Taiwan, and Australia. Therefore, I hypothesized that the bacterial community composition varies with host phylogeny, habitat, season and dietary manipulation in the bivalve gut and surrounding environment.

1.10. Overall Study Objectives

The main research aims to understand bacterial community composition and assess if the gut microbial change is related to species, habitat, season, or feed composition. To achieve this aim, I outline my thesis into the following four specific objectives. The objectives are,

1. To compare gut bacteria between two inter-generic bivalve species.
2. To compare gut bacterial community between two different habitats.
3. To investigate seasonal pattern in gut bacterial community composition.
4. To identify the impact of different feed composition on gut bacteria.

1.11. Summary of Thesis

This thesis is represented six chapters with a general introduction (Chapter 1), four data chapters and a general discussion (Chapter 6).

Chapter 1 is outlined as a general introduction that mainly includes research background based on microbial community composition, gut bacterial composition and its role in invertebrates, factors regulating the gut bacterial dynamics, and major challenges invertebrate aquaculture.

Chapter 2 addresses how host phylogeny, seasonality, and aging shape the gut microbiota of cohabiting marine bivalves - Pacific oyster and Mediterranean mussel (Objective -1). There is a lack of information on host preference for colonization of certain groups of bacteria among inter-generic marine bivalves. This study correlates the influence of host genetics to identify host-specificity in marine bivalves.

Chapter 3 reports the comparison of oyster gut microbial community between two different habitats – two premier oyster culture regions of Southern Australia Coffin Bay and Franklin Harbor (Objective -2). Coffin Bay is maily based on oceanic nutrient because of its high-water exchange and upwelling system, whereas Franklin Harbor is mainly based on local nutrient and food sources. The microbial symbiosis in host tissue relating to nutrient availability in a marine environment has never been examined. This research was carried out to explore how the microbial symbiosis changes due to food availability and how they respond to future environmental changes.

Chapter 4 illustrates seasonal pattern of oyster gut microbial diversity and identifies seasonal symbiosis throughout the year from one winter to another (Objective - 3). There is a lack of information on microbiota in different physiological

and environmental conditions. Therefore, this study describes microbial symbiosis in different seasons.

Chapter 5 clarifies the impact of feed composition on the gut microbial community. There is a lack of information on invertebrate microbiota related to dietary manipulation. The two-month feeding trial was carried out using two diets (microalgae and macroalgae) to compare the gut microbial diversity between oysters and mussels. This lab experiment was run to predict bivalve microbial symbiosis in different feed types and food availability in different ecosystems.

Chapter 6 is a general discussion and conclusion highlighting the major findings of four different objectives, and their implications to the bivalve industry. Major limitations of this research are also included, and future research directions are proposed.

1.12. References

Amaro, T., Witte, H., Herndl, G. J., Cunha, M. R., & Billett, D. S. (2009). Deep-sea bacterial communities in sediments and guts of deposit-feeding holothurians in Portuguese canyons (NE Atlantic). *Deep Sea Research Part I: Oceanographic Research Papers*, 56(10), 1834-1843.

Bairagi, A., Ghosh, K. S., Sen, S. K., & Ray, A. K. (2002). Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture International*, 10(2), 109-121.

Becker, P. T., Samadi, S., Zbinden, M., Hoyoux, C., Compère, P., & De Ridder, C. (2009). First insights into the gut microflora associated with an echinoid from wood falls environments. *Cahiers de Biologie Marine*, 50(4), 343.

Burgess, J. G., Jordan, E. M., Bregu, M., Mearns-Spragg, A., & Boyd, K. G. (1999). Microbial antagonism: a neglected avenue of natural products research. *Journal of Biotechnology*, 70(1), 27-32.

Cavanaugh, C. M. (1983). Symbiotic chemoautotrophic bacteria in marine invertebrates from sulphide-rich habitats. *Nature*, *302*(5903), 58-61.

Cheung, M. K., Yip, H. Y., Nong, W., Law, P. T. W., Chu, K. H., Kwan, H. S., & Hui, J. H. L. (2015). Rapid change of microbiota diversity in the gut but not the hepatopancreas during gonadal development of the new shrimp model *Neocaridina denticulata*. *Marine Biotechnology*, 17(6), 811-819.

Cox, C. R., & Gilmore, M. S. (2007). Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infection and Immunity*, 75(4), 1565-1576.

Desriac, F., Defer, D., Bourgougnon, N., Brillet, B., Le Chevalier, P., & Fleury, Y. (2010). Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic. *Marine Drugs*, 8(4), 1153-1177.

DoBell C. (1932). AntonVon Leewenhoek and Big 'LittleAnimals'. Harcourt Brace and Company, New York, NY, USA.

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin production: a probiotic trait. *Applied and Environmental Microbiology*, 78(1), 1-6.

Dubilier, N., Bergin, C., & Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology*, 6(10), 725-740.

Durand, L., Zbinden, M., Cueff-Gauchard, V., Duperron, S., Roussel, E. G., Shillito, B., & Cambon-Bonavita, M. A. (2010). Microbial diversity associated with the hydrothermal shrimp *Rimicaris exoculata* gut and occurrence of a resident microbial community. *FEMS Microbiology Ecology*, 71(2), 291-303.

Erasmus, J. H., Cook, P. A., & Coyne, V. E. (1997). The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture*, 155(1), 377-386.

Erwin, P. M., Pita, L., López-Legentil, S., & Turon, X. (2012). Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance. *Applied and Environmental Microbiology*, 78(20), 7358-7368.

Fan, L., Liu, M., Simister, R., Webster, N. S., & Thomas, T. (2013). Marine microbial symbiosis heats up: the phylogenetic and functional response of a sponge holobiont to thermal stress. *The ISME Journal*, 7(5), 991-1002.

Fernández, N. T., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Ascencio-Valle, F., & Romero, J. (2014). Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiology Ecology*, 88(1), 69-83.

Frank, U., Oren, U., Loya, Y., & Rinkevich, B. (1997). Alloimmune maturation in the coral *Stylophora pistillata* is achieved through three distinctive stages, 4 months post–metamorphosis. Proceedings of the Royal Society of London B: *Biological Sciences*, 264(1378), 99-104.

Fraune, S., & Bosch, T. C. (2007). Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. *Proceedings of the National Academy of Sciences*, 104(32), 13146-13151.

Gao, F., Li, F., Tan, J., Yan, J., & Sun, H. (2014). Bacterial community composition in the gut content and ambient sediment of sea cucumber *Apostichopus japonicus* revealed by 16S rRNA gene pyrosequencing. *PloS one*, *9*(6), e100092.

Gibson, L. F., Woodworth, J., & George, A. M. (1998). Probiotic activity of Aeromonas media on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. *Aquaculture*, 169(1), 111-120.

Gomez-Gil, B., Tron-Mayen, L., Roque, A., Turnbull, J. F., Inglis, V., & Guerra-Flores, A. L. (1998). Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. *Aquaculture*, 163(1), 1-9.

Gómez-León, J., Villamil, L., Lemos, M. L., Novoa, B., & Figueras, A. (2005). Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. *Applied and Environmental Microbiology*, 71(1), 98-104.

Green, T. J., & Barnes, A. C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology*, 109(2), 613-622.

Harris, J. M., Seiderer, L. J., & Lucas, M. I. (1991). Gut microflora of two saltmarsh detritivore thalassinid prawns, *Upogebia africana* and *Callianassa kraussi*. *Microbial Ecology*, 21(1), 277-296.

Hentschel, U., Piel, J., Degnan, S. M., & Taylor, M. W. (2012). Genomic insights into the marine sponge microbiome. *Nature Reviews Microbiology*, 10(9), 641-654.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B. E., & Kautsky, N. (2003). Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International Journal of Food Science & Technology*, 38(3), 255-266.

Hoyoux, C., Zbinden, M., Samadi, S., Gaill, F., & Compère, P. (2009). Wood-based diet and gut microflora of a galatheid crab associated with Pacific deep-sea wood falls. *Marine Biology*, 156(12), 2421-2439.

Iehata, S., Valenzuela, F., & Riquelme, C. (2015). Analysis of bacterial community and bacterial nutritional enzyme activity associated with the digestive tract of wild Chilean octopus (*Octopus mimus* Gould, 1852). *Aquaculture Research*, *46*(4), 861-873.

Kimes, N. E., Grim, C. J., Johnson, W. R., Hasan, N. A., Tall, B. D., Kothary, M. H., Kiss, H., Munk, A. C., Tapia, R., Green, L., Detter, C., Bruce, D. C., Brettin, T. S., Colwell, R. R., & Morris, P. J. (2012). Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. *The ISME Journal*, 6(4), 835-846.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One*, *7*(12), e51475.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., & Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology*, 6(10), 776-788.

Li, K., Guan, W., Wei, G., Liu, B., Xu, J., Zhao, L., & Zhang, Y. (2007a). Phylogenetic analysis of intestinal bacteria in the Chinese mitten crab (*Eriocheir sinensis*). *Journal of Applied Microbiology*, 103(3), 675-682.

Li, M., Yang, H., & Gu, J. D. (2009). Phylogenetic diversity and axial distribution of microbes in the intestinal tract of the polychaete *Neanthes glandicincta*. *Microbial Ecology*, 58(4), 892-902.

Liu, Q., Ren, P., Piao, M., & Yang, J. Y. (2013). Protease Properties of Protease-Producing Bacteria Isolated from the Digestive Tract of *Octopus vulgaris*. *Journal of Life Science*, 23(12), 1486-1494.

Loker, E. S., Adema, C. M., Zhang, S. M., & Kepler, T. B. (2004). Invertebrate immune systems–not homogeneous, not simple, not well understood. *Immunological Reviews*, 198(1), 10-24.

Long, R. A., Rowley, D. C., Zamora, E., Liu, J., Bartlett, D. H., & Azam, F. (2005). Antagonistic interactions among marine bacteria impede the proliferation of *Vibrio cholerae*. *Applied and Environmental Microbiology*, 71(12), 8531-8536.

MacDonald, N. L., Stark, J. R., & Austin, B. (1986). Bacterial microflora in the gastro-intestinal tract of Dover sole (*Solea solea* L.), with emphasis on the possible role of bacteria in the nutrition of the host. *FEMS Microbiology Letters*, 35(1), 107-111.

Meziti, A., Mente, E., & Kormas, K. A. (2012). Gut bacteria associated with different diets in reared *Nephrops norvegicus*. *Systematic and Applied Microbiology*, 35(7), 473-482.

Mohapatra, S., Chakraborty, T., Kumar, V., Deboeck, G., & Mohanta, K. N. (2013). Aquaculture and stress management: a review of probiotic intervention. *Journal of Animal Physiology and Animal Nutrition*, 97(3), 405-430.

Morrow, K. M., Moss, A. G., Chadwick, N. E., & Liles, M. R. (2012). Bacterial associates of two Caribbean coral species reveal species-specific distribution and geographic variability. *Applied and Environmental Microbiology*, 78(18), 6438-6449.

Moss, S. M., LeaMaster, B. R., & Sweeney, J. N. (2000). Relative abundance and species composition of gram-negative, aerobic bacteria associated with the gut of juvenile white shrimp *litopenaeus vannamei* reared in oligotrophic well water and eutrophic pond water. *Journal of the World Aquaculture Society*, 31(2), 255-263.

Musso, G., Gambino, R., & Cassader, M. (2011). Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annual Review of Medicine*, 62, 361-380.

Nakagawa, S., & Takai, K. (2008). Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance. *FEMS Microbiology Ecology*, *65*(1), 1-14.

Ninawe, A. S., & Selvin, J. (2009). Probiotics in shrimp aquaculture: avenues and challenges. *Critical Reviews in Microbiology*, 35(1), 43-66.

Oxley, A. P. A., Shipton, W., Owens, L., & McKay, D. (2002). Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *Journal of Applied Microbiology*, 93(2), 214-223.

Pierce, M. L., Ward, J. E., Holohan, B. A., Zhao, X., & Hicks, R. E. (2016). The influence of site and season on the gut and pallial fluid microbial communities of the eastern oyster, *Crassostrea virginica* (Bivalvia, Ostreidae): community-level physiological profiling and genetic structure. *Hydrobiologia*, 765(1), 97-113.

Plotieau, T., Lavitra, T., Gillan, D. C., & Eeckhaut, I. (2013). Bacterial diversity of the sediments transiting through the gut of *Holothuria scabra* (Holothuroidea; Echinodermata). *Marine biology*, 160(12), 3087-3101.

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, 101(13), 4596-4601.

Ray, A. K., Ghosh, K., & Ringø, E. (2012). Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition*, 18(5), 465-492.

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., Griffin, N. W., Lombard, V., Henrissat, B., Bain, J. R., & Muehlbauer, M. J. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science*, 341(6150), 1241214.

Rinkevich, B. (1996). Immune responsiveness in marine invertebrates revisited: the concourse of puzzles. *New Directions in Invertebrate Immunology*, 55-90.

Roberts, D., Moore, H. M., Berges, J., Patching, J. W., Carton, M. W., & Eardly, D. F. (2001). Sediment distribution, hydrolytic enzyme profiles and bacterial activities in the guts of *Oneirophanta mutabilis*, *Psychropotes longicauda* and *Pseudostichopus villosus*: what do they tell us about digestive strategies of abyssal holothurians. *Progress in Oceanography*, 50(1), 443-458.

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., & Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5(10), 1595-1608.

Rosenberg, E., Koren, O., Reshef, L., Efrony, R., & Zilber-Rosenberg, I. (2007). The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology*, 5(5), 355-362.

Rosenberg, E., Kushmaro, A., Kramarsky-Winter, E., Banin, E., & Yossi, L. (2009). The role of microorganisms in coral bleaching. *The ISME Journal*, 3(2), 139-146.

Rosenberg, E., Kushmaro, A., Kramarsky-Winter, E., Banin, E., & Yossi, L. (2009). The role of microorganisms in coral bleaching. *The ISME Journal*, 3(2), 139-146.

Rungrassamee, W., Klanchui, A., Maibunkaew, S., Chaiyapechara, S., Jiravanichpaisal, P., & Karoonuthaisiri, N. (2014). Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). *PloS One*, 9(3), e91853.

Rypien, K. L., Ward, J. R., & Azam, F. (2010). Antagonistic interactions among coral-associated bacteria. *Environmental Microbiology*, 12(1), 28-39.

Sakai, T., Ishizuka, K., & Kato, I. (2003). Isolation and characterization of a fucoidan-degrading marine bacterium. *Marine Biotechnology*, 5(5), 409-416.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., & Garcia, C. (2010). A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. *Microbial Ecology*, 59(4), 787-798.

Sawabe, T., Oda, Y., Shiomi, Y., & Ezura, Y. (1995). Alginate degradation by bacteria isolated from the gut of sea urchins and abalones. *Microbial Ecology*, 30(2), 193-202.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M., & Ezura, Y. (2003). Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone *V. halioticoli* association. *Aquaculture*, 219(1), 671-679.

Schöttner, S., Hoffmann, F., Wild, C., Rapp, H. T., Boetius, A., & Ramette, A. (2009). Inter-and intra-habitat bacterial diversity associated with cold-water corals. *The ISME Journal*, 3(6), 756-759.

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host & Microbe*, 12(3), 277-288.

Soonthornchai, W., Rungrassamee, W., Karoonuthaisiri, N., Jarayabhand, P., Klinbunga, S., Söderhäll, K., & Jiravanichpaisal, P. (2010). Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Developmental & Comparative Immunology*, 34(1), 19-28.

Stewart, F. J., Newton, I. L., & Cavanaugh, C. M. (2005). Chemosynthetic endosymbioses: adaptations to oxic–anoxic interfaces. *Trends in Microbiology*, 13(9), 439-448.

Sugita, H., & Ito, Y. (2006). Identification of intestinal bacteria from Japanese flounder (*Paralichthys olivaceus*) and their ability to digest chitin. *Letters in Applied Microbiology*, 43(3), 336-342.

Sugita, H., Miyajima, C., & Deguchi, Y. (1991). The vitamin B 12-producing ability of the intestinal microflora of freshwater fish. *Aquaculture*, 92, 267-276.

Sweet, M. J., Croquer, A., & Bythell, J. C. (2011). Bacterial assemblages differ between compartments within the coral holobiont. *Coral Reefs*, 30(1), 39-52.

Tanaka, R., Sugimura, I., Sawabe, T., Yoshimizu, M., & Ezura, Y. (2003). Gut microflora of abalone *Haliotis discus hannai* in culture changes coincident with a change in diet. *Fisheries Science*, 69(5), 951-958.

Tanaka, R., Sugimura, I., Sawabe, T., Yoshimizu, M., & Ezura, Y. (2003). Gut microflora of abalone *Haliotis discus hannai* in culture changes coincident with a change in diet. *Fisheries Science*, 69(5), 951-958.

Tang, K., Dziallas, C., Hutalle-Schmelzer, K., & Grossart, H. P. (2009). Effects of food on bacterial community composition associated with the copepod *Acartia tonsa* Dana. *Biology Letters*, *5*(4), 549-553.

Thompson, F. L., Abreu, P. C., & Cavalli, R. (1999). The use of microorganisms as food source for *Penaeus paulensis* larvae. *Aquaculture*, 174(1), 139-153.

Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., Lim, E., & Polz, M. F. (2004). Diversity and dynamics of a North Atlantic coastal *Vibrio* community. *Applied and Environmental Microbiology*, 70(7), 4103-4110.

Thorsen, M. S. (1998). Microbial activity, oxygen status and fermentation in the gut of the irregular sea urchin *Echinocardium cordatum* (Spatangoida: Echinodermata). *Marine Biology*, 132(3), 423-433.

Thorsen, M. S. (1999). Abundance and biomass of the gut-living microorganisms (bacteria, protozoa and fungi) in the irregular sea urchin *Echinocardium cordatum* (Spatangoida: Echinodermata). *Marine Biology*, 133(2), 353-360.

Trabal, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Asencio-Valle, F., Morales-Bojórquez, E., & Romero, J. (2012). Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. *Microbial Ecology*, 64(2), 555-569.

Tremaroli, V., & Bäckhed, F. (2012). Functional interactions between the gut microbiota and host metabolism. *Nature*, 489(7415), 242-249.

Turnbaugh, P. J., Bäckhed, F., Fulton, L., & Gordon, J. I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host & Microbe*, 3(4), 213-223.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., & Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science Translational Medicine*, 1(6), 6ra14-6ra14.

Weiland-Bräuer, N., Neulinger, S. C., Pinnow, N., Künzel, S., Baines, J. F., & Schmitz, R. A. (2015). Composition of bacterial communities associated with *Aurelia aurita* changes with compartment, life stage, and population. *Applied and Environmental Microbiology*, 81(17), 6038-6052.

Wilson, K. H., Brown, R. S., Andersen, G. L., Tsang, J., & Sartor, B. (2006). Comparison of fecal biota from specific pathogen free and feral mice. *Anaerobe*, 12(5), 249-253.

Yamamoto, M., & Takai, K. (2011). Sulfur metabolisms in epsilon-and gamma-Proteobacteria in deep-sea hydrothermal fields. *Frontiers in Microbiology*, 2.

Zbinden, M., Pailleret, M., Ravaux, J., Gaudron, S. M., Hoyoux, C., Lambourdière, J., Warén, A., Lorion, J., Halary, S., & Duperron, S. (2010). Bacterial communities associated with the wood-feeding gastropod *Pectinodonta* sp. (Patellogastropoda, Mollusca). *FEMS Microbiology Ecology*, 74(2), 450-463.

Zhang, M., Sun, Y., Chen, K., Yu, N., Zhou, Z., Chen, L., Du, Z., & Li, E. (2014). Characterization of the intestinal microbiota in Pacific white shrimp, *Litopenaeus vannamei*, fed diets with different lipid sources. *Aquaculture*, 434, 449-455.

Zhao, J., Shi, B., Jiang, Q. R., & Ke, C. H. (2012). Changes in gut-associated flora and bacterial digestive enzymes during the development stages of abalone (*Haliotis diversicolor*). *Aquaculture*, 338, 147-153.

Zimmer, M., Danko, J. P., Pennings, S. C., Danford, A. R., Ziegler, A., Uglow, R. F., & Carefoot, T. H. (2001). Hepatopancreatic endosymbionts in coastal isopods (Crustacea: Isopoda), and their contribution to digestion. *Marine Biology*, 138(5), 955-963.

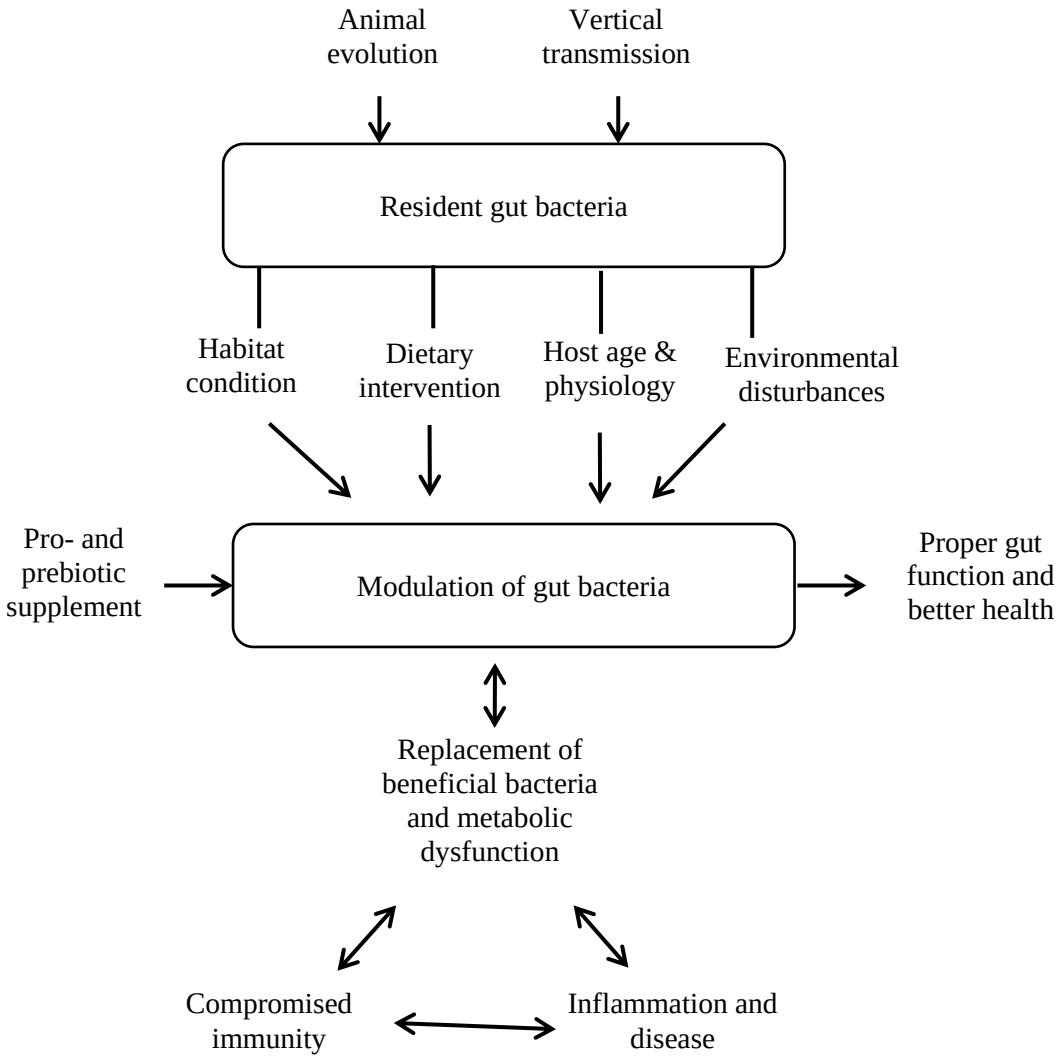


Figure 1.1. The acquisition of gut bacteria and the consequences of their modulations in marine invertebrates. The resident gut microbe is determined through animal evolution and vertical transmission from parents to offspring. The resident bacteria can be accompanied/ outcompeted by the pathogenic bacteria depending on the habitat condition, dietary intervention, host age and physiological condition, and environmental disturbances. The supplementation of pro- and pre-biotics can also modulate gut bacteria for proper gut function and better animal health. When the pathogenic bacteria outcompete beneficial bacteria, the immune homeostasis disrupts.

CHAPTER 2

Host phylogeny and seasonality shape the gut microbiota of commercially important cohabiting marine bivalves

2.1. Abstract

The Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) are two commercially important marine bivalves that frequently coexist and have overlapping feeding ecologies. The gut-associated microbial community of these bivalves is thought to play an important role in supporting their health and nutrition. Yet, despite their commercial importance and varied susceptibility to disease (notably seasonal mass mortality events), little is known regarding the role of host and environment in driving gut microbiota composition of cohabiting, intergeneric populations. Here, bacterial assemblages were surveyed from DNA extracts obtained from seawater and gut aspirates of farmed *C. gigas* and co-occurring wild *M. galloprovincialis* collected in summer and winter from Coffin Bay, South Australia, by constructing Illumina 16S rDNA gene amplicon libraries. Unlike seawater, which was dominated by Proteobacteria, samples from bivalves largely consisted of Tenericutes (Mollicutes) and accounted for >50% of the total OTU abundance. Oysters and mussels shared a large number of common (core) bacterial taxa, though bivalve-specific species (OTUs) were also evident and largely associated with taxa representing *Mycoplasamataceae* (notably *Mycoplasma* spp.), as well as *Anaplasmataceae*, *Spirochaetaceae* and *Spiroplasmataceae*. Bacterial community composition was seasonally influenced, whereby an increase in diversity (though with varied taxonomic evenness) was observed in winter for both bivalves and was associated with changes in abundance of both core and bivalve-specific taxa, including several representing typically host-associated and environmental (free-living or particle-diet associated) organisms. Our findings highlight the contribution

of both environment and host in defining gut microbiota composition. This study represents seasonal differences in the gut microbiota of cohabiting marine bivalves, revealing constituents that may support their ecology and ability to share same environment, which can be used to infer potentially disparate changes arising from future stressors.

Keywords: Gut microbiota, seasonal changes, Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis*, Mycoplasma, 16S rDNA.

2.2. Introduction

Microbes are ubiquitous and vital components of marine ecosystems that interact and form various, often intimate, relationships with an array of marine animal life (Fraune and Bosch 2010). Those associated with the gastrointestinal (GI) tract are considered to be of particular importance in supporting the animal's health and nutrition and are driven by an array of both intrinsic factors such as host physiology, genetics, age, growth, sex, immune status and life stage, and extrinsic factors such as diet and environmental conditions (Bajinka *et al*. 2020; Moran-Ramos *et al*. 2020). The GI tract of healthy animals is thought to harbour rich and diverse populations of both resident and transient microbes, of which bacteria are predominant constituents. During physiological and environmental stress, opportunistic microbes may outcompete resident populations for resources, leading to an imbalance in community composition, possible immune suppression, and disease emergence (Webster *et al*. 2008; Belikov *et al*. 2019; Muñoz *et. al*. 2019). For sessile, suspension-feeding marine invertebrates, who actively ingest and are subject to local fluctuations in the surrounding environmental microbial consortia, host-microbe interactions and relationships are likely to be of particular importance in supporting and influencing

their ecology. However, unlike vertebrates, our understanding of marine invertebrate host-microbe interactions is limited (Petersen and Osvatic 2018), though it is thought to be similarly shaped through co-evolutionary pressures, leading to the selection of species that support host health and metabolism (O'Brien *et al*. 2019).

While recent efforts have focused on the association and contributions of microbes belonging to particular invertebrate hosts (notably sponge or coral holobiont systems), our knowledge of other important marine invertebrate species like bivalves is gaining momentum due to their commercial significance and the tremendous risks posed by various pathogens (Paillard *et al*. 2004; Travers *et al*. 2015; Pierce and Ward 2018; King *et al*. 2019). Like in other marine animals, microbial community composition in bivalves varies across the different body regions, with the GI tract, gills, pallial fluid or haemolymph supporting distinct, tissue-specific assemblages (Dubé *et al*. 2019; Pathirana *et al*. 2019; Musella *et al*. 2020). Variations in the physicochemical characteristics and underlying immune functions within these regions are likely contributing features in the selection and enrichment of these assemblages (Meisterhans *et al*. 2016; Leite *et al*. 2017; Dubé *et al*. 2019; Musella *et al*. 2020), which together are thought to drive the host phenotype as constituent members of the bivalve microbiome (Pierce and Ward 2018; Simons *et al*. 2018; Sepulveda and Moeller, 2020). Microbes in the gut can tolerate low pH and can support the host through contribution to, among others, the synthesis of digestive enzymes, utilisation of energy, nutrient absorption, metabolism and immune homeostasis (Marques *et al*. 2006; Xiong *et al*. 2019; Lindsay *et al*. 2020). As reported for particular bivalves like oysters, these communities may, however, be influenced by particular stressors (e.g., elevated water temperatures), leading to microbiome imbalances that have the capacity to influence normal host functioning

and susceptibility to pathogen infection (Lokmer and Wegner 2015; King *et al*. 2019; Li *et al*. 2019; Scanes *et al*. 2021). This is of particular concern for oysters and other commercially important species like mussels, where the threat of a changing climate, seasonal mass mortality events, and population decline is becoming increasingly apparent (Thomas *et al*. 2018; Green *et al*. 2019; Soon and Zheng, 2019; Capelle *et al*. 2021). Our ability to gauge the magnitude that such risks represent though is largely dependent upon our understanding of the natural dynamics of the microbiome and the factors influencing its composition. This includes the role that host genetics plays in the selection of particular (core) constituents, their contribution to key host processes, and the impact of environmental change at both a spatial and temporal scale. For most bivalve species, such knowledge currently remains limited, though has the capacity to further support efforts that seek to use the microbiome as a predictive marker of environmental stress and disease susceptibility (Clerissi *et al*. 2020).

The Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) are two globally important species of significant economic value, accounting for up to ~30% of the world's commercial bivalve production (Wijsman *et al*. 2019). Like other bivalves, these species occupy bays, estuaries and near shore coastal waters and, at least in their native range, are also considered important for supporting the broader dynamics of marine ecosystems through the roles they play in nutrient cycling, habitat formation and modification, and trophodynamics (Vaughn and Hoellein, 2018). Having been introduced in other parts of the world like Australia through farming and early immigration (Gillies *et al*. 2018), these species are able to translocate into and cohabit surrounding areas (Svane 2011; Hedge and Johnston 2014) where they interact with competing for similar food sources (Rahman *et al*. 2019). In parts of the Northern Hemisphere, the invasion and subsequent cohabitation

of *C. gigas* with other related mussel species (namely *M. edulis*) has led to the formation of 'oyssel' reef systems (Reise *et al*. 2017). Alongside the valuable insights that this has been suggested to present for elucidating the functioning of species assemblages (Reise *et al*. 2017), the cohabitation of bivalves also offers a prospect for delineating and exploring the role of host genetics and environment on the gut microbiome. However, while current insights from these species suggest a likely role for the host in the occurrence of select bacterial taxa (including the differential enrichment of potentially pathogenic *Vibrio* spp.) (Vezulli *et al*. 2018), little is known regarding the influence of seasonality on these communities.

This study aims to understand the influence of host phylogeny and seasonality on the gut microbiome (bacterial assemblages) of the intergeneric, cohabiting marine bivalves *C. gigas* (Ostreidae) and *M. galloprovincialis* (Mytilidae). Specifically, comparative evaluations of the gut bacterial assemblages from farmed *C. gigas* and wild *M. galloprovincialis* collected from the same site in summer and winter were performed using an Illumina 16S rDNA gene deep-sequencing approach. The impact of the surrounding environmental bacterial consortia on these gut bacterial communities was also evaluated by collecting and comparing samples obtained from seawater from the same site during summer and winter. This study represents the seasonal differences in cohabiting bivalves. Such knowledge could be used to further support our current understanding of host-specific microbiomes and inferring potentially disparate changes in health and disease may arise from future stressors.

2.3. Material and Methods

2.3.1. Oyster, Mussel and Seawater sampling

Pacific oyster, Mediterranean mussel and seawater samples were collected from Coffin Bay, South Australia, Australia in February and August 2017 (mid-summer and late winter in the Southern Hemisphere, respectively). The oysters were farmed using the longline method, where four parallel lines were strung between wooden posts, and oysters were hung in plastic baskets. Oysters were collected from baskets graded for market size at the farm and likely represent mixed genetic cohorts, while wild mussels were randomly collected from the wooden posts at the same farm. Seawater samples were collected at the farm at a depth of ~1 m using 2L sterile glass bottles. In summer and winter, 30 Pacific oysters, 30 Mediterranean mussels, and 3×2L seawater samples were collected (total = 60 Pacific oysters, 60 Mediterranean mussels and six seawater samples) (Table 2.1). All samples were stored at 4°C immediately upon collection and transported to the Lincoln Marine Science Centre (Port Lincoln, South Australia, Australia) for further processing. Oysters and mussels were cleaned of fouling organisms (barnacles) and blotted with a paper towel for weight measurements. Oysters and mussels with a shell length of ~30-70 mm from the anterior to the posterior of the shell were used in this study and were absent of any obvious (symptomatic) features of the disease.

Oysters and mussels were cleaned with 70% ethanol to minimise potential contamination arising from bacteria on the outer shell surfaces. Gut contents from the oysters and mussels were collected by carefully prying open the shells and inserting a sterile glass pasture pipette fitted with a rubber bulb (Wheaton, DWK) through the mouth and applying gentle suction. The gut content from each individual (~200µl) was dispensed into sterile cryovials and stored in liquid nitrogen for downstream DNA extraction. Gut and water samples were then transported to the Molecular Science Laboratory at South Australian Research and Development Institute (West

Beach, South Australia, Australia) under temperature-controlled conditions for further analysis.

2.3.2. DNA Extraction from Gut and Water Samples

According to the manufacturer's instructions, DNA was extracted from gut samples using the FastDNATM spin kit for soil (MP Biomedicals). Water samples were also extracted using the same kit but were first filtered using Nalgene™ Rapid-Flow™ filter units with a sterile disposable bottle on the top (filter capacity 500ml, pore size 0.2µm, 45mm bottle neck, Sigma®) and the filter paper cut into pieces and placed within the accompanying lysing matrix tubes. All DNA samples were concentrated by ethanol precipitation using standard procedures, quantified using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and stored at -20°C until downstream library preparation.

2.3.3. PCR amplification, Library Preparation and Sequencing

The V1-V2 hypervariable region of the 16S rDNA gene was amplified from DNA extracts using a multi-step PCR procedure with the universal eubacterial primers 27F and 338R according to Camarinha-Silva *et al*. (2014) and Legrand *et al*. (2018). Specifically, for library generation, 25ng of sample DNA was subjected to an initial 20 cycles of PCR comprising 2.5mM deoxynucleoside triphosphates, 2.5U/µl PrimeSTAR® HS DNA Polymerase (Takara Bio), 5×PrimeSTAR® Buffer (Takara Bio) and 10µm of each primer, with cycling consisting of initial denaturation at 95°C for 3 min, followed by consecutive rounds of 98°C for 10s, 55°C for 10 s and 72°C for 45s. One microliter from this reaction was used as a template for a further 15

cycles of PCR (using the same conditions and cycling parameters) for incorporating individual 6nt barcodes and Illumina specific adaptors. A final 10 cycles of PCR were conducted using 1μl from this second reaction for incorporating the Illumina multiplexing sequencing and indexing primers. PCR products were visualised by agarose gel electrophoresis, and those of the expected size (~438bp) were subsequently purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies). Amplicons were pooled in equimolar ratios and sequenced on the Illumina MiSeq platform (Illumina) using 250nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF, North Melbourne, VIC, Australia). Amplicons obtained from gDNA extracts of *Lactobacillus reuteri* were sequenced alongside the samples as a control.

2.3.4. Bioinformatics Analysis

A total of ~12.5 million raw sequence reads were obtained from a total of 122 samples (n=60/60 oyster; n=56/60 mussel; n=6/6 seawater). Reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang *et al*. 2014), and the primers identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology - QIIME (version 1.8; Caporaso *et al*. 2010), USEARCH (version 8.0.1623; Edgar, 2010), and UPARSE software (Edgar, 2013). Using USEARCH tools, sequences were quality filtered to remove low-quality reads, full-length duplicate sequences and singletons. Sequences were clustered into operational taxonomic units (OTUs) at a minimum identity of 97%, with putative chimeras removed using the RDP-gold database as a reference (Cole *et al*. 2014).

A total of 5,517,945 high quality, paired-end reads (mean=45,229 ± 17,036 reads/sample; min=18,427; max=112,618) were clustered into 22,402 OTUs. These OTUs were further filtered as conducted previously (Legrand *et al*. 2018) where only those that contributed to >0.01% of the bivalve-associated (n=116) or >0.01% of the seawater dataset (n=6) were retained. The resultant OTUs were interrogated using the Seqmatch function of the RDP database (Wang *et al*. 2007) as well as SILVA (Quast *et al*. 2013), whereby taxonomic lineages based on the SILVA taxonomy and the best hit from RDP were assigned for each OTU. Those OTUS representing chloroplast or fungi were removed from the dataset, leaving a total of 659 OTUs for downstream analysis (**Supplementary Datasheet 1**). Rarefaction curves were used to assess (retrospectively) sampling depth (Supplementary Figure S1).

2.3.5. Statistical Analysis

The final dataset comprised 659 OTUs from 122 samples (60 oyster gut, 56 mussel gut and six water samples) was used for statistical analysis using Primer-E version 7.0.11 (Clarke *et al*. 2014). Non-metric multidimensional scaling (nMDS) ordination plots were generated to visualise the global bacterial community structures using Bray-Curtis similarity resemblance (Bray and Curtis, 1957; Clarke *et al*. 2014). Bubble overlays were incorporated in the ordination plot, comparing mussels and oysters to indicate variations in weight. Two-way and one-way permutational multivariate analysis of variance, PERMANOVA was used to assess differences between groups of samples such as oyster vs mussel, summer vs winter, species vs season, large vs small mussels allowing for type III (partial) sums of squares, fixed effects of sum to 0 for mixed terms. The *p*-values were generated using unrestricted permutations of raw data (Anderson, 2001; Clarke *et. al*. 2014) and were considered significantly different if $p < 0.05$. Diversity measures for each group of samples were

generated as box plots in Primer-E, and included species/OTU richness (S), Pielou's evenness (J′), Shannon index (H′), Simpson index (1-λ), average Taxonomic Distinctness – avTD (delta+) and variation in Taxonomic Distinctness – varTD (lambda+). The latter two measures are respectively used to gauge the average taxonomic distance between all pairs of species (as an indicator of the taxonomic breadth of the OTUs) and how consistently each taxonomic lineage is represented (as an indicator of the taxonomic evenness of the OTUs) (Warwick and Clarke 1995; Pienkowski *et al*. 1998). When comparing differences in the diversity indices between small and large mussels, an independent t-test was performed, while for comparisons between bivalve species and season, a two-way crossed ANOVA was used, where alpha was set to 0.05 (GraphPad Prism, version 8.1.1). In both cases, distribution (normality) was first assessed using the D'Agostino and Pearson and the Shapiro-Wilk algorithms. Variations in the abundance of bacterial taxa were visualised using stacked bar charts in Primer-E, with Venn diagrams used to display the numbers of unique and shared OTUs among oyster, mussel and seawater samples. Differential abundance analysis based on Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was conducted in Microbiome Analyst (Dhariwal *et al*. 2017; Chong *et al*. 2020) to discern the significant phyla, families and/or OTUs contributing to the observed differences among treatments; as determined using the Kruskal-Wallis rank test (unadjusted/adjusted *p*-value cut off = 0.01), with the Log LDA Score value set to 2.0 and significant taxa/OTUs given in descending order from the highest to lowest LDA score. Univariate measures of shell length and weight were visualised using a scatter plot in Primer-E. The OTU tables from which the analyses were derived are given in Supplementary Datasheet 1.

2.3.6. Data Deposition

The OTU table used for the associated analyses is presented in Supplementary Datasheet S1. Sequences from individual samples were deposited within the NCBI SRA repository under accession numbers SAMN23375497 – SAMN23375618.

2.4. Results

To characterise the gut bacterial community structure of two cohabiting marine bivalves (oysters and mussels) across two different seasons (summer and winter), the V1-V2 region of 16S rDNA gene was profiled from 116 bivalves (60 oysters and 56 mussels, ~30 per species/season; with four mussel winter samples failing library preparation and sequencing). Comparison of length and weight measurements from individuals provided size class information that was used to infer potential cohort differences. Distinct size classes were observed for both oysters and mussels between the sampling periods (Figure 2.1). In summer, oysters comprised smaller shell lengths and weights (mean 45.9 ± SD 3.6 mm; mean 35.2 ± SD 1.9 g), while in winter they had larger mean shell lengths and weights (64.9 ± 3.9 mm; 52.3 ± 10.5 g). Similar differences were also observed for mussels, though those collected in summer appeared to consist of at least two separate size classes (and thus likely cohorts); one group with smaller shell lengths of <40 mm (18 mussels: 33.6 ± 2.8 mm; 16.5 ± 4.0 g) and one with larger shell lengths of >60 mm (12 mussels: 73.0 ± 12.0 mm; 83.8 ± 33.5 g). Mussels in winter comprised a mean shell length of 64.0 ± 3.2 mm and a mean weight of 20.5 ± 3.3g. Accordingly, only matched size classes were used for bacterial community comparisons between bivalve species (small oysters vs small mussels in

summer, and large oysters vs large mussels in winter). An independent comparison between mussels with different shell lengths obtained in summer (<40 mm and >60 mm) was conducted to explore possible size class differences.

The bacterial communities of farmed oysters and cohabiting wild mussels were surveyed from aspirated gut contents and six seawater samples obtained from the same farm site (3 per season). Of the 659 OTUs obtained for analysis, 105 were unique to bivalves, 15 to seawater, and 539 shared between bivalves and seawater (Supplementary Datasheet 1). Despite the large number of shared OTUs, the ordination of the samples revealed that the bivalve samples clustered independently to those obtained from seawater (Figure 2.2). Furthermore, samples from oysters and mussels clustered independently of one another and in association with the season in which they were obtained (summer or winter). This observation was confirmed by two-way PERMANOVA, which crossed bivalve species with the season, revealing highly significant differences between oysters and mussels and between seasons (pseudo-F = 66.31, p-value = 0.0001; pseudo-F = 40.92, p-value = 0.0001 respectively). However, there was a significant interaction effect between species and season (pseudo-F = 18.74, p-value = 0.0001), indicating that seasonal changes were species-specific.

In evaluating all samples, OTUs represented bacterial taxa belonging to 17 phyla, 28 classes, 90 orders, 150 families, and 285 genera, of which the two phyla Tenericutes and Proteobacteria accounted for >80% of the total OTU abundance (Figure 2.3A). Unlike seawater, which was dominated by Proteobacteria (α- and γ-proteobacteria) and to a lesser extent Bacteroidetes (Bacteroidia) and Actinobacteria (Acidimicrobiia and Actinobacteria), samples from bivalves largely consisted of Tenericutes (Mollicutes) as well as Proteobacteria (α-, γ- and δ-proteobacteria),

Spirochaetes (Spirochaetia) and Cyanobacteria (Oxyphotobacteria and Sericytochromatia). The Tenericutes (Mollicutes) were largely associated with bivalve samples, accounting for ~52% of the total OTU abundance; as derived from a total of 36 OTUs, two most closely representing *Spiroplasmataceae* (<72% identity) – which were almost exclusively associated with mussels, and 34 most closely representing *Mycoplasmataceae* (<83% identity) – of which 31 were shared between both bivalve species (Supplementary Datasheet 1). The greatest proportion of Tenericutes occurred in summer for both oysters (mean 64.2 ± SD 16.9%) and mussels (80.9 ± 8.7%). In contrast, the phyla Proteobacteria had a lower mean abundance in summer compared to winter for both oysters (21.3 ± 12.5% vs 56.7 ± 18.4%) and mussels (6.3 ± 3.0% vs 36.0 ± 12.1%). These findings were supported by differential abundance analysis (as determined using the Kruskal-Wallis rank test, adjusted *p*-value cut-off = 0.01). These results also revealed a seasonal trend in the proportions of other phyla, including a higher abundance of Spirochaetes in both bivalves in summer and, conversely, a higher proportion of Bacteroidetes, Actinobacteria, Firmicutes, Chloroflexi, Epsilonbacteraeota and Acidobacteria in winter (Figure 2.3B, Supplementary Table S1). Two phyla, however, appeared to have seasonally disparate abundances, with Fusobacteria more abundant in summer in mussels and winter in oysters and Verrucomicrobia in winter in mussels and summer in oysters. The 10 most dominant bivalve associated OTUs accounted for >50% of the total standardised sequence reads and included taxa largely related to Mollicutes, including *Mycoplasma* spp. (OTU 7, mean abundance of 9.2%; OTU 6, 6.4%; OTU 4, 4.8%; OTU 51, 3.3%; OTU 19, 3.1%), *Candidatus* Bacilloplasma sp. (OTU 11, 5.1%) and uncultured *Mycoplasmataceae* spp. (OTU 2, 3.9%; OTU 14, 3.9%; OTU 17,

2.6%), as well as the γ-proteobacteria *Halioglobus* sp. (OTU 1, 8.9%) (Supplementary Datasheet 1).

2.4.1. Defining a Role for Host Phylogeny in Gut Bacterial Community Composition in Bivalves

To understand the influence of host phylogeny on bivalve gut bacterial communities, core and unique bacterial constituents were first evaluated from comparisons between all samples (irrespective of season). Of the 644 OTUs that were detected from bivalves, only 35 were unique to oysters and 28 to mussels, with the majority (~90%) being shared (Figure 2.4A). Of these, in seawater, 13 OTUs from oysters and 7 OTUs from mussels were not detected (or occurred in very low abundance). The top three most prevalent in oysters related to taxa belonging to *Anaplasmataceae* (α-proteobacteria) (OTU 140 – *Candidatus* Neoehrlichia, min. 0 – max. 0.2%), *Spirochaetaceae* (Spirochaetes) (OTU 263 – *Spirochaeta* 2, 0 – 1.9%) and *Mycoplasmataceae* (Mollicutes) (OTU 9073 – *Mycoplasma* sp., 0 – 1.4%) (Supplementary Table S2), while in mussels the most prevalent were *Mycoplasmataceae* (OTU 115 – *Mycoplasma* sp., min. 0 – max. 3.6%; OTU 261 – *Mycoplasma* sp., 0 – 1.5%) and *Spiroplasmataceae* (OTU 180 – *Spiroplasma* sp., 0 – 1.4%) (Supplementary Table S3). Alongside this, in assessing the differentially abundant families and OTUs associated with these samples, certain taxa also appeared to be preferentially more abundant in one of the two bivalve species – as determined using the Kruskal-Wallis rank test (adjusted *p*-value cut-off = 0.01) (Figure 5A and B). In oysters, this included a total of 7 families, including the γ- and α-proteobacteria families *Halieaceae* (OTU 1 – *Halioglobus* sp.), *Kiloniellaceae* (OTU 22 – *Kiloniella* sp.) and *Pseudomonadaceae* (OTU 16 – *Pseudomonas alcaligenes*), as well as an unclassified *Sericytochromatia* (Cyanobacteria), Epsilonbacteraeota families

*Helicobacteracea*e and *Campylobacteraceae*, and a number of other OTUs belonging to Mycoplasma/uncultured *Mycoplasmataceae* (OTUs 6, 4, 2, 13, 1474, 281, 3 and 27) and *Spirochaetaceae* (OTU 26 – *Salinispira* sp., OTU 38 – uncultured *Spirochaetaceae*). In contrast, taxa belonging to 18 different families appeared to contribute to the differences observed for mussels. The most notable of these included *Flavobacteriaceae* (OTU 71 – *Polaribacter* sp., OTU 41 – *Ulvibacter* sp.), *Rhodobacteraceae* (OTU 59 – *Sulfitobacter* sp., OTU 43 – *Planktomarina* sp.) and *Fusobacteriaceae* (OTU 37 – *Psychrilyobacter* sp.). In addition, like that observed for oysters, a number of OTUs belonging to the *Mycoplasmataceae* and *Spirochaetaceae* also appeared to contribute to the observed differences, including OTU 11 (*Candidatus* Bacilloplasma sp.), OTU 14 and 17 (uncultured *Mycoplasmataceae*), OTU 51 (*Mycoplasma* sp.) and OTU 28 (*Spirochaeta* 2).

Alongside the evaluation of possible intergeneric differences, samples obtained from likely individual cohorts of mussels (based on the occurrence of distinct size classes, as indicated above) also provided an opportunity to seek possible intraspecific differences. Ordination of samples from mussels obtained in summer with large (>60 mm) and small (<40mm) shell lengths, and in comparison, to samples from mussels in winter with large shell lengths (>60mm), revealed the independent clustering and likely differences in the global bacterial community compositions of these size classes (Figure 2.6A). This observation was confirmed by one-way PERMANOVA, revealing a significant difference between the large and small summer mussels (pseudo-F = 4.5604, p-value = 0.0028). No significant differences were observed, however, between these groups for measures of species/OTU richness (p-value = 0.2253; large mussels: mean 244 ± SD 48, small mussels: 263 ± 37), Shannon diversity (p-value = 0.6574; large mussels: 2.13 ± 0.59, small mussels: 2.04

± 0.48) and Pielou's evenness (*p*-value = 0.5610; large mussels: 0.39 ± 0.10, small mussels: 0.37 ± 0.09). Furthermore, though some (albeit slight) changes were observed in the mean abundances of various bacterial classes (Figure 2.6B), differential abundance analysis revealed the occurrence of only five significantly different OTUs – as determined using the Kruskal-Wallis rank test (unadjusted *p*-value cut-off = 0.01) (Supplementary Table S4). Based on Log LDA scores, the two with the largest effect size included those most closely related to *Mycoplasma* spp. – OTU 115 (LDA -4.99), which had a higher abundance in samples from small mussels, and OTU 51 (LDA 3.48), which had a higher abundance in samples from large mussels (Figure 2.6C).

2.4.2. Seasonal Drivers of Gut Bacterial Community Composition in Bivalves

With the occurrence of notable phyla-level shifts in the bacterial community composition of oysters and mussels apparent between summer and winter (as indicated above), the impact of the season was further explored between bivalves and in relation to samples obtained from the surrounding environment (seawater). At a global level, while samples from mussels comprised a greater number of OTUs compared to oysters in both summer (263 ± 37 vs. 184 ± 66) and winter (349 ± 46 vs. 240 ± 60) (Figure 2.7A), changes in species/OTU richness and diversity (Shannon and Simpson's diversity and Pielou's evenness) were apparent for both bivalves across seasons. Most notably, was a marked increase in these measures in winter (Figure 2.7A-D). This observation was confirmed by two-way ANOVA, which crossed bivalve species with the season, revealing highly significant differences between summer and winter (*p*-value <0.0001). However, there was a significant interaction effect between species and season (*p*-value <0.0001) for all measures except for

species/OTU richness, indicating that while a similar increase in the number of OTUs occurred for both oysters and mussels in winter, there were likely species-specific differences in the types and relative abundances of these OTUs. A similar trend for richness and diversity measures was also observed for the seawater samples between summer and winter, though the differences were not significantly different (*p*-value >0.05). In comparing variation in taxonomic distinctness (varTD, lambda+) as a function of average taxonomic distinctness (avTD, delta+), which assesses the breadth and evenness of the taxonomic diversity of the OTUs within each sample, a significant difference was observed between bivalve species and season (Figure 2.7E). Samples from oysters typically comprised OTUs covering a greater breadth of taxa in summer compared to winter (based on a higher mean value for delta+: 91.39 ± SD 0.49 vs 90.16 ± 0.67 respectively), though they were similarly evenly distributed across taxonomic lineages in both seasons (based on similarly low mean values for lambda+: 259.97 ± 21.98 and 258.29 ± 15.72 respectively). In contrast, samples from mussels comprised OTUs covering a similar breadth of taxa in both summer and winter (based on similar mean values for delta+: 90.33 ± 0.38 and 90.12 ± 0.31, respectively). However, they were more unevenly distributed across taxonomic lineages in winter compared to summer (based on a higher mean value for lambda+: winter 282.27 ± 10.84 vs summer 266.81 ± SD 11.50). This observation was supported by the occurrence of a significant interaction effect between species and season (for both measures of delta+ and lambda+), indicating that seasonal changes were species-specific. Despite seawater samples comprising the greatest number of OTUs (Figure 2.7A), these OTUs represented a substantially lower breadth of taxa and were more unevenly distributed across taxonomic lineages in both summer and

winter compared to those from bivalves (based on lower values for delta+ and higher values for lambda+) (Figure 2.7E).

Differential abundance analysis was performed to identify the features contributing to these observed seasonal differences by comparing oyster and mussel gut and seawater samples in summer and winter. A total of 29 families and 151 OTUs were observed to be significantly different as determined using the Kruskal-Wallis rank test (adjusted *p*-value cut-off = 0.01). In evaluating the top 20 most differentially abundant families and OTUs with the greatest effect size (based on the Log LDA scores), distinct seasonal patterns were observed whereby a concomitant increase in the abundance of certain taxa was observed in either summer or winter in both bivalve species (Figure 2.8, Supplementary Tables S5 and S6). This included five families in summer (*Mycoplasmataceae*, *Spirochaetaceae*, *Cyanobiaceae* [*Synechococcus* and *Cyanobium* spp.], *Methylophilaceae* [OM43 clade] and *Pseudoalteromonadaceae* [*Pseudoalteromonas* spp.]) and 12 in winter (α-proteobacteria SAR11 clade 1a, *Halieaceae*, *Rhodobacteraceae*, *Flavobacteriaceae*, *Burkholderiaceae*, *Pseudomonadaceae*, *Rhizobiaceae*, *Cryomorphaceae*, *Microbacteriaceae*, *Desulfobulbaceae*, unclassified Sericytochromatia, and γ-proteobacteria SAR86 clade). Of these, five also had an associated seasonally high abundance in the seawater, including *Pseudoaltermonadaceae* in summer, and α-proteobacteria SAR11 clade 1a (OTU 8), *Rhodobacteraceae* (OTU 43 and 65, *Planktomarina* spp.), *Flavobacteriaceae* (OTU 44, unclassified NS5 marine group) and γ-proteobacteria SAR86 clade in winter (as marked by asterisks in Figure 2.8A and B). The majority of OTUs contributing to the observed seasonal differences included those most closely related to members of the *Mycoplasmataceae* (notably Mycoplasma), whereby in mussels OTUs 7, 17, 19 and 51 were more abundant in summer, and OTUs 11

(*Candidatus* Bacilloplasma sp.) and 19 (*Mycoplasma* sp.) were more abundant in winter. A similar trend was observed for *Mycoplasmataceae* related taxa in oysters, whereby OTUs 6, 4 and 13 were more abundant in summer and OTUs 2 and 1474 in winter. Other OTUs with a notable seasonal increase in abundance included OTU 1 (*Halioglobus* sp. 79.46% identity), OTU 16 (*Pseudomonas pseudoalcaligenes*, 99.08% identity) and OTU 22 (*Kiloniella* sp., 80.33% identity) in oysters in winter, and OTU 28 (Spirochaeta 2 sp., 77.24% identity) in mussels in summer (Figure 2.8B, Supplementary Table S6 and Supplementary Datasheet 1).

2.5. Discussion

The gut bacterial assemblages of two intergeneric cohabiting marine bivalves *Crassostrea gigas* and *Mytilus galloprovincialis* were revealed in summer and winter. Though each harboured distinct communities that differed from that of the surrounding environment, many common (core) bacterial OTUs were observed between bivalves, suggesting a role of both the environment and the host in determining the bacterial community composition of the gut. Similar findings have been reported elsewhere in comparative studies of the haemolymph and digestive gland of *C. gigas* and *M. galloprovincialis* (Vezulli *et al*. 2018), as well as the gut of the eastern oyster (*Crassostrea virginica*) and blue mussel (*Mytlis edulis*) (Pierce and Ward, 2019), and is thought to occur as a result of the ingestion of common planktonic or aggregate-associated environmental consortia through filter feeding (Paillard *et al*. 2022). Here, OTUs typically representing environmental taxa such as the γ-proteobacteria family *Halieaceae* (namely *Halioglobus* sp.) (Spring *et al*. 2015)

and Cyanobacteria (which are likely to be fed upon by bivalves, (Pierce and Ward, 2019) were found to occur in the gut aspirates of both bivalves, and thus likely reflect such constituents. However, other likely environmental associated OTUs were also observed though were found to be more predominant in either oysters or mussels. Specifically, alongside *Halioglobus*, the α-proteobacteria family *Kiloniellaceae* (*Kiloniella* sp.) and the Cyanobacteria *Sericytochromatia* were more abundant in oysters, while *Flavobacteriaceae* (namely *Polaribacter* and *Ulvibacte*r spp.) as well as *Rhodobacteraceae* (*Planktomarina* and *Sulfitobacter* spp.) and *Fusobacteriaceae* (*Psychrilyobacter* sp.) were more abundant in mussels. Furthermore, as reported for other Mytilids (Pierce and Ward, 2019), a greater number of OTUs occurred in samples from mussels compared to oysters across the seasons. These bivalves selectively feed on various spectrums of particle sizes. For example, *M. galloprovincialis* can access a wider range of food particle sizes than *C. gigas*) (Rahman *et al*. 2020). Microbial diversity and species richness also change with particle size (Mestre *et al*. 2017). Such a finding reflects the variation of host feeding ecology and the types of bacteria introduced into the gut. The prevalence of OTUs in mussels representing particular organisms like *Rhodobacteraceae,* which have been reported as constituents of larger particles sizes (Mestre *et al*. 2017), as well as *Psychrilyobacter* spp., which are among some of the most significant degraders of detrital matter (Yadav *et al*. 2021), may further support the influence that particle size has on gut microbiota composition. The filter-feeding organisms may select food to enable cohabitation via resource partitioning (Tran 2017) or obtain energy from microbiota associated with pseudofeces. Among microorganisms, cyanobacteria play a role in reducing susceptibility to disease in oysters as endosymbionts (Avila-Poveda

et al. 2014; Clerissi *et al*. 2020). Therefore, establishing such organisms as gut microbiota components would be of considerable value.

Of particular importance in this study was the occurrence of key groups of bacteria that occurred predominantly in association with bivalve rather than seawater samples. Specifically, unlike seawater, which comprised larger proportions of OTUs associated with α- and γ-proteobacteria as well as Bacteroidetes and Actinobacteria, more than half of the OTUs derived from bivalve samples appeared to be exclusively associated with Mollicutes (notably members of the family *Mycoplasmataceae* as well as *Spiroplasmataceae* and *Candidatus* Bacilloplasma). Though *Candidatus* Bacilloplasma was originally reported from the hindgut of terrestrial isopods (Kostanjsek *et al*. 2007), this and other members of the *Mycoplamsmataceae* (particularly *Mycoplasma* spp.) have been detected and may represent key gut constituents in other marine organisms, including in oysters and mussels (Green and Barnes, 2010; King *et al*. 2012; Wegner *et al*. 2013; Cleary *et al*. 2015; Arfken *et al*. 2017; Pierce and Ward 2019; Offret *et al*. 2020; Pimentel *et al*. 2021). Bacteria belonging to the class Mollicutes are generally considered host-associated (parasitic) organisms. These bacteria have undergone substantial reductive evolution and lack cell walls. Therefore, they have become reliant upon the host for supporting their metabolic processes (Trachtenberg, 2005). The prevalence of certain taxa such as *Mycoplamsa* in marine animals (particularly in fish) (Llewellyn *et al*. 2016; Legrand *et al*. 2020) and the occurrence of several pathogenic species (Paillard *et al*. 2004) has attracted considerable attention, though recent studies point towards a more mutualistic relationship. As inferred from metagenome-assembled genomes (MAGs) of Mollicutes associated with the gut of the eastern oyster (*C. virginica*), it was

reported that such organisms (as being most closely related to *Mycoplasma* spp.) might also confer a benefit to the host by reducing parasite infection through the competitive sequestration of arginine (Pimentel *et al*. 2021). However, given that increased abundances of particular Mollicutes related taxa like members of *Mycoplasmataceae* have also been observed to occur in oysters that are more susceptible to disease (namely in Pacific Oyster Mortality Syndrome, POMS) (Clerissi *et al*. 2020), their roles here in farmed oysters and cohabiting mussels requires further examination. This should be extended to include other taxa like *Helicobacteraceae* and *Campylobacteraceae* whose roles in the bivalve host, to the best of our knowledge, remain unclear, and may represent potential foodborne pathogens and/or environmental indicator organisms of human fecal pollution (Wilson and Moore 1996; Frühe *et al*. 2021).

That Mollicutes may, in particular, be relevant in the bivalve host was further evident here from the recovery of sequences belonging to a considerable number of related OTUs. Specifically, a total of 38 Mollicutes OTUs were detected from the bivalve gut samples, of which 36 were most closely related to *Mycoplasmataceae* (*Mycoplasma* spp.), with the majority (31) being shared between the two bivalve species. Interestingly, similarly diverse populations of Mollicutes related taxa have also been previously reported for oysters (namely *C. virginica*), with a total of 36 distinct amplicon sequence variants (ASVs) found to belong to four major clades based on groups of environmental 16S rRNA gene reference sequences (Pimentel *et al*. 2020). While *Mycoplasma* spp. are generally considered to have established unique relationships with individual hosts over extended periods of coevolution, they are thought to have descended from multiple bacterial lineages (rather than a single

common ancestor) and appear to undergo rapid and divergent evolution, allowing them to rapidly adapt to changing microenvironments (Woese *et al*. 1980; Chen *et al*. 2022). With mussels (Family Mytilidae) and oysters (Family Ostreidae) having evolved at different times from a common ancestor (Combosch *et al*. 2017), the detection of shared, diverse *Mycoplasmataceae*-associated OTUs is thus not surprising, and given their low sequence identities (~72-83%) may represent different (and likely novel) species that have emerged from multiple bacterial lineages within the gut of these bivalves. Of course, more detailed, targeted analyses need to be conducted to verify the diversity of these organisms to discount biases that may arise from the presence of pseudogenes (Amikam *et al*. 1984).

The presence of various bivalve-specific OTUs in this study suggests that more explicit intrinsic (host) selection pressures may also be important in determining gut microbiota composition. Alongside the occurrence of various species-specific *Mycoplasmataceae*-related OTUs this included OTUs representing the α-proteobacteria *Anaplasmataceae* (Rickettsiales) and *Spirochaetaceae* (Spirochaetes) in oysters and *Spiroplasmataceae* (namely *Spiroplasma* sp.) in mussels. Several of these organisms, such as *Anaplasmataceae*, *Mycoplasma* and Spirochaetes have been associated with infections arising from intracellular microcolonies of bacteria (IMC) in bivalves (Cano *et al*. 2020). In some cases, IMC infections have the potential to cause widespread disease in certain farmed bivalve species (Wu *et al*. 2000; Zhu *et al*. 2012), though in earlier health surveys of *C. gigas* and *M. galloprovincialis*, the presence of specific IMC-related organisms (namely Rickettsia and Mycoplasma-like colonies) has not been associated with underlying pathology (Villalba *et al*. 1997; Hine, 2002). Furthermore, in *M. galloprovincialis*, such organisms increased in

prevalence with growth during cultivation, where adults comprised the greatest proportion of these organisms compared to the seed (Villalba *et al*. 1997). Together, this supports earlier notions from other related bivalves of a perhaps more symbiotic relationship (Lohrmann *et al*. 2019), which is likely established during host development.

The host-associated OTUs observed here were derived from samples from seemingly healthy individuals, and such a relationship may appear more likely. Furthermore, with the occurrence of varied abundances of select host-associated OTUs between small and large mussels (notably those belonging to certain Mycoplasma-related taxa), these organisms' relevance may also vary with age and cohort-specific genetics. Further insights into the presence and role of these taxa are thus warranted and may be expedited through *in situ* studies and phylogenomic-based investigations as conducted elsewhere (Cano *et al.* 2020; Wang *et al*. 2020).

In this study, the gut microbiota composition of oysters and mussels also appeared to be influenced by season. Most notable was an increase in species (OTU) richness and diversity in both oysters and mussels during winter and was associated with a variety of taxa, including several that exhibited a concomitant increase in prevalence in the bivalves and the corresponding seawater samples. In particular, during winter this included OTUs associated with largely heterotrophic taxa such as α-proteobacteria SAR11 clade 1a, *Rhodobacteraceae* (*Planktomarina* spp.), *Flavobacteriaceae* (NS5 marine group) and γ-proteobacteria SAR86 clade. As significant free-living or particle-associated constituents found in coastal and open-ocean waters throughout the world, and whose populations are well known to vary temporally (Brown *et al*. 2014; Ward *et al*. 2017; Chun *et al*. 2021), the occurrence of

such taxa may reflect the common ingestion of local, environmentally driven microbial consortia, as discussed earlier. Indeed, bivalves in this study were sampled from a particularly dynamic region that, as part of the broader eastern Great Australian Bight (GAB), is marked by significant wind-driven summer upwelling and winter downwelling events that influence nutrient availability and mixing (Kämpf *et al*. 2004; van Ruth *et al*. 2018). During these periods, changes in the rates of productivity are observed and are highest in the late summer upwelling season (typically Jan-Apr) when nutrient-rich waters favour productivity and lowest during winter downwelling conditions (van Ruth *et al*. 2018). This is likely reflected here by the increased prevalence of particular cyanobacterial taxa in the bivalves during summer (e.g., *Synechococcus* spp.). These species are associated with nutrient-rich coastal waters (Partensky *et al*. 1999) and have been found to be significant seasonal components of the environmental consortia that may be ingested by these bivalves in the region (Rahman *et al*. 2020). However, while an increase in their prevalence was not reflected in the seawater in summer, other OTUs such as Methylophilaceae (OM43 clade) were associated with blooms (Morris *et al*. 2006; Salcher *et al*. 2019) may support their seasonal relevance here. Nevertheless, as discussed above, whether these organisms represent common seasonally ingested components of the diet or play a more direct role as part of the gut microbiota requires further examination. This may be particularly important for organisms like *Pseudoalteromonadaceae* (*Pseudoalteromonas* spp.), which also increased in abundance in bivalve and seawater samples during summer, and which are associated with a variety of eukaryotic hosts (including oysters and mussels) (Holmström and Kjelleberg, 1999; Vezzulli *et al*. 2018) and may confer a benefit through e.g., antibiotic activity against pathogens (Desriac *et al*. 2020).

Though extrinsic, seasonally relevant (environmental/particle diet) factors can shape the gut microbiota of bivalves (Pierce and Ward 2019; Conceição *et al*. 2021), the season impacts on these communities vary between host species. Specifically, in this study, samples from oysters typically comprised a greater breadth of taxa in summer compared to winter, despite similar distributions across taxonomic lineages in both seasons. In contrast, samples from mussels comprised a similar breadth of taxa in both summer and winter, but they were more unevenly distributed across taxonomic lineages in winter than in summer. Such disparity has also been previously reported to occur between other intergeneric bivalves held in the same environment, namely the eastern oyster (*C. virginica*) and blue mussel (*M. edulis*) (Pierce and Ward, 2019). However, unlike this study, more evenness was observed in mussels than oysters across the seasons. Other studies measured functional diversity (catabolic activity), but this may fail to assess community components that require unusual substrates (Konopka et al. 1998; Garland, 1999). Nevertheless, at a holistic level, such differences may reflect the unique combinations of extrinsic and host-specific intrinsic factors that together shape the gut microbiota, which underpin their ecology and ability to share the same environment. Indeed, for oysters (*C. gigas*) it has been recently reported that high microbial evenness may confer enhanced resistance to infection by pertinent viral infections (namely ostreid herpesvirus 1, OsHV-1 µVar), possibly through the promotion of homeostasis (Clerissi *et al*. 2020). However, the seasonal prevalence of select taxa in the individual bivalves may also confer specific benefits. For oysters, this may be reflected in the increased prevalence of certain taxa like *Pseudomonas pseudoalcaligenes* in winter, which is known for its capacity to accumulate or breakdown harmful compounds (e.g., mercury, polychlorinated biphenyls) (Triscari-Barberi *et al*. 2012; Liu *et al*. 2016) may help to overcome

potential stressors posed by such compounds for growth and reproduction (Encomio and Chu, 2000). Conversely, for mussels, a greater proportion of Spirochaeta in summer, as a saccharolytic organism thought to mediate the turnover of algal detritus (Morrison *et al*. 2017), may support nutrition by breaking down the cellulolytic components of the diet.

The majority of OTUs contributing to the observed seasonal differences in oysters and mussels, however, included those most closely related to members of the *Mycoplasmataceae*, whereby each comprised a number of distinct OTUs that were either more prevalent in summer or winter. Given that such organisms are not typically considered free-living but instead rely upon their host to support their metabolic requirements (Trachtenberg, 2005), such changes were somewhat unexpected. Interestingly, alongside their reported absence from suspended marine aggregates (particle diets), seasonal changes in the abundance of the Mycoplasmatales have also been observed previously for oysters (*C. virginica*) and mussels (*M. edulis*) (Pierce and Ward, 2019). While it is impossible to exclude other factors that may influence the prevalence of these organisms (e.g., environmental conditions or competition with other microbes), such a finding raises some intriguing questions. Specifically, whether the host may be directly controlling these populations to support seasonal changes in its physiology or metabolic requirements, a feature observed for lucinid bivalves where its symbionts are enzymatically digested during starvation (König *et al*. 2015). Further, more detailed, investigations are thus needed to determine the association and dynamics these organisms share with the bivalve host.

2.6. Acknowledgements

This work was funded by Fisheries Research and Development Corporation (FRDC) (Project No. 2014/027), with tuition support provided to the first author (S. Akter) by

the Flinders University Research Scholarship (FURS) program. We wish to thank Dr. Md. Mahbubul Hassan, Dr. Maziidah Ab Rahman and Shaun Henderson their kind assistance with field sampling.

2.7. Author's Contributions

Experimental design: JQ, AO, XL and SA; Sample collection: MH; Sample processing and library preparation: SA and SC; Data analysis: SA, SC, MO and AO; Paper writing: SA, AO.

2.8. References

Amikam, D., Glaser, G. and Razin, S.H.M.U.E.L., 1984. *Mycoplasmas* (Mollicutes) have a low number of rRNA genes. *Journal of Bacteriology*, *158*(1), pp.376-378.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, *26*(1), pp.32-46.

Arfken, A., Song, B., Bowman, J.S. and Piehler, M., 2017. Denitrification potential of the eastern oyster microbiome using a 16S rRNA gene based metabolic inference approach. *PloS One*, *12*(9), p.e0185071.

Avila-Poveda, O.H., Torres-Arino, A., Girón-Cruz, D.A. and Cuevas-Aguirre, A., 2014. Evidence for accumulation of *Synechococcus elongatus* (Cyanobacteria: Cyanophyceae) in the tissues of the oyster *Crassostrea gigas* (Mollusca: Bivalvia). *Tissue and Cell*, *46*(5), pp.379-387.

Bajinka, O., Tan, Y., Abdelhalim, K.A., Özdemir, G. and Qiu, X., 2020. Extrinsic factors influencing gut microbes, the immediate consequences and restoring eubiosis. *AMB Express*, *10*(1), pp.1-11.

Belikov, S., Belkova, N., Butina, T., Chernogor, L., Martynova-Van Kley, A., Nalian, A., Rorex, C., Khanaev, I., Maikova, O. and Feranchuk, S., 2019. Diversity and shifts of the bacterial community associated with Baikal sponge mass mortalities. *PLoS One*, *14*(3), p.e0213926.

Bray, J.R. and Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs*, *27*(4), pp.326-349.

Brown, M.V., Ostrowski, M., Grzymski, J.J. and Lauro, F.M., 2014. A trait-based perspective on the biogeography of common and abundant marine bacterioplankton clades. *Marine Genomics*, *15*, pp.17-28.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A.P., Schaumburg, F., Becker, K., Wos-Oxley, M.L. and Pieper, D.H., 2014. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environmental Microbiology*, *16*(9), pp.2939-2952.

Cano, I., Ryder, D., Webb, S.C., Jones, B.J., Brosnahan, C.L., Carrasco, N., Bodinier, B., Furones, D., Pretto, T., Carella, F. and Chollet, B., 2020. Cosmopolitan distribution of *Endozoicomonas*-like organisms and other intracellular microcolonies of bacteria causing infection in marine mollusks. *Frontiers in Microbiology*, p.2778.

Capelle, J.J., Garcia, A.B., Kamermans, P., Engelsma, M.Y. and Jansen, H.M., 2021. Observations on recent mass mortality events of marine mussels in the Oosterschelde, the Netherlands. *Aquaculture International*, *29*(4), pp.1737-1751.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I. and Huttley, G.A., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), pp.335-336.

Chen, R., Zhao, L., Gan, R., Feng, Z., Cui, C., Xie, X., Hao, F., Zhang, Z., Wang, L., Ran, T. and Wang, W., 2022. Evidence for the rapid and divergent evolution of mycoplasmas: structural and phylogenetic analysis of enolases. *Frontiers in Molecular Biosciences*, *8*, p.811106.

Chong, J., Liu, P., Zhou, G. and Xia, J., 2020. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature Protocols*, *15*(3), pp.799-821.

Chun, S.J., Cui, Y., Baek, S.H., Ahn, C.Y. and Oh, H.M., 2021. Seasonal succession of microbes in different size-fractions and their modular structures determined by both macro-and micro-environmental filtering in dynamic coastal waters. *Science of The Total Environment*, *784*, p.147046.

Clarke, K.R., Gorley, R.N., Somerfield, P.J. and Warwick, R.M., 2014. Change in marine communities: an approach to statistical analysis and interpretation.

Cleary, D.F., Becking, L.E., Polónia, A.R., Freitas, R.M. and Gomes, N., 2015. Composition and predicted functional ecology of mussel-associated bacteria in Indonesian marine lakes. *Antonie van Leeuwenhoek*, *107*(3), pp.821-834.

Clerissi, C., De Lorgeril, J., Petton, B., Lucasson, A., Escoubas, J.M., Gueguen, Y., Dégremont, L., Mitta, G. and Toulza, E., 2020. Microbiota composition and

evenness predict survival rate of oysters confronted to Pacific oyster mortality syndrome. *Frontiers in Microbiology*, *11*, p.311.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R. and Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, *42*(D1), pp.D633-D642.

Combosch, D.J., Collins, T.M., Glover, E.A., Graf, D.L., Harper, E.M., Healy, J.M., Kawauchi, G.Y., Lemer, S., McIntyre, E., Strong, E.E. and Taylor, J.D., 2017. A family-level tree of life for bivalves based on a Sanger-sequencing approach. *Molecular Phylogenetics and Evolution*, *107*, pp.191-208.

Conceição, M.V.R., Costa, S.S., Schaan, A.P., Ribeiro-dos-Santos, Â.K.C., Silva, A., das Graças, D.A., Schneider, M.P.C. and Baraúna, R.A., 2021. Amazonia Seasons Have an Influence in the Composition of Bacterial Gut Microbiota of Mangrove Oysters (*Crassostrea gasar*). *Frontiers in Genetics*, p.1784.

Desriac, F., El Harras, A., Simon, M., Bondon, A., Brillet, B., Le Chevalier, P., Pugnière, M., Got, P., Destoumieux-Garzon, D. and Fleury, Y., 2020. Alterins produced by oyster-associated *Pseudoalteromonas* are antibacterial Cyclolipopeptides with LPS-binding activity. *Marine Drugs*, *18*(12), p.630.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B. and Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, *45*(W1), pp. W180-W188.

Dubé, C.E., Ky, C.L. and Planes, S., 2019. Microbiome of the black-lipped pearl oyster *Pinctada margaritifera*, a multi-tissue description with functional profiling. *Frontiers in Microbiology*, p.1548.

Edgar, R., 2010. *Usearch*. Lawrence Berkeley National Lab. (LBNL), Berkeley, CA (United States).

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods*, *10*(10), pp.996-998.

Encomio, V. and Chu, F.L.E., 2000. The effect of PCBs on glycogen reserves in the eastern oyster *Crassostrea virginica*. *Marine Environmental Research*, *50*(1-5), pp.45-49.

Fraune, S. and Bosch, T.C., 2010. Why bacteria matter in animal development and evolution. *Bioessays*, *32*(7), pp.571-580.

Frühe, L., Dully, V., Forster, D., Keeley, N.B., Laroche, O., Pochon, X., Robinson, S., Wilding, T.A. and Stoeck, T., 2021. Global trends of benthic bacterial diversity and community composition along organic enrichment gradients of Salmon farms. *Frontiers in Microbiology*, *12*, p.853.

Garland, J.L., 1999. Potential and limitations of BIOLOG for microbial community analysis. In *Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium on Microbial Ecology* (pp. 1-7). Halifax, NS: *Atlantic Canada Society for Microbial Ecology*.

Gillies, C.L., McLeod, I.M., Alleway, H.K., Cook, P., Crawford, C., Creighton, C., Diggles, B., Ford, J., Hamer, P., Heller-Wagner, G. and Lebrault, E., 2018.

Australian shellfish ecosystems: Past distribution, current status and future direction. *PLoS One*, 13(2), p.e0190914.

Green, T.J. and Barnes, A.C., 2010. Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology*, *109*(2), pp.613-622.

Green, T.J., Siboni, N., King, W.L., Labbate, M., Seymour, J.R. and Raftos, D., 2019. Simulated marine heat wave alters abundance and structure of *Vibrio* populations associated with the Pacific oyster resulting in a mass mortality event. *Microbial Ecology*, *77*(3), pp.736-747.

Hedge, L.H. and Johnston, E.L., 2014. Colonisation of the non-indigenous Pacific oyster *Crassostrea gigas* determined by predation, size and initial settlement densities. *PloS One*, *9*(3), p.e90621.

Hine, P.M., 2002. Results of a survey on shellfish health in New Zealand in 2000. *Surveillance*, *29*(1), pp.3-7.

Holmström, C. and Kjelleberg, S., 1999. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiology Ecology*, *30*(4), pp.285-293.

Kämpf, J., Doubell, M., Griffin, D., Matthews, R.L. and Ward, T.M., 2004. Evidence of a large seasonal coastal upwelling system along the southern shelf of Australia. *Geophysical Research Letters*, *31*(9).

King, G.M., Judd, C., Kuske, C.R. and Smith, C., 2012. Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One*, *7*(12), p.e51475.

King, W.L., Jenkins, C., Seymour, J.R. and Labbate, M., 2019. Oyster disease in a changing environment: decrypting the link between pathogen, microbiome and environment. *Marine Environmental Research*, *143*, pp.124-140.

König, S., Le Guyader, H. and Gros, O., 2015. Thioautotrophic bacterial endosymbionts are degraded by enzymatic digestion during starvation: Case study of two lucinids *Codakia orbicularis* and *C. orbiculata*. *Microscopy Research and Technique*, *78*(2), pp.173-179.

Konopka, A., Oliver, L. and Turco Jr, R.F., 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial Ecology*, *35*(2), pp.103-115.

Kostanjsek, R., Strus, J. and Avgustin, G., 2007. "*Candidatus Bacilloplasma*," a novel lineage of Mollicutes associated with the hindgut wall of the terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda). *Applied and Environmental Microbiology*, *73*(17), pp.5566-5573.

Legrand, T.P., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A., Qin, J.G. and Oxley, A., 2018. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. *Frontiers in Microbiology*, *8*, p.2664.

Legrand, T.P., Catalano, S.R., Wos-Oxley, M.L., Wynne, J.W., Weyrich, L.S. and Oxley, A., 2020. Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota. *Animal Microbiome*, *2*(1), pp.1-16.

Leite, L., Jude-Lemeilleur, F., Raymond, N., Henriques, I., Garabetian, F. and Alves, A., 2017. Phylogenetic diversity and functional characterization of the Manila clam microbiota: a culture-based approach. *Environmental Science and Pollution Research*, *24*(27), pp.21721-21732.

Li, Y.F., Xu, J.K., Chen, Y.W., Ding, W.Y., Shao, A.Q., Liang, X., Zhu, Y.T. and Yang, J.L., 2019. Characterization of gut microbiome in the mussel *Mytilus galloprovincialis* in response to thermal stress. *Frontiers in Physiology*, p.1086.

Lindsay, E.C., Metcalfe, N.B. and Llewellyn, M.S., 2020. The potential role of the gut microbiota in shaping host energetics and metabolic rate. *Journal of Animal Ecology*, *89*(11), pp.2415-2426.

Liu, B., Bian, C., Huang, H., Yin, Z., Shi, Q. and Deng, X., 2016. Complete genome sequence of a marine bacterium, *Pseudomonas pseudoalcaligenes* strain S1, with high mercury resistance and bioaccumulation capacity. *Genome Announcements*, *4*(3), pp.e00381-16.

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S. and Derome, N., 2016. The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal*, *10*(5), pp.1280-1284.

Lohrmann, K.B., Bustos, E., Rojas, R., Navarrete, F., Robotham, H. and Bignell, J., 2019. Histopathological assessment of the health status of *Mytilus chilensis* (Hupé 1854) in southern Chile. *Aquaculture*, *503*, pp.40-50.

Lokmer, A. and Mathias Wegner, K., 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *The ISME Journal*, *9*(3), pp.670-682.

Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P. and Bossier, P., 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host–microbe interactions. *Journal of Applied Microbiology*, *100*(5), pp.903-918.

Meisterhans, G., Raymond, N., Girault, E., Lambert, C., Bourrasseau, L., De Montaudouin, X., Garabetian, F. and Jude-Lemeilleur, F., 2016. Structure of Manila clam (*Ruditapes philippinarum*) microbiota at the organ scale in contrasting sets of individuals. *Microbial Ecology*, *71*(1), pp.194-206.

Mestre, M., Borrull, E., Sala, M.M. and Gasol, J.M., 2017. Patterns of bacterial diversity in the marine planktonic particulate matter continuum. *The ISME Journal*, *11*(4), pp.999-1010.

Moran-Ramos, S., Lopez-Contreras, B.E., Villarruel-Vazquez, R., Ocampo-Medina, E., Macias-Kauffer, L., Martinez-Medina, J.N., Villamil-Ramirez, H., León-Mimila, P., Del Rio-Navarro, B.E., Ibarra-Gonzalez, I. and Vela-Amieva, M., 2020. Environmental and intrinsic factors shaping gut microbiota composition and diversity and its relation to metabolic health in children and early adolescents: A population-based study. *Gut Microbes*, *11*(4), pp.900-917.

Morris, R.M., Longnecker, K. and Giovannoni, S.J., 2006. *Pirellula* and OM43 are among the dominant lineages identified in an Oregon coast diatom bloom. *Environmental Microbiology*, *8*(8), pp.1361-1370.

Morrison, J.M., Murphy, C.L., Baker, K., Zamor, R.M., Nikolai, S.J., Wilder, S., Elshahed, M.S. and Youssef, N.H., 2017. Microbial communities mediating algal detritus turnover under anaerobic conditions. *PeerJ*, *5*, p.e2803.

Muñoz, K., Flores-Herrera, P., Gonçalves, A.T., Rojas, C., Yáñez, C., Mercado, L., Brokordt, K. and Schmitt, P., 2019. The immune response of the scallop *Argopecten purpuratus* is associated with changes in the host microbiota structure and diversity. *Fish & Shellfish Immunology*, *91*, pp.241-250.

Musella, M., Wathsala, R., Tavella, T., Rampelli, S., Barone, M., Palladino, G., Biagi, E., Brigidi, P., Turroni, S., Franzellitti, S. and Candela, M., 2020. Tissue-scale microbiota of the Mediterranean mussel (*Mytilus galloprovincialis*) and its relationship with the environment. *Science of the Total Environment*, *717*, p.137209.

O'Brien, P.A., Webster, N.S., Miller, D.J. and Bourne, D.G., 2019. Host-microbe coevolution: applying evidence from model systems to complex marine invertebrate holobionts. *MBio*, *10*(1), pp.e02241-18.

Offret, C., Paulino, S., Gauthier, O., Château, K., Bidault, A., Corporeau, C., Miner, P., Petton, B., Pernet, F., Fabioux, C. and Paillard, C., 2020. The marine intertidal zone shapes oyster and clam digestive bacterial microbiota. *FEMS Microbiology Ecology*, *96*(8), p.fiaa078.

Paillard, C., Gueguen, Y., Wegner, K.M., Bass, D., Pallavicini, A., Vezzulli, L. and Arzul, I., 2022. Recent advances in bivalve-microbiota interactions for disease prevention in aquaculture. *Current Opinion in Biotechnology*, *73*, pp.225-232.

Paillard, C., Le Roux, F. and Borrego, J.J., 2004. Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. *Aquatic Living Resources*, *17*(4), pp.477-498.

Partensky, F., Blanchot, J. and Vaulot, D., 1999. Differential distribution and ecology of *Prochlorococcus* and *Synechococcus* in oceanic waters: a review. *Bulletin de L'institut Océanographique de Monaco*, (spécial 19), pp.457-475.

Pathirana, E., McPherson, A., Whittington, R. and Hick, P., 2019. The role of tissue type, sampling and nucleic acid purification methodology on the inferred composition of Pacific oyster (*Crassostrea gigas*) microbiome. *Journal of Applied Microbiology*, *127*(2), pp.429-444.

Petersen, J.M. and Osvatic, J., 2018. Microbiomes in natura: importance of invertebrates in understanding the natural variety of animal-microbe interactions. *Msystems*, *3*(2), pp.e00179-17.

Pienkowski, M.W., Watkinson, A.R., Kerby, G., Clarke, K.R. and Warwick, R.M., 1998. A taxonomic distinctness index and its statistical properties. *Journal of Applied Ecology*. 35, pp.523–531.

Pierce, M.L. and Ward, J.E., 2018. Microbial ecology of the Bivalvia, with an emphasis on the family Ostreidae. *Journal of Shellfish Research*, *37*(4), pp.793-806.

Pierce, M.L. and Ward, J.E., 2019. Gut microbiomes of the eastern oyster (*Crassostrea virginica*) and the blue mussel (*Mytilus edulis*): Temporal variation and the influence of marine aggregate-associated microbial communities. *Msphere*, *4*(6), pp.e00730-19.

Pimentel, Z.T., Dufault-Thompson, K., Russo, K.T., Scro, A.K., Smolowitz, R.M., Gomez-Chiarri, M. and Zhang, Y., 2020. Diversity and function of the eastern oyster (*Crassostrea virginica*) microbiome. *bioRxiv*.

Pimentel, Z.T., Dufault-Thompson, K., Russo, K.T., Scro, A.K., Smolowitz, R.M., Gomez-Chiarri, M. and Zhang, Y., 2021. Microbiome analysis reveals diversity and function of mollicutes associated with the eastern oyster, *Crassostrea virginica*. *Msphere*, *6*(3), pp.e00227-21.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), pp. D590-D596.

Rahman, M.A., Henderson, S., Miller-Ezzy, P., Li, X.X. and Qin, J.G., 2019. Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*. *Fish & Shellfish Immunology*, *86*, pp.868-874.

Rahman, M.A., Henderson, S., Miller-Ezzy, P.A., Li, X.X. and Qin, J.G., 2020. Analysis of the seasonal impact of three marine bivalves on seston particles in water column. *Journal of Experimental Marine Biology and Ecology*, *522*, p.151251.

Reise, K., Buschbaum, C., Büttger, H., & Wegner, K. M. (2017). Invading oysters and native mussels: from hostile takeover to compatible bedfellows. *Ecosphere*, *8*(9), e01949.

Salcher, M.M., Schaefle, D., Kaspar, M., Neuenschwander, S.M. and Ghai, R., 2019. Evolution in action: habitat transition from sediment to the pelagial leads to genome streamlining in *Methylophilaceae*. *The ISME Journal*, *13*(11), pp.2764-2777.

Scanes, E., Parker, L.M., Seymour, J.R., Siboni, N., King, W.L., Danckert, N.P., Wegner, K.M., Dove, M.C., O'Connor, W.A. and Ross, P.M., 2021. Climate change alters the haemolymph microbiome of oysters. *Marine Pollution Bulletin*, *164*, p.111991.

Sepulveda, J. and Moeller, A.H., 2020. The effects of temperature on animal gut microbiomes. *Frontiers in Microbiology*, *11*, p.384.

Simons, A.L., Churches, N. and Nuzhdin, S., 2018. High turnover of faecal microbiome from algal feedstock experimental manipulations in the Pacific oyster (*Crassostrea gigas*). *Microbial Biotechnology*, *11*(5), pp.848-858.

Soon, T.K. and Zheng, H., 2019. Climate change and bivalve mass mortality in temperate regions. *Reviews of environmental contamination and toxicology volume 251*, pp.109-129.

Spring, S., Scheuner, C., Göker, M. and Klenk, H.P., 2015. A taxonomic framework for emerging groups of ecologically important marine gamma-proteobacteria based on the reconstruction of evolutionary relationships using genome-scale data. *Frontiers in Microbiology*, *6*, p.281.

Svane, I., 2011. An overview of the blue mussel in southern Australia–a serial invader, a blind passenger, or just a welcome addition to the menu. *Transactions of the Royal Society of South Australia*, *135*(2), pp.134-139.

Thomas, Y., Cassou, C., Gernez, P. and Pouvreau, S., 2018. Oysters as sentinels of climate variability and climate change in coastal ecosystems. *Environmental Research Letters*, *13*(10), p.104009.

Trachtenberg, S., 2005. Mollicutes. *Current Biology*, *15*(13), pp.R483-R484.

Tran, K., 2017. Selective feeding of freshwater mussels: Implications for resource partitioning (Doctoral dissertation, University of Guelph).

Travers, M.A., Miller, K.B., Roque, A. and Friedman, C.S., 2015. Bacterial diseases in marine bivalves. *Journal of Invertebrate Pathology*, *131*, pp.11-31.

Triscari-Barberi, T., Simone, D., Calabrese, F.M., Attimonelli, M., Hahn, K.R., Amoako, K.K., Turner, R.J., Fedi, S. and Zannoni, D., 2012. Genome sequence of the polychlorinated-biphenyl degrader *Pseudomonas pseudoalcaligenes* KF707, 4426-4427

van Ruth, P.D., Patten, N.L., Doubell, M.J., Chapman, P., Rodriguez, A.R. and Middleton, J.F., 2018. Seasonal-and event-scale variations in upwelling, enrichment and primary productivity in the eastern Great Australian Bight. *Deep Sea Research Part II: Topical Studies in Oceanography*, *157*, pp.36-45.

Vaughn, C.C. and Hoellein, T.J., 2018. Bivalve impacts in freshwater and marine ecosystems. *Annual Review of Ecology, Evolution, and Systematics*, *49*, pp.183-208.

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L. and Pruzzo, C., 2018. Comparative 16S rDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microbial Ecology*, *75*(2), pp.495-504.

Villalba, A., Mourelle, S.G., Carballal, M.J. and Lopez, C., 1997. Symbionts and diseases of farmed mussels *Mytilus galloprovincialis* throughout the culture process in the Rias of Galicia (NW Spain). *Diseases of Aquatic Organisms*, *31*(2), pp.127-139.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), pp.5261-5267.

Wang, Y., Huang, J.M., Zhou, Y.L., Almeida, A., Finn, R.D., Danchin, A. and He, L.S., 2020. Phylogenomics of expanding uncultured environmental Tenericutes provides insights into their pathogenicity and evolutionary relationship with *Bacilli*. *Bmc Genomics*, *21*(1), pp.1-12.

Ward, C.S., Yung, C.M., Davis, K.M., Blinebry, S.K., Williams, T.C., Johnson, Z.I. and Hunt, D.E., 2017. Annual community patterns are driven by seasonal switching between closely related marine bacteria. *The ISME Journal*, *11*(6), pp.1412-1422.

Warwick, R.M. and Clarke, K.R., 1995. New\'biodiversity\'measures reveal a decrease in taxonomic distinctness with increasing stress. *Marine Ecology Progress Series*, *129*, pp.301-305.

Webster, N.S., Xavier, J.R., Freckelton, M., Motti, C.A. and Cobb, R., 2008. Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. *Environmental Microbiology*, *10*(12), pp.3366-3376.

Wegner, K.M., Volkenborn, N., Peter, H. and Eiler, A., 2013. Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiology*, *13*(1), pp.1-12.

Wijsman, J.W.M., Troost, K., Fang, J. and Roncarati, A., 2019. Global production of marine bivalves. Trends and challenges. In *Goods and services of marine bivalves* (pp. 7-26). *Springer*, Cham.

Wilson, I.G. and Moore, J.E., 1996. Presence of *Salmonella* spp. and *Campylobacter* spp. in shellfish. *Epidemiology & Infection*, *116*(2), pp.147-153.

Woese, C.R., Maniloff, J. and Zablen, L.B., 1980. Phylogenetic analysis of the mycoplasmas. *Proceedings of the National Academy of Sciences*, *77*(1), pp.494-498.

Wu, X.Z. and Pan, J.P. 2000. An intracellular prokaryotic microorganism associated with lesions in the oyster, *Crassostrea ariakensis* Gould. *Journal of Fish Diseases*, *23*, pp.409-414.

Xiong, J.B., Nie, L. and Chen, J., 2019. Current understanding on the roles of gut microbiota in fish disease and immunity. *Zoological Research*, *40*(2), p.70.

Yadav, S., Koenen, M., Bale, N., Sinninghe Damste, J.S. and Villanueva, L., 2021. The physiology and metabolic properties of a novel, low-abundance *Psychrilyobacter* species isolated from the anoxic Black Sea shed light on its ecological role. *Environmental Microbiology Reports*, *13*(6), pp.899-910.

Zhang, J., Kobert, K., Flouri, T. and Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*, *30*(5), pp.614-620.

Zhu, Z., Xu, T., He, Z., Wu, X., Wu, L., Meng, Q. and Huang, J., 2012. Rickettsia-like organism infection associated with mass mortalities of blood clam, *Tegillarca granosa*, in the Yueqing Bay in China. *Acta Oceanológica Sinica*, *31*(1), pp.106-115.

Table 2.1. Samples collected and sequenced for bacterial community comparisons from Coffin Bay, South Australia. Monthly water temperatures (mean ± SD) are provided.

Species	Oyster		Mussel	
Season	Summer (Feb)	Winter (Aug)	Summer (Feb)	Winter (Aug)
No. of bivalve samples collected/sequenced	30/30	30/30	30/30	30/26
Water samples (2 L)	3	3	3	3
Collection Date	07.02.17	07.08.17	07.02.17	07.08.17
Water temp. (°C)†	18.6 ± 1.9	13.6 ± 0.6	18.6 ± 1.9	13.6 ± 0.6

†Average water temperatures were derived from data obtained from the Australian Ocean Data Network (AODN) Portal - Integrated Marine Observing System (IMOS) [https://imos.org.au/facilities/aodn].

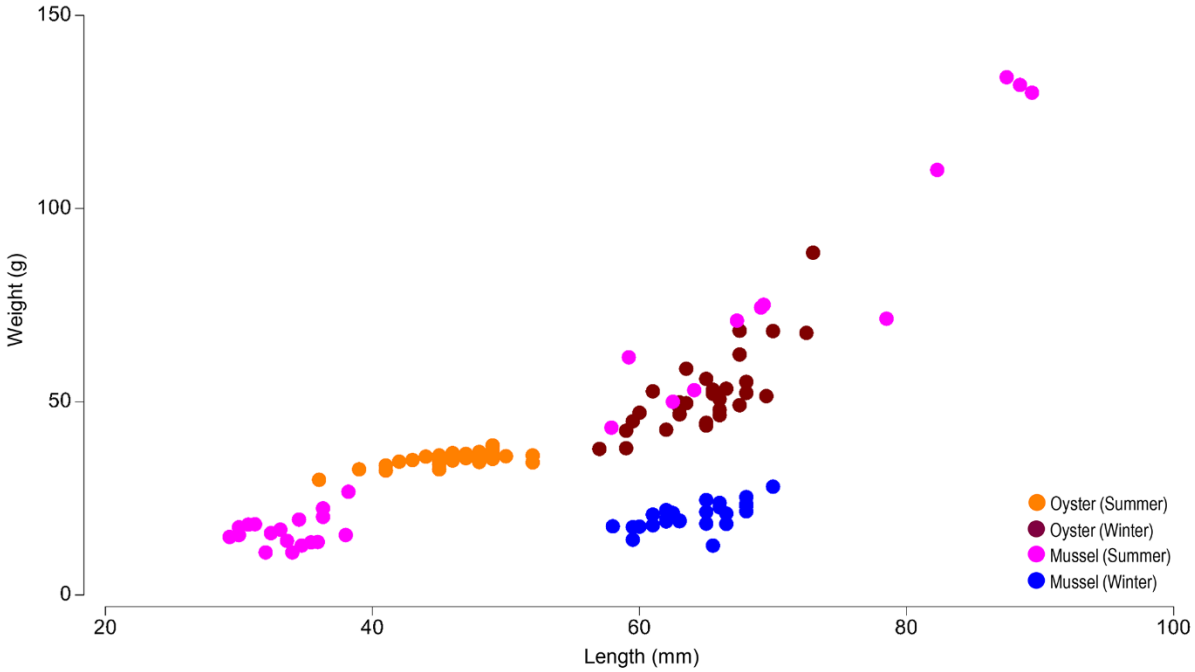


Figure 2.1. Scatter plot displaying the lengths vs weights of oyster and mussels collected in summer and winter for bacterial community analysis from Coffin Bay, South Australia.

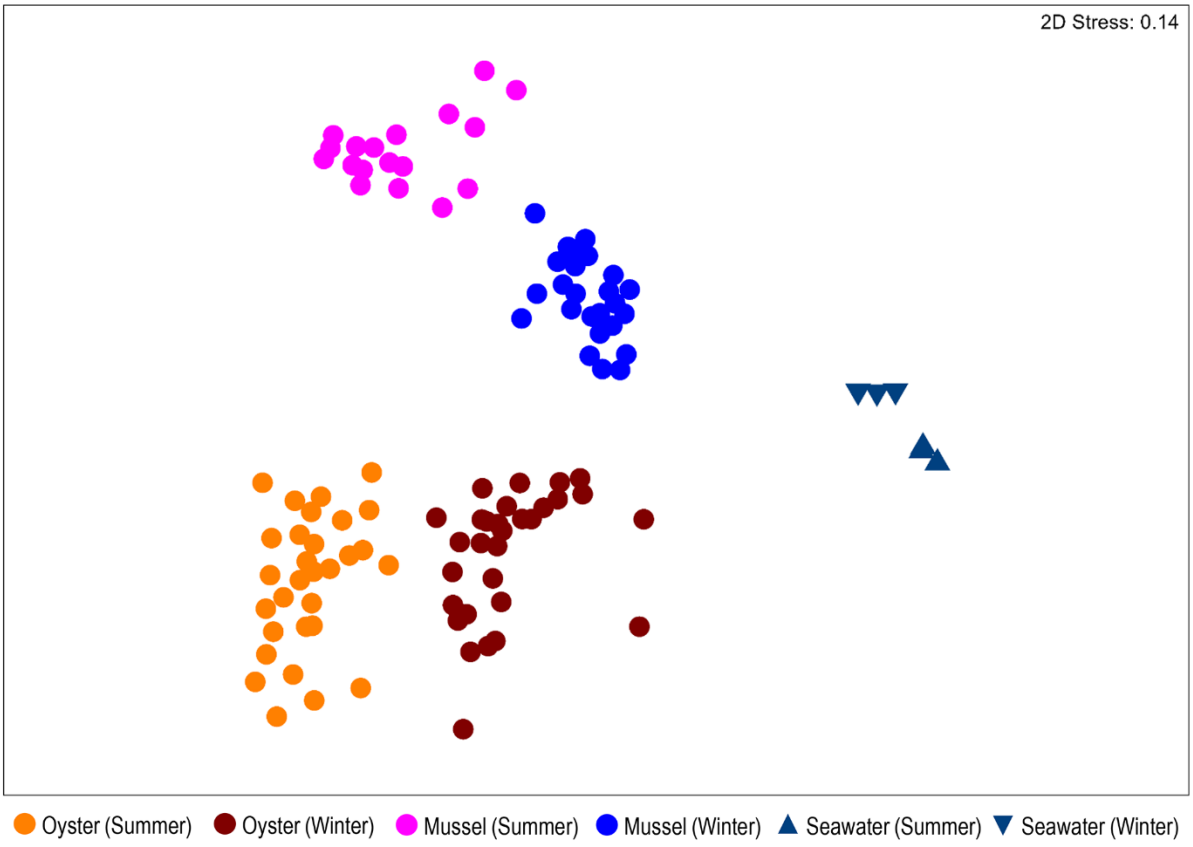


Figure 2.2. Ordination plot depicting the global differences in the bacterial community composition between matched oyster and mussel gut and seawater samples collected in summer and winter from Coffin Bay (South Australia), as assessed by non-metric multi-dimensional scaling (nMDS) using Bray-Curtis dissimilarity.

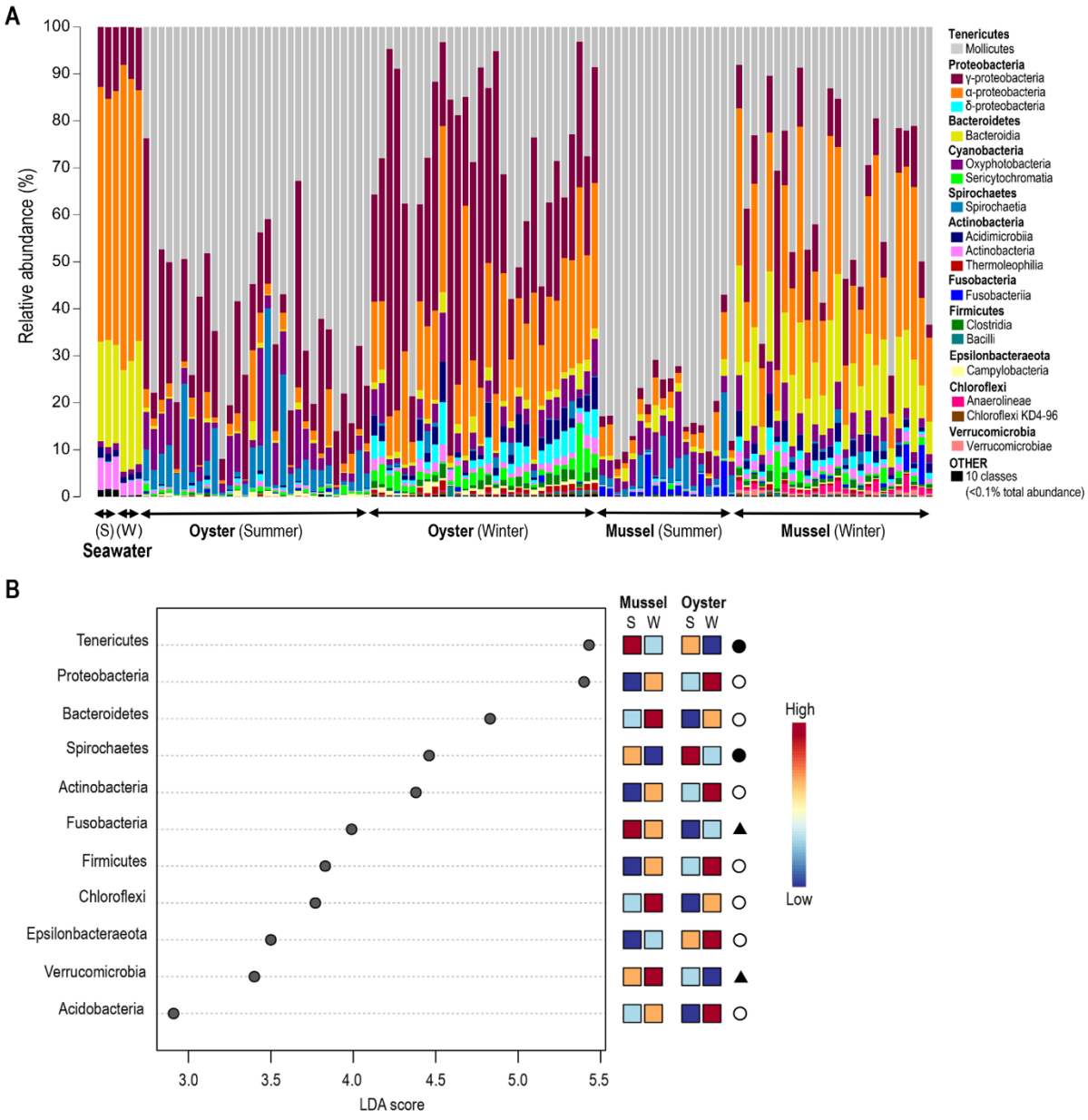


Figure 2.3. Mean relative abundances of bacterial classes (A), and Linear Discriminant Analysis (LDA) Effect Size (LEfSe) plot (B) displaying the differentially abundant bacterial phyla associated with seawater and matched oyster and mussel gut samples collected in summer (S) and winter (W) from Coffin Bay, South Australia. Differentially abundant features were determined using the Kruskal-Wallis rank test (adjusted p-value cut off = 0.01), with the Log LDA Score value adjusted to 2.0 and significant taxa given in descending order from the highest to lowest LDA score. The heat key denotes the rank ordered abundance of each phylum. Symbols represent phyla that occurred in higher

abundance in summer (●) or in winter (○), or which had notably disparate seasonal abundances between bivalves (▲).

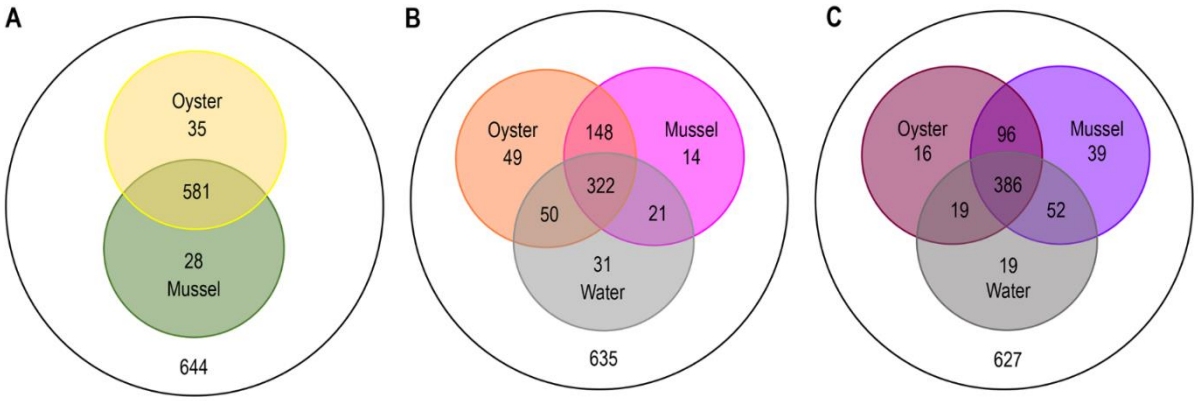


Figure 2.4. Venn diagrams indicate the distribution of unique and shared bacterial OTUs in (A) oyster and mussel gut - irrespective of season; (B) oyster and mussel gut and seawater in summer; and (C) oyster and mussel gut and seawater in winter. Values inside the outermost circles indicate the total number of observed OTUs.

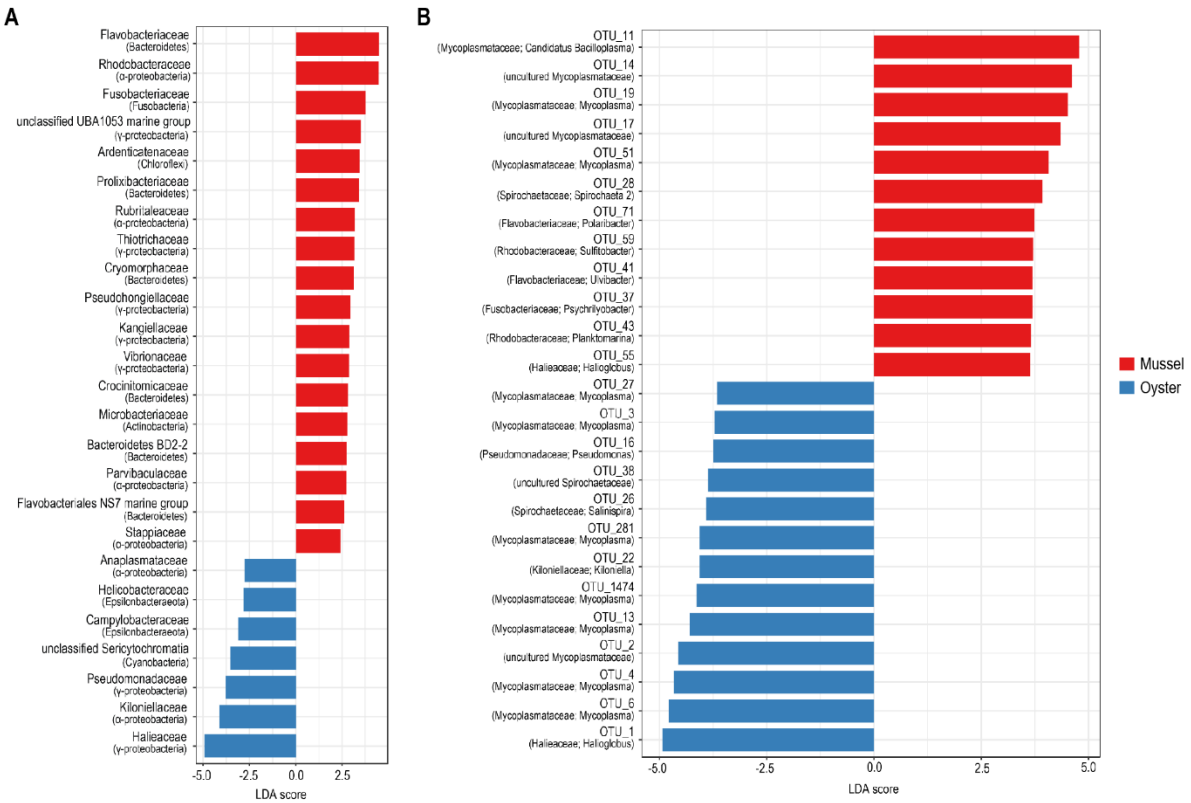


Figure 2.5. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) bar plots displaying the differentially abundant bacterial families (A) and top 25 OTUs (B) from mussel and oyster gut samples obtained from Coffin Bay, South Australia (irrespective of season). Differentially abundant features were determined using the Kruskal-Wallis rank test (adjusted *p*-value cut off = 0.01), with the Log LDA Score value adjusted to 2.0 and significant taxa given in descending order from the highest to lowest LDA score.

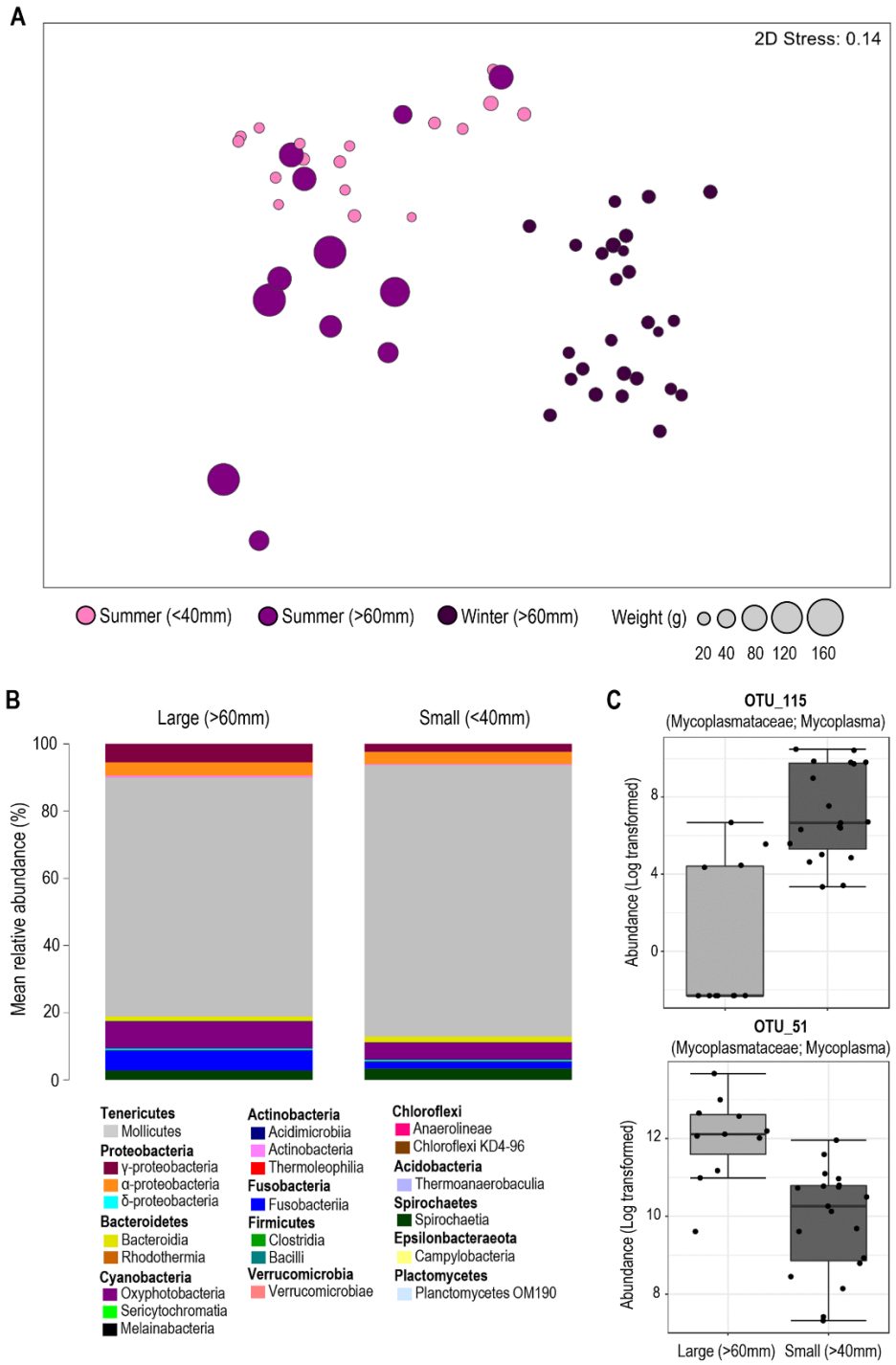


Figure 2.6. Comparison of the bacterial compositional differences between gut samples obtained from mussels with large (>60mm) and small (<40mm) shell lengths. (A) Global differences in the bacterial community composition between large and small mussels collected in summer (and in comparison, to large mussels in winter), as assessed by non-metric multi-dimensional scaling (nMDS) using Bray-Curtis dissimilarity. Bubble overlays represent mussel weight (g). (B) mean relative

abundance of bacterial classes from large and small summer mussel samples; and (C) differentially abundant OTUs observed from large and small summer mussel samples as determined using the Kruskal-Wallis rank test (adjusted p-value cut off = 0.01).

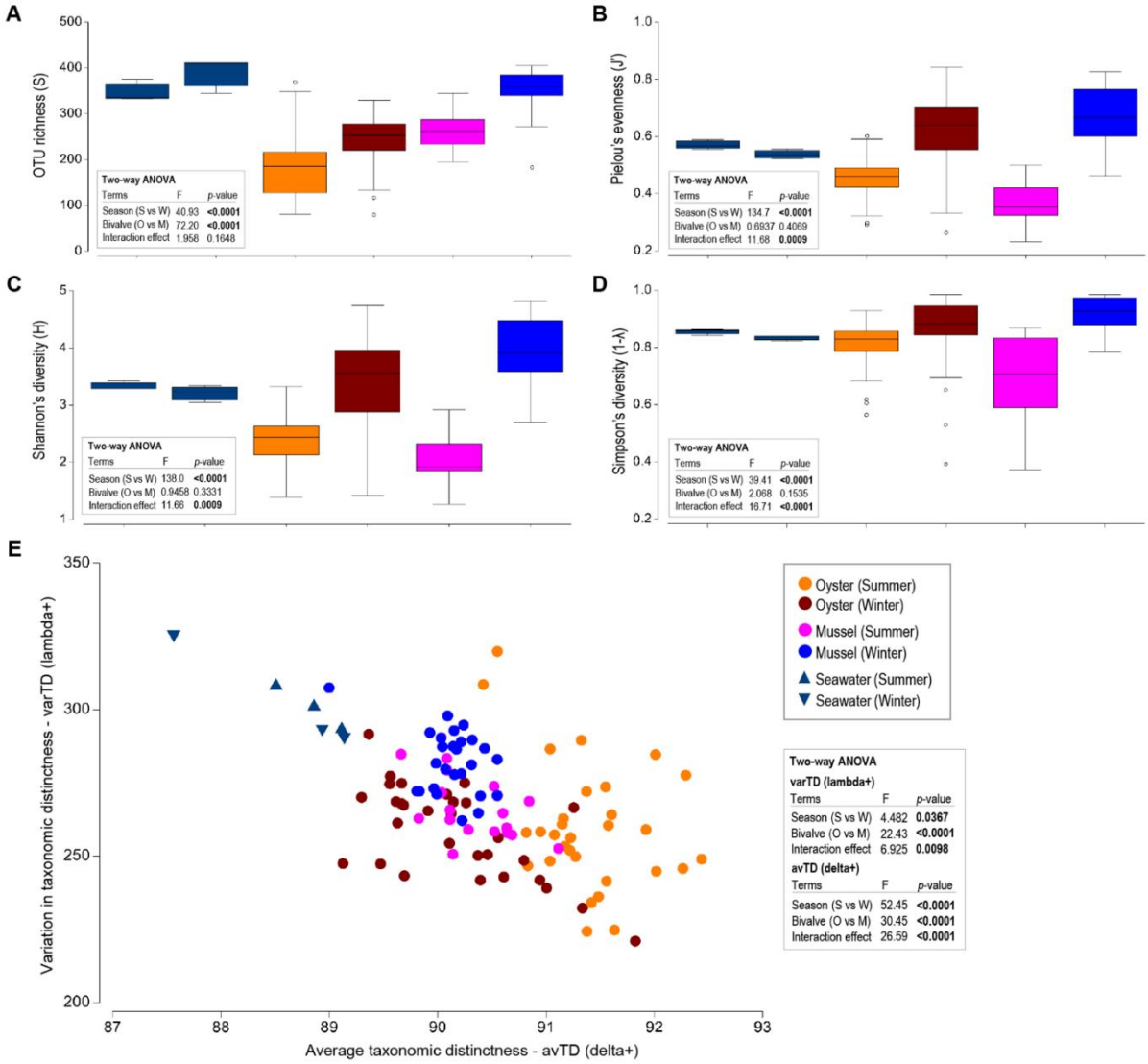


Figure 2.7. Scatter plot representing measures of Taxonomic distinctness (TD) measures such as the average taxonomic distinctness (avTD, delta+) as a function of variation in taxonomic distinctness (varTD, lambda+) in the gut bacterial communities in oyster and mussel.

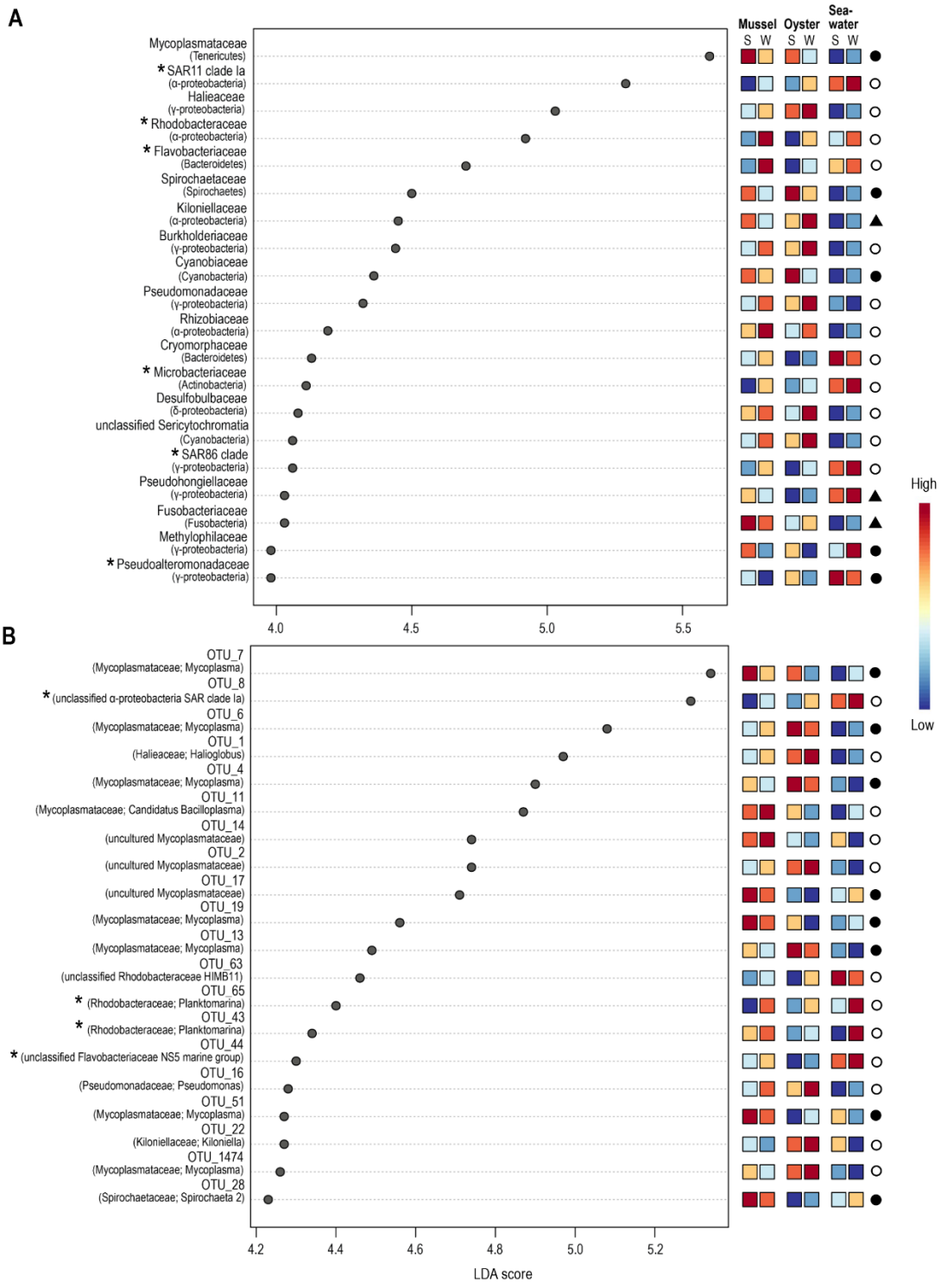
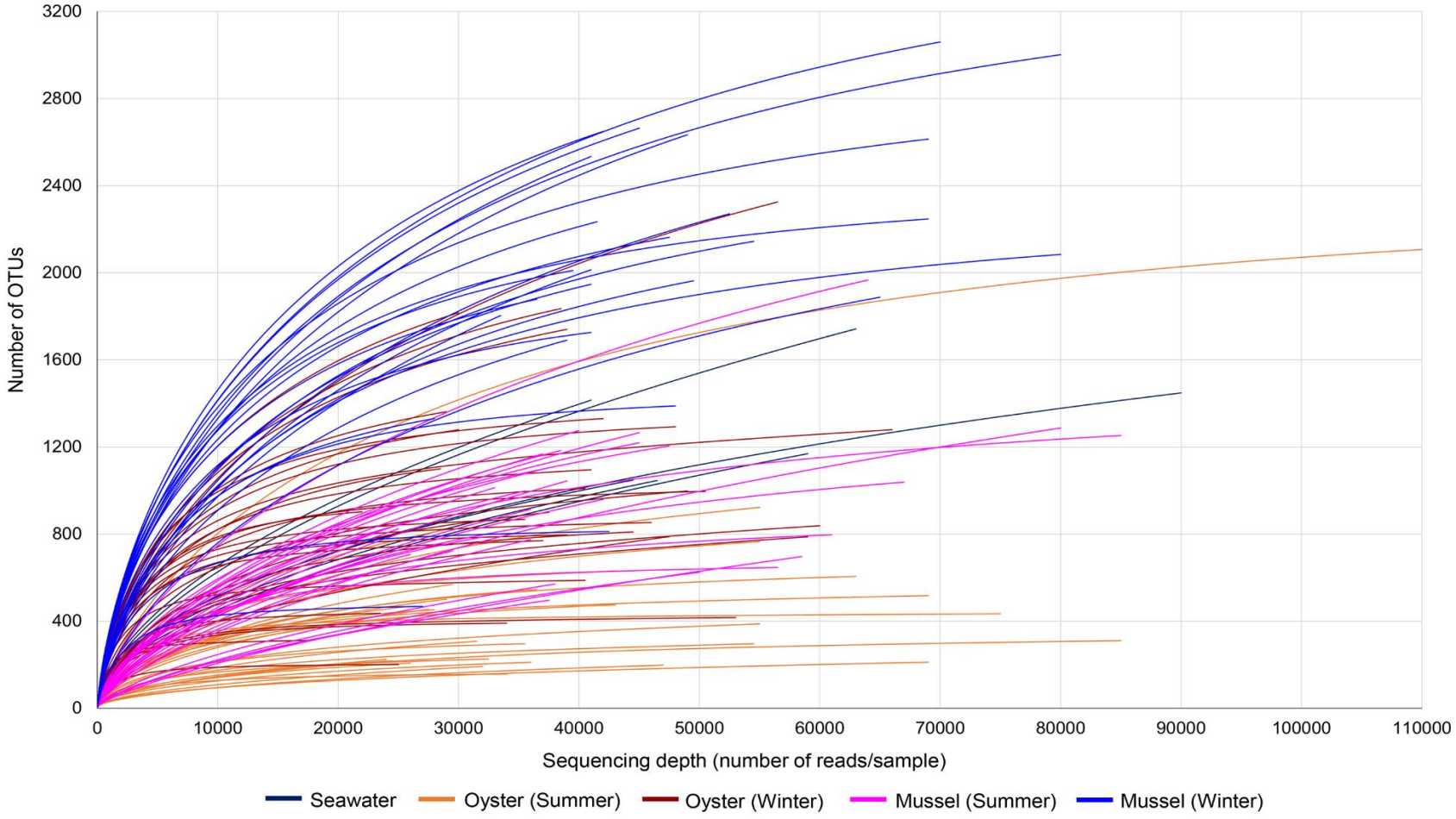


Figure 2.8. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) plots displaying the top 20 differentially abundant bacterial families (A) and OTUs (B) from mussel and oyster gut and seawater samples obtained in summer (S) and winter (W) from Coffin Bay, South Australia. Differentially abundant features were determined using the Kruskal-Wallis rank test (adjusted *p*-value cut off = 0.01), with the Log LDA Score value adjusted to 2.0 and significant taxa given in descending order

from the highest to lowest LDA score. The heat key denotes the rank ordered abundance of each taxa. Symbols represent taxa with the highest abundance in summer (●) or in winter (○), or which had notably disparate seasonal abundances between bivalves (▲). Taxa marked with an asterisk represent those that had a corresponding high seasonal abundance in seawater.


3200
2800
2400
2000
1600
1200
800
400
0
Number of OTUs
0
10000
20000
30000
40000
50000
60000
70000
80000
90000
100000
110000
Sequencing depth (number of reads/sample)
Seawater
Oyster (Summer)
Oyster (Winter)
Mussel (Summer)
Mussel (Winter)

Supplementary Figure S1. Rarefaction curves depicting the number of resolved OTUs against sequencing depth of the 122 samples collected in summer and winter (6 seawater, and 60 oyster and 56 mussel gut samples).

CHAPTER 3

Dynamics of bacterial communities in the water and the gut of Pacific oyster (*Crassostrea gigas*) between two different habitats in cold and warm seasons

Highlight

1. In both oceanic nutrient based (Coffin Bay) and local nutrient based habitats (Franklin Harbor), Mollicutes were dominant in oyster guts and decreased in winter but almost disappeared in the water.
2. Coffin Bay water was alpha-proteobacteria rich, regardless of the season. Franklin Harbor was alpha-proteobacteria rich in summer and gamma-proteobacteria rich in winter.
3. Bacteria colonized in the oyster gut are host-specific, and their abundance was season-dependent.
4. Bacterial species richness was higher in winter than in summer in both fertile and lean habitats.
5. The bacterial diversity in oyster gut showed seasonal differences in both fertile and lean habitats.
6. *Vibrio* was the most dominant bacteria in Franklin Harbor in summer, accounting for 24.25%.

3.1. Abstract

The Pacific oyster (*Crassostrea gigas*) has been widely cultured worldwide, but the variation of seasonal temperature and nutrient supply can influence bacterial population in water, thus affecting gut bacteria in a filter-feeding oyster. This study compares bacterial community in water and oyster gut between a oceanic nutrient based habitat (Coffin Bay) and a local nutrient based habitat (Franklin Harbor) in summer and winter. The Illumina sequencing of V1-V2 of 16S rDNA was used to differentiate gut bacteria of oysters in Coffin Bay and Franklin Harbor in South Australia. The gut bacterial community in oceanic nutrient based Coffin Bay oysters had higher species diversity than in local nutrient based Franklin Harbor oysters, and the bacterial diversity in winter was higher than that in summer. The relative abundance of different bacteria varied with seasons, but oysters in both habitats accumulated a taxonomically similar group of bacteria. Besides, the venn diagram displayed 498 and 346 shared OTUs in summer, while 497 and 419 OTUs in winter between oysters and water samples in Coffin Bay and Franklin Harbor. Tenericutes was dominant in summer, whereas Proteobacteria was dominant in winter oysters. In Coffin Bay, alpha-proteobacteria was dominant in water in both seasons, but in Franklin Harbor, the ambient alpha-proteobacteria were abundant in winter, and gamma-proteobacteria were dominant in summer. Noticeably, *Vibrio* was most dominant (24.25%) in water of Franklin Harbor in summer. Finally, oysters colonized host-specific bacteria in their gut, and its abundance and pattern of bacterial diversity was both season and habitat-dependent. This study provides valuable information on the difference in bacterial community between oyster gut and ambient water in two nutrient-contrasting habitats in summer and winter.

Keywords: Gut bacteria, Pacific oyster *Crassostrea gigas*, Nutrient, Habitat

3.2. Introduction

Nutrient availability is a crucial factor driving the ecological process in marine ecosystems, and nutrients could affect niche structure and food web dynamics through resource partitioning in marine environment (Fisher *et al*., 2000; Church, 2008). As such, nutrient availability and food web interactions can strongly influence distribution and abundance of marine bacteria (Calbet and Landry, 1999). Previous studies have demonstrated a strong impact of temperature and nutrients on microbial diversity in marine environment and host-microbial interaction through symbiosis (Morris *et al*., 2005, Fuhrman *et al*., 2006, Gilbert *et al*., 2009). Temperature is the major abiotic factor regulating microbial symbiosis and other biological processes in marine ecosystem. Two consensuses have been generated on the relationship between temperature and microbes: (1) climate change can affect stability of marine microbial symbiosis (Webster *et al*., 2008; Mouchka *et al*., 2010) and (2) the shift of microbiota can cause mass mortality in marine bivalves during temperature elevation (Paillard *et al*., 2004). In the marine coastal site off Plymouth, UK, microbial community was regulated by environmental variables such as temperature, salinity and nutrients (nitrogen, carbon, phosphate) with peak microbial diversity in winter (Gilbert *et al*., 2012). Cliff (1982) found comparatively higher nitrate, silicate and particulate matter in winter than that in summer in the ocean regarding nutrient availability. In the marine environment ocean, some autotrophic bacteria such as Cyanobacteria are capable of photosynthesis to obtain energy from organic carbon in environment. In contrast, most heterotrophic bacteria get energy from other autotrophic organisms and the environment. In turn, heterotrophic bacteria re-mineralise organic carbon into the inorganic matter in the cycling of oxygen, CO_2 and nitrogen and phosphorus (Yuan *et*

al., 2010; Yuan *et al*, 2011). However, very little is known on the impact of nutrient availability and seasonal temperature on symbiotic relationship between microbiota and host in a marine environment.

The symbiotic relationship between bacteria and host differs among species in aquatic ecosystem, and bacteria can flourish a symbiotic relationship with their hosts (Pinhassi et al., 2004). In the microbial food web, bacteria are a portion of food for planktonic organisms and other filter feeders such as oysters. Bacteria also help nutrient recycling through organic matter decomposition and degeneration of hazardous pollutants to a safe form (Bentzon-Tilia *et al*., 2016). The productivity of an aquatic ecosystem largely depends on bacterial abundance and species composition. Nutrient recycling and food web interaction can influence bacterial abundance in the aquatic environment (Pernthaler *et al*., 2004; Kent *et al*., 2006). On the other hand, habitat influences a symbiotic relationship between bacteria and their host, which is particularly important in oysters because of filter-feeding. The potential influence of habitat on shaping symbiotic microbiota has been observed in many marine invertebrates such as eastern oyster (LaValley *et al*., 2009), coral (Klaus et al., 2005), shrimp *Litopenaeus vannamei* (Luo *et al*., 2009), Chinese mitten crab *Eriocheir sinensis* (Zhang *et al*., 2016), and sea cucumber *Apostichopus japonicas* (Gao *et al*., 2014). The gills collect environmental bacteria and store them in the gut during respiration and feeding. Under a natural condition, invertebrates selectively accumulate symbiotic microbes from water into different organs according to their respective physiological functions (Meisterhans *et al*., 2016). Thus, the microbiota communities differ in an organism between body parts such as the surface mucus layer and skeleton in a coral (Sweet *et al*., 2011), intestine and gills in crab *Eriocheir sinensis* (Zhang *et al*., 2016), stomach and intestine in eastern oyster *Crassostrea*

virginica (King *et al*., 2012), anterior and posterior parts of the gastrointestinal tract in yellow grouper *Epinephelus awoara* (Zhou *et al*., 2009), different gut sections in sea cucumber *Apostichopus japonicus* (Wang *et al*., 2018), and intestine, rectum and gills in Yesso scallop *Patinopecten yessoensis* (Lu *et al*., 2017). In addition, a spatial variation of microbial composition was reported in similar sites on a yesso scallop *Patinopecten yessoensis* farm in Changhai, China (Lu *et al*., 2017). However, little attention has been paid to compare host-associated symbiotic microbiota on the same invertebrate species between two habitats with different upwelling conditions.

The Pacific oyster (*Crassostrea gigas*, Thunberg, 1793) is a leading shellfish in world aquaculture because of its taste, consumer demand, and ease of production. This species has been widely cultured worldwide using natural food to sustain its growth without any nutritional supplementation. Coffin Bay and Franklin Harbor are two premier oyster growing regions in Southern Australia, having similar level of nutrients. However, Coffin-bay is mainly based on oceanic nutrient, whereas, Franklin harbor is based on local nutrient supply. The wind-driven upwelling events that occurred in Coffin Bay during summer and autumn (Kämpf et al., 2004) could result in nutrient-rich upwelled surface water with high primary producers (Ward et al., 2006) and deep water with high nitrate, phosphate and silicic acid (Kämpf et al., 2004). On the other hand, Franklin Harbor is a swampy, low tide shallow water area enclosed with coastal mangroves seagrass beds dominated by brown algae (Aquaculture policy-Franklin Harbor, 2015), resulting in low water exchange (Lower spencer gulf assessment report, 2010). The sustainable development of oyster industry has now been heavily threatened worldwide due to mass mortality in summer. The Pacific oyster mortality syndrome (POMS) outbreak is mainly caused by the ostreid herpes virus (OsHV-1 µvar) and *Vibrio* bacteria when seawater temperature reaches

>19°C (Samain and McCombie, 2008). In a recent study, Petton et al. (2015) reported that infection by *Vibrio* is sufficient to cause the death of juvenile oysters irrespective of the presence of herpes virus. As *Vibrio* spp. are more common in coastal and marine environments from surface to deep water (Thompson & Swings, 2006), it becomes an emerging urgency to understand microbial community associated with oysters and their environments.

Pacific oysters being an ecosystem engineer can consume a wide variety of phytoplankton/ microalgae, bacteria and viruses from the surrounding water through filter-feeding, so the gut of oysters should harbour a variety of different bacterial flora. As variations in seawater conditions such as seasonal temperature, natural nutrient availability in environment can influence environmental bacterial population, which is expected to influence oysters' gut bacteria in different habitats. Characterizing resident microbiota in healthy Pacific oysters in different habitats could be an essential step to learn how the microbial symbiosis is changing due to food availability of habitats and to predict susceptibility to bacterial infections and disease outbreaks (Moriarty, 1997). Gut microbes play a functionally important role in digestion, immunity, disease resistance and maintenance of microbial homeostasis (Moriarty, 1990; Harris, 1993; Moriarty, 1997; Fraune & Bosch, 2010). The current understanding of the bacterial communities in aquatic systems and host tissues such as gut microbiota between two different environments is fragmental.

This study aims to explore seasonal bacterial symbiosis in a filter-feeding invertebrate in different habitats. The Pacific oyster has been used as a model species to compare oyster gut bacterial composition between habitats based on oceanic nutrients and local nutrients in two distinct seasons (summer and winter). This study

was conducted in two premier oyster culture regions of South Australia (Coffin Bay and Franklin Harbour). Comparatively, the water in Coffin Bay is ocenic nutrient as it locates in a strong nutrient upwelling area, whereas the water in Franklin Harbor is local nutrient based as nutrient input is mainly from watershed in a barren region. We hypothesize that benign bacteria dominate gut microbial communities in Pacific oysters of a oceanic nutrient habitat over a local nutrient based habitat, and pathogen bacteria dominate gut of oysters in a warm season over a cold season. We applied Illumina sequencing in the current study based on highly conserved hypervariable V1-V2 region of 16S rDNA to compare gut microbial diversity of Pacific oysters from the above habitats in two seasons to build a theoretical basis for a healthy aquaculture system of Pacific oysters.

3.3. Materials and Methods

3.3.1. Experimental Design and Sample Collection

Oysters and seawater were sampled from two premier oyster farming regions of South Australia: Coffin Bay and Franklin Harbor in summer (February 17) and in winter (August 17). A total of 30 Pacific oysters and three replicates of water samples (2L each) were collected from each habitat and in each season (Table 3.1). The water temperature, salinity, dissolved oxygen (DO) and pH of both sites were measured during sampling. After collection, samples were stored rapidly at 4^{o}C and then brought to the laboratory of Lincoln Marine Science Centre, Coffin Bay, to collect gut content on sampling day.

3.3.2. Oyster Gut Collection and Water Filtration

After being brought to the lab, the oysters were rinsed with tap water and wiped with a paper towel for length-weight measurement. Coffin Bay and Franklin Harbor's

oysters were 40–70 mm and 50–80 mm shell length, respectively. Oyster shells were rinsed with 70% ethanol to kill bacteria attached to shells. Then shells were opened with an oyster knife, and gut contents were sucked through the mouth using a sterile glass pasture pipette fitted with a rubber head (Wheaton, DWK). The gut content was stored in a separate sterile cryovial for each individual with a sampling code on it, and then vials were stored immediately in liquid nitrogen. Finally, the collected gut and water samples were transported to South Australian Research and Development Institute (SARDI) laboratory the next day. The gut samples were then transferred from liquid nitrogen to -80 ^{o}C for further processing. The water samples kept at 4^{o}C were then filtered using a separate Nalgene™ Rapid-Flow™ filter unit (pore size 0.2µm, filter capacity 500mL, Sigma®). The filter paper was cut into small pieces using a scalpel blade and stored into separate cryovials for each water sample at -80^{o}C for further processing.

3.3.3. DNA Extraction

For extracting DNA, firstly, the oyster gut and filtered water samples were defrosted at room temperature. FastDNA™ spin kits for soil (MP biomedicals) were used to extract DNA from gut and water samples. In brief, samples were taken into a Lysing Matrix E tube with sodium phosphate buffer and MT buffer and then homogenized in the FastPrep® instrument for 40sec followed by several centrifugation steps at 14,000×g following manufacturer's instructions. Next, the resuspended binding matrix was added to supernatant where DNAs bound to the silica matrix and then DNA was purified using a spin filter™ followed by centrifugation and air dry. Finally, extracted DNA was resuspended into DES (DNase/ Pyrogen Free Water). After extraction, DNA quantification (ng/µl) was measured using a NanoDrop 2000 spectrophotometer. The absorbance was measured at $A_{260/280\ nm}$ and $A_{260/230\ nm}$

wavelengths. Then extracted DNA was further purified by ethanol precipitation, and precipitated DNA concentration (ng/µl) was measured again using NanoDrop spectrophotometer. The precipitated DNA was then stored at -20 ^{o}C for further downstream analysis.

3.3.4. Library preparation and Illumina amplicon sequencing

The extracted DNA was further amplified by polymerase chain reaction (PCR) targeting highly conserved hypervariable region V1-V2 of 16S rDNA following Camarinha-Silva *et al.* (2014) and Legrand *et al.* (2018). Amplification was performed using universal eubacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 338R (GCTGCCTCCCGTAGGAGT). The sequences of both primers are complementary to the 5'-ends of Illumina adaptors (Camarinha-Silva *et al.*, 2014). In the screening PCR of 35 cycles, a total volume of 25µl master was mixed with 5×primeStar buffer, 2.5mM de-oxynucleoside triphosphate (dNTPs), 2.5U/µl taq primeStar, 10µm of each primer (27F and 338R) and 25ng of DNA template. The PCR cycles were set at an initial denaturation step for 3min at 95°C with further denaturation for 10s at 98°C, elongation for 10s at 55°C, for 45 s at 72°C, and a final elongation for 2min at 72°C in the Master cycler (Eppendorf, Germany). The PCR products were screened on 1% agarose gel electrophoresis (pre-stain) with GelRed^{TM} at 85V for 45min. Then, the same PCR conditions were followed by a first-round PCR with the same reaction mixture of screening PCR to 20 cycles, a second-round PCR for 15 cycles using 1µl template (round 1) with 10 µm IlluminaFBC 27F, 10µm 338R adapter and the final third-round PCR for 10 cycles using 1µl template (round 2) with 10µm Illumina Multiplex and 10µm Illumina Index. The *Lactobacillus reuteri* were sequenced as a positive control and water as a negative control. The final resultant PCR amplicons were visualized

again in 1% pre-stain gel electrophoresis to cross-check all samples (expected size ~438 bp). In the next step, Agencourt PCR Clean-up protocol (Promega, Madison, WI) was followed for bead purification of the PCR products. The purified PCR amplicons were then quantified by Quant-iT™ Picogreen® dsDNA kit (Life Technologies). The amplicons library was prepared by pooling in equimolar ratios of 20ng of each sample with a unique barcode, and subsequently the pooled library was quantified in PicoPlate. The science primer website was used to calculate template concentration (nM), template volume to add and EB buffer volume. The concentration (ng/µl) of final library was calculated according to the Australian Genome Research Facility (AGRF) recommendation (5-10nM in 20-30µl EB buffer). The library size was confirmed on 1% gel electrophoresis. Finally, the cDNA library was sent to AGRF (Melbourne, Vic, Australia) for Illumina MiSeq sequencing using 250bp paired-end sequencing.

3.3.5. Bioinformatics

A total of ~13.1 million raw reads were obtained from a total of 131 samples (n=60/60 oyster; n=59/60 mussel; n=12/12 seawater). According to Zhang *et al*., 2014, the forward and reverse paired-end reads were aligned using PEAR (version 0.9.5). After identification, primers were trimmed, and the trimmed sequences were dealt with Quantitative Insights into Microbial Ecology, QIIME 1.8 (Caporaso *et al.*, 2010) and assigned by Greengenes (Version13_8, Aug 2013) and SILVA database. The sequencing amplicons were then clustered into OTUs through UPARSE software. The sequence quality was checked by USEARCH (version 8.0.1623; Edgar, 2010), and UPARSE software (Edgar, 2013) software, and duplicate sequences (full length) were discarded and sorted by abundance. Unique reads (singletons) were removed from the dataset, and reads were recorded to OTUs with 97% minimum identity (Cole

et al., 2014). Using "RDP-gold" database as a reference, sequences were filtered for removing putative Chimeras. The resultant OTUs were taxonomically assigned through the Seqmatch function in RDP and SILVA. Then each OTU was named according to the SILVA taxonomy and best match from RDP linages.

Using PEAR, a total of 5,651,091 high quality paired-end reads were clustered into a total 22402 OTUs with a minimum library size 16,820 and a maximum library size 112,618 (Zhang *et al*. 2016). The gut only reads for Coffin Bay oysters (60 oysters) and Franklin Harbor oysters (59 oysters) were 2,568,546 and 2,492,165 respectively, whereas seawater only reads were 346,731 (6 CB water) and 243,649 (6 FH water), respectively. The OTUs were standardized and filtered (Zhang et al., 2016 and Legrand *et al*., 2018), and the host-associated datasets with the contribution >0.01% were finally used. The total OTUs were then percent standardized and filtered with the contribution of >0.01% in the datasets and fungi containing OTUs were removed from the datasets, and finally a total of 662 OTUs were used for downstream analysis. A rarefaction curve was calculated from raw sequences of each individual to assess the sampling depth (Figure 3.10)

3.3.6. Statistical analysis

The unique and shared OTUs between oysters and water of each habitat in each season were visualized in venn diagrams. According to Clarke *et al*. (2014), the consequential filtered dataset of 662 OTUs from 60 Coffin Bay oysters, 59 Franklin Harbour oysters and six seawater samples from each habitat were finally used in Primer-E (version 7.0.11) for further analysis. Firstly, the global bacterial community structure was visualized through non-metric multidimensional scaling (nMDS) plots with stress value using Bray-Curtis dissimilarity resemblance (Bray and Curtis, 1957; Clarke *et al.*, 2014). The main test one-way permutational multivariate analysis of

variance (PERMANOVA) was generated using unrestricted permutations of raw data to evaluate the differences among *a priori* groups of the oyster gut and water from Coffin Bay and Franklin Harbor. Besides, two-way pairwise PERMANOVA was also calculated to assess the difference among gut and water samples in summer and winter. Season-wise, the relative abundance (%) of different phyla and classes found in gut and water samples were plotted as a stacked bar chart in Primer (Anderson, 2001). The univariate diversity measures such as Species richness (S), Pielou's evenness (J′), Shannon index (H′), Simpson index (1-λ), Average Taxonomic distinctness (Delta+) and Variation in Taxonomic Distinctness (Lambda+) were analysed in Primer between oysters of two habitats, and then unpaired Welch t-test of each diversity index was generated in Graphpad prism (version 8.1.1). Considering season as another factor with habitat, multivariate diversity indices (S, J′, H′, 1-λ, Delta+ and Lambda+) were also evaluated, and subsequently these indices were analysed using one-way ANOVA and Turkey's multiple comparison test in Graphpad prism. Variation in all statistical tests was measured statistically different at $p < 0.05$.

3.4. Results

To differentiate seasonal bacterial symbiosis in filter-feeding Pacific oysters from a nutrient-rich habitat, Coffin Bay and a nutrient-lean habitat, Franklin Harbour across two seasons, the V1-V2 16S rDNA was characterized from 60 individuals of each site (Table 3.1). The scatter plot of length-weight ordinations of oysters from both habitats revealed that Coffin Bay summer group was comparatively smaller than winter group. However, the Franklin Harbor oyster samples were of similar size classes in both seasons (Figure 3.1), though the size classes were not considered in the study. The pattern of OTU reads of all oysters, collected from Coffin Bay and Franklin Harbor

showed a plateau for each sample, indicating maximum sequencing depth read for all samples used (Figure 3.9).

3.4.1. Global Bacterial Diversity

Ordinations of all oysters and their surrounding water samples revealed that oyster samples clustered quite independently to water samples in both habitats across seasons. Season-wise, the global bacterial community structures of oyster and water samples showed an entirely different pattern in both habitats. Despite some shared OTUs in other three groups, Franklin Harbor oyster samples in summer presented quite different patterns (Figure 3.2). Season separated on the PCO1 axis (variation explained), while habitat separated on the PCO2 axis only in summer, not in winter (variation explained) (Figure 3.3), indicating that seasonality is a more significant driver than habitat. The gut bacterial communities of oysters of Coffin Bay and Franklin Harbor showed similarity in winter rather than in summer. This cluster was then verified by PERMANOVA, suggesting significant differences in the global microbial communities between two habitats (pseudo-F = 12.491 and *p*-value = 0.0001) across two seasons (pseudo-F = 30.319 and *p*-value = 0.0001).

3.4.2. Taxonomic Profile

The microbial community composition revealed 17 bacterial phyla, which belonged to 28 classes. In Coffin Bay oysters, Mollicutes (Phylum: Tenericutes) was the dominant bacterial class (64%) in summer, while gamma-proteobacteria (33%) and Mollicutes (26%) were dominant in winter (Figure 3.4). On the other hand, the dominant class in Franklin Harbour oysters was Mollicutes (76%) in summer. However, the change of relative abundances of Mollicutes (40%) and Gamma-proteobacteria (36%) suggested a decline of Mollicutes in both habitats. The abundance of alpha-proteobacteria was

higher in the Coffin Bay water, regardless of seasons (53% in summer and 59% in winter), whereas Franklin Harbor waters represented gamma-proteobacteria in summer (66%) and alpha-proteobacteria in winter (61%) (Figure 3.4). Although Mollicutes was the dominant class in oyster gut, it was almost absent in water samples in both habitats regardless of seasons. Besides, Spirochaetia and Sericytochromatia (Cyanobacteria) were almost absent in both habitats' water samples, though it was found in oyster guts of both habitats. Our results indicate that oysters may colonize host-specific bacteria in their gut, and its abundance is season-dependent. We also studied the relative abundance of *Vibrio,* which was our *species of interest* known as a causative agent for oyster summer mortality worldwide, revealed extremely high in Franklin harbor water in summer, accounting for about 25% though its abundance was found relatively low in other three *priori groups* (Figure 3.4).

3.4.3. Alpha-diversity Measures

Though Coffin Bay and Franklin Harbor are two different habitats, we found a total of 498 and 346 common OTUs between oysters and waters of Coffin Bay and Franklin Harbor in summer (Fig 3.5A and 3.5B) and 497 and 419 OTUs in winter, respectively (Fig 3.5C and 3.5D), indicating some similarities in bacterial communities in both habitats. Moreover, the season-wise alpha-diversity measures across two habitats showed that number of bacterial species in oyster gut increased from 185 OTUs in summer to 255 OTUs in winter in Coffin Bay. This was also consistent with Franklin Harbor oysters, increasing from 135 OTUs in summer to 210 OTUs in winter (Figure 3.6A). Thus, overall, the species richness was higher in winter in both habitats. In addition, other classic diversity indices such as Species Evenness, Shannon and Simpson index was found higher in winter in Coffin Bay oysters. However, these indices were similar in both seasons in Franklin Harbor oysters (Figure 3.6B - 3.6D).

In Coffin Bay oysters, though we found that species diversity (T) increased in winter, taxonomic diversity (Delta+) was decreased in winter, and taxonomic evenness (Lambda+) was similar in both seasons. However, Franklin Harbor oysters showed an increase in both species diversity (T) and taxonomic diversity (Delta+), and a decrease in taxonomic evenness (Lambda+) in winter compared to that in summer. (Figure 3.6A, 3.6E and 3.6F). The Taxonomic distinctness measurements represented that Coffin Bay oysters were more diverse with comparatively higher Delta+ value in summer and less diverse with lower Delta+ value in winter, which was unequally weighted, less even with lower Lambda+ value, regardless of the season. On the other hand, Franklin Harbor oysters were less diverse, more evenly distributed in summer, and more diverse with less evenness in winter (Figure 3.7). The present results suggest seasonal differences in bacterial diversity across both habitats.

The bacterial diversity in oyster gut responded differently with seasonal changes in different habitats. The Coffin Bay oysters were more taxonomically diverse in summer but species were richer in winter. In contrast, the Franklin Harbor oysters were less diverse in taxonomic and species richness in summer than winter (Figure 3.8A). Finally, number of oyster gut bacterial species in both habitats was higher in winter than that in winter, indicating obvious seasonal impact on oyster gut bacterial diversity. However, the season impacts differently in taxonomic diversity and taxonomic evenness in oyster gut across two studied habitats, Coffin Bay and Franklin Harbor (Figure 3.8B).

3.5. Discussion

I found, as expected, more bacterial diversity in the oyster gut of nutrient-rich habitat (Coffin Bay) than nutrient lean habitat (Franklin Harbor) in terms of species richness, species diversity, species evenness, and Shannon and Simpson index. Mean OTUs were higher in Coffin Bay oysters than in Franklin Harbor oysters. Besides, the oysters of Coffin Bay showed a higher level of bacterial species diversity than oysters of Franklin Harbor and bacterial species were more evenly distributed in gut community of Coffin Bay than that of Franklin Harbor. The higher gut bacterial species diversity in Coffin Bay in both seasons suggests that Coffin Bay oysters are more resistant to any future environmental changes than Franklin Harbor oysters. In both habitats, a comparatively low level of bacterial diversity occurred in summer oysters than in winter. Our result was consistent with previous study where low bacterial diversity was also documented in mussel, *Mytilus coruscus* gut with increasing temperature from 27 °C to 31 °C (Li et al., 2018) and high bacterial diversity found in the digestive gland of Mania clam, *Ruditapes philippinarum* in winter (Milan et al., 2018). Some studies reported a comparatively high level of particulate matter, nitrate and sulphate in calmer sea in winter than in summer (Cliff, 1982). Thus, low nutrients at high temperatures might result in low bacterial diversity, sometimes triggering environmental stress and leading to disease caused by opportunistic pathogens and invertebrate mass mortality in summer.

The bacterial diversity in oyster gut responded quite differently with seasonal changes across two habitats. For instance, Coffin Bay oysters were more taxonomically diverse in summer but more species-rich in winter. In contrast, Franklin Harbor oysters were less diverse in taxonomic and species richness in summer than in winter. Though there were variations in the gut bacterial diversity of oysters from Coffin Bay and Franklin Harbor, a similar taxonomic group of bacteria

was characterized in oysters from both habitats. Aggregation in primer revealed a similarity in most abundant bacterial phyla and class despite its relative abundance (%) in summer and winter. The most dominant phylum found in oyster gut is Tenericutes, whereas Proteobacteria are in both habitats' water. The other abundant phylum in oyster gut was Proteobacteria, Cyanobacteria and Spirochaetes; whereas Bacteroidetes, Cyanobacteria, and Actinobacteria in both the water of both habitats. Tenericutes was also dominant in the digestive gland of eastern oyster (*Crassostrea virginca*) and Proteobacteria in sediment, which is consistent with our result (Arfken *et al*., 2017). Despite a large number of shared OTUs between oysters of two habitats and waters of two habitats in summer and winter, the nMDS plot showed a distinct clustering pattern of oyster samples to water samples in both seasons. This result indicates seasonal differences in oyster gut microbial communities from their habitats.

In the current study, Bacteroidetes and Actinobacteria were almost absent in the oyster gut, whereas Mollicutes (Tenericutes), Sericytochromatia (Cyanobacteria) and Spirochaetia were almost absent in water of both habitats indicating that host specificity in colonizing certain bacteria in oyster gut, different from habitats. Previous studies have also reported microbial communities in marine invertebrates different from their surrounding habitat (Prieur *et al*., 1990; Harris, 1993), such as in eastern oyster, *Crassostrea virginica* from two harvest areas on the coast of Maine, USA (La Valley *et al*., 2009), Mediterranean oyster, *Ostrea edulis* from their growing seawater in Mediterranean coast (Pujalte *et al*., 1999), two saltmarsh prawns, *Upogebia africana* and *Callianassa kraussi* collected from two sites of Langebaan lagoon in south Africa (Harris *et al*., 1991). Oyster-bacterial assemblages in Coffin Bay and Franklin Harbor are pretty similar. However, relative abundance of different bacteria appeared seasonally linked and showed some differences that suggest core

bacteria can maintain throughout the year and show species-specificity. Season-wise differences were also revealed in the gut and pallial fluid microbiota in eastern oysters collected from the Long Island Sound estuary (Pierce *et al.,* 2016). In addition, the nutrient availability in seawater somehow controls the summer community and winter community (Pinhassi, J., & Hagström, Å. 2000).

Our dataset indicated similarity in colonizing selective groups of bacteria in the gut of oysters from their surrounding waters of both habitats in both seasons. According to dominancy in the oyster gut, we found that Tenericutes (Mollicutes) and Proteobacteria were the most dominant phylum in summer, whereas Proteobacteria and Tenericutes in winter in both habitats. Our results were consistent with previous findings such as Mollicutes, and Proteobacteria found dominant in the intestine of the small abalone, *Haliotis diversicolor* (Huang *et al*., 2010); Proteobacteria in *Crassostrea gigas* (Fernandez-Piquer *et al*., 2012); Proteobacteria and Bacteroidetes in gastrointestinal tract of *Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea* (Trabal *et al*., 2014); Proteobacteria, Firmicutes and Tenericutes in mussel *Brachidontes* (Cleary *et al*., 2015); Proteobacteria and Tenericutes in the intestine of wild largemouth bronze gudgeon, *Coreius guichenoti* (Li *et al*., 2016); Proteobacteria and Tenericutes in clam, *Chamelea gallina* (Milan *et al*., 2019). These stable microbiomes in different bivalves, including oysters in different habitats, can be established as species-specific host-associated assemblages. Previous literature has reported that some marine invertebrates can inhabit a permanent resident microbiota in their gut such as in *Crassostrea gigas* (Hernández-Zárate & Olmos-Soto, 2006), eastern oyster *Crassostrea virginica* (La Valley *et al.*, 2009), abalone *Haliotis discus hannai* (Tanaka *et al*., 2004). This result directs specific functional roles of those bacteria, such as gut development, digestion into the oyster gut. In the present study,

the abundance of Spirochaetes was relatively low (0.5-6%) in oyster guts of both habitats. Small quantities of Spirochetes were also reported in *Crassostrea gigas* (Husmann *et al*., 2010); in a comparative study among three oyster species (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) (Trabal *et al*., 2014) and Spirochetes helps in digestion (Green and Barnes, 2010). It is previously well-established that host can selectively colonize symbiotic bacteria into their organs according to the respective physiological functions (Harris, 1993; Meisterhans *et al*., 2016; Lu *et al*., 2017). So, future functional metagenomics might reveal the link of maintaining gut bacterial communities in oysters of different habitats with their specific functions, which will, in turn, give possible insight into host-microbiota symbiosis.

In summer, we found Mollicutes, members of Tenericutes phyla, were the most predominant phylum in the oyster gut in summer and gamma-proteobacteria, members of Proteobacteria, dominant in winter Coffin Bay. Roterman *et al*. (2015) also reported gamma-proteobacteria was comparatively higher in winter in Northern Red Sea oysters *Spondylus spinous* and lower in summer. Mycoplasma belongs to Mollicutes found prevalent in oysters of both habitats in summer, with comparatively higher abundance in Franklin Harbor summer (76%) than Coffin Bay summer (64%). Mycoplasma was also dominant (80%) in the stomach microbiota of eastern oyster, *Crassostrea virginica* in Lake Caillou (King *et al*., 2012); Mussel, *Brachidontes* in Indonesian lake (Cleary *et al*., 2015); in the digestive gland of calm *Chamelea gallina* (Milan *et al*., 2019). This result suggests *Mycoplasma* spp. as a core gut microbiome and represents symbiosis with oysters. In Coffin Bay water, alpha-proteobacteria was dominant in both seasons, whereas in Franklin Harbor water, gamma-proteobacteria were dominant in summer and alpha-proteobacteria in winter. Alpha-proteobacteria

was also found prevalent in seawater of South Pacific Gyre (Yin et al., 2013) and in the mesotrophic Lake Biwa, Japan, throughout seasons (Nishimura & Nagata, 2007). We found that gamma-proteobacteria is prevalent in summer of Franklin Harbor water (66%). Previous studies reported that most members of gamma-proteobacteria are sulfer-oxidizing (Sakami *et al*., 2008; Yamamoto and Takai, 2011; Patwardhan *et al.,* 2018), and waters in mangrove areas are predominant in sulfer-oxidizing bacteria (Vethanayagam 1991; Al-Sayed *et al.,* 2005). This might be linked with our findings, as Franklin Harbor is enclosed with a large mangrove area. Therefore, it might be a reason for getting a higher abundance of gamma-proteobacteria in Franklin Harbor.

In comparison, the abundance of *Vibrio*, a gamma-proteobacteria, was also very low in oyster gut of both habitats (0.03 to 1.7%) in both seasons. Particularly, the occurrence of *Vibrio* in oyster gut is a foremost concern as it was evident as a causative agent in diseases and summer mass mortality of many bivalves, including Pacific oysters (Samain and McCombie, 2008; Garnier *et al.*, 2008; Petton *et al.*, 2015). However, a small number of *Vibrio* in healthy oyster gut is not surprising in our study, because the similar result has also been reported in many other kinds of literature, such as in digestive tract of healthy oysters, *Crassostrea gigas* (Hernández-Zárate & Olmos-Soto, 2006), Sydney rock oysters *Saccostrea glomerata* (Green and Bernes, 2010), in haemolymph and digestive glands of *Crassostrea gigas* and *Mytilus galloprovincialis* (Vezzulli *et al.,* 2018). Usually, the abundance of *Vibrios* increased in oysters at high temperatures (Deepanjali *et al*., 2005; Parveen *et al.,* 2008), which is consistent with our observation. In addition, not all *Vibrio* spp is pathogenic, and some of them are resident and beneficial in marine bivalves, such as most *Vibrio* spp., which can produce hydrolytic exoenzymes (Pujalte *et al.,* 1999).

On another particular note, our study underlined exclusively higher abundance of *Vibrio* (24.65%), a member of gamma-proteobacteria, in Franklin Harbor water in summer, though its abundance was very low in winter water of Franklin Harbor (0.09%), whereas in Coffin Bay water in both seasons, in summer (0.09%) and winter (0.47%). It is well established that *Vibrios* are widespread free-living resident bacteria in the coastal and marine waters (Thompson *et al*., 2004), and their abundances increased in seawater with increasing temperature (Vezzuli *et al*., 2010). Thus, our results are consistent with previous studies, as we found comparatively higher abundances in summer. However, our major concern is an exclusively higher abundance of *Vibrio* in Franklin Harbor water in summer (24.65%). Pinhassi and Berman (2003) found that iron-enrichment of the North Sea and East Mediterranean seawater stimulated gamma-proteobacteria such as *Vibrio* spp. Though the mangrove sediments are low-nutrient environments (Alfaro-Espinoza and Ullrich, 2015), it is rich in iron (Kristensen *et al*., 2000). The study site, Franklin Harbor, was shallow coastal water with a mangrove ecosystem. Thus, it might be enriched with iron and favoured the higher abundance of *Vibrio,* a gamma-proteobacteria) in our current study. In the mangrove ecosystem, the required nitrogen derives through nitrogen fixation (N2) via bacterial activity (Van der Valk and Attiwill, 1984). Besides, the nitrogen fixation rate found increased in summer (Vovides *et al*., 2011) and some *Vibrio* spp. such as *Vibrio aesturianus* helps in N2 fixation (Bashan and Holguin, 2002; Ravikumar *et al*., 2004). So, our findings may be co-related with the above evidence. *Vibrio* might help in nitrogen fixation in Franklin Harbor, and as the fixation rate increased in summer, we found higher abundance of *Vibrio* in summer water of Franklin Harbor. However, this assumption needs to be further addressed in future studies.

3.6. Conclusion

Taken together, our results define that oceanic nutrient based Coffin Bay oysters showed more diversity in gut bacteria than local nutrient based Franklin Harbor oysters. In the current study, the distinct microbial community in oyster gut and water revealed similarity in bacterial taxonomic groups, despite differences in relative proportion of abundances, regardless of seasons and habitats. This study confirms species-specific colonization of bacteria in oyster gut. When season changes, the gut bacteria respond differently in different habitats, suggesting both seasonal and habitat impacts in bacterial assemblages in oyster gut. This study builds a baseline of a healthy oyster aquaculture system.

3.7. References

Alfaro-Espinoza, G., & Ullrich, M. S. (2015). Bacterial N2-fixation in mangrove ecosystems: insights from a diazotroph–mangrove interaction. *Frontiers in Microbiology*, *6*, 445.

Al-Sayed, H. A., Ghanem, E. H., & Saleh, K. M. (2005). Bacterial community and some physico-chemical characteristics in a subtropical mangrove environment in Bahrain. *Marine Pollution Bulletin*, *50*(2), 147-155.

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26(1), 32-46.

Arfken, A., Song, B., Bowman, J. S., & Piehler, M. (2017). Denitrification potential of the eastern oyster microbiome using a 16S rRNA gene based metabolic inference approach. *PloS One*, 12(9).

Bashan, Y., & Holguin, G. (2002). Plant growth-promoting bacteria: a potential tool for arid mangrove reforestation. *Trees*, *16*(2), 159-166.

Bentzon-Tilia, M., Sonnenschein, E. C., & Gram, L. (2016). Monitoring and managing microbes in aquaculture–Towards a sustainable industry. *Microbial Biotechnology*, *9*(5), 576-584.

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, 27(4), 325-349.

Calbet, A., & Landry, M. R. (1999). Mesozooplankton influences on the microbial food web: direct and indirect trophic interactions in the oligotrophic open ocean. *Limnology and Oceanography*, 44, 1370-1380.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A. P., Schaumburg, F., Becker, K., Wos-Oxley, M. L., & Pieper, D. H. (2014). Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environmental Microbiology*, 16(9), 2939-2952.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., & Huttley, G. A. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5), 335.

Church, M. J. (2008). Resource control of bacterial dynamics in the sea. *Microbial Ecology of the Oceans*, *1*, 335-382.

Clarke, K. R., Gorley, R. N., Somerfield, P. J., & Warwick, R. M. (2014). Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd.

Cleary, D. F., Becking, L. E., Polónia, A. R., Freitas, R. M., & Gomes, N. C. (2015). Composition and predicted functional ecology of mussel-associated bacteria in Indonesian marine lakes. *Antonie van Leeuwenhoek*, 107(3), 821-834.

Cliff, G. (1982). Seasonal variation in the contribution of phytoplankton, bacteria, detritus and inorganic nutrients to a rocky shore ecosystem. *Transactions of the Royal Society of South Africa*, *44*(4), 523-538.

Cole J. R., Wang Q., Fish J. A., Chai B., McGarrell D. M., Sun Y., et al. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42 D633–D642.

Deepanjali, A., Kumar, H. S., Karunasagar, I., & Karunasagar, I. (2005). Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. *Applied and Environmental Microbiology*, *71*(7), 3575-3580.

Edgar R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10 996–998. 10.1038/nmeth.2604.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

Fernandez-Piquer, J., Bowman, J. P., Ross, T., & Tamplin, M. L. (2012). Molecular analysis of the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of postharvest temperature on its structure. *Journal of Applied Microbiology*, *112*(6), 1134-1143.

Fisher, M. M., Klug, J. L., Lauster, G., Newton, M., & Triplett, E. W. (2000). Effects of resources and trophic interactions on freshwater bacterioplankton diversity. *Microbial Ecology*, *40*(2), 125-138.

Fraune, S., & Bosch, T. C. (2010). Why bacteria matter in animal development and evolution. *Bioessays*, *32*(7), 571-580.

Fuhrman, J. A., Hewson, I., Schwalbach, M. S., Steele, J. A., Brown, M. V., & Naeem, S. (2006). Annually reoccurring bacterial communities are predictable from ocean conditions. *Proceedings of the National Academy of Sciences*, *103*(35), 13104-13109.

Gao, F., Tan, J., Sun, H., & Yan, J. (2014). Bacterial diversity of gut content in sea cucumber (*Apostichopus japonicus*) and its habitat surface sediment. *Journal of Ocean University of China*, *13*(2), 303-310.

Garnier, M., Labreuche, Y., & Nicolas, J. L. (2008). Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. *Systematic and Applied Microbiology*, *31*(5), 358-365.

Gaylard, S., Nelson, M., & Noble, W. (2010). Nearshore marine aquatic ecosystem condition reports: Lower Spencer Gulf Assessment Report. Environment Protection Authority, Adelaide, Australia. 102p.

Gilbert, J. A., Field, D., Swift, P., Newbold, L., Oliver, A., Smyth, T., Somerfield, P.J., Huse, S. & Joint, I. (2009). The seasonal structure of microbial communities in the Western English Channel. *Environmental Microbiology*, *11*(12), 3132-3139.

Gilbert, J. A., Steele, J. A., Caporaso, J. G., Steinbrück, L., Reeder, J., Temperton, B., Huse, S., McHardy, A. C., Knight, R., Joint, I. & Somerfield, P. (2012).

Defining seasonal marine microbial community dynamics. *The ISME Journal*, *6*(2), 298-308.

Green, T. J., & Barnes, A. C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology*, 109(2), 613-622.

Harris, J. M. (1993). The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. *Microbial Ecology*, *25*(3), 195-231.

Harris, J. M., Seiderer, L. J., & Lucas, M. I. (1991). Gut microflora of two saltmarsh detritivore thalassinid prawns, *Upogebia africana* and *Callianassa kraussi*. *Microbial Ecology*, *21*(1), 277-296.

Hernández-Zárate, G., & Olmos-Soto, J. (2006). Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. *Journal of Applied Microbiology*, *100*(4), 664-672.

Huang, Z. B., Guo, F., Zhao, J., Li, W. D., & Ke, C. H. (2010). Molecular analysis of the intestinal bacterial flora in cage-cultured adult small abalone, *Haliotis diversicolor*. *Aquaculture Research*, *41*(11), e760-e769.

Husmann, G., Gerdts, G., & Wichels, A. (2010). Spirochetes in crystalline styles of marine bivalves: Group-specific PCR detection and 16S rRNA sequence analysis. *Journal of Shellfish Research*, *29*(4), 1069-1075.

Kämpf, J., Doubell, M., Griffin, D., Matthews, R. L., & Ward, T. M. (2004). Evidence of a large seasonal coastal upwelling system along the southern shelf of Australia. *Geophysical Research Letters*, *31*(9).

Kent, A. D., Jones, S. E., Lauster, G. H., Graham, J. M., Newton, R. J., & McMahon, K. D. (2006). Experimental manipulations of microbial food web interactions

in a humic lake: shifting biological drivers of bacterial community structure. *Environmental Microbiology*, *8*(8), 1448-1459.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One*, *7*(12), e51475.

Klaus, J. S., Frias-Lopez, J., Bonheyo, G. T., Heikoop, J. M., & Fouke, B. W. (2005). Bacterial communities inhabiting the healthy tissues of two Caribbean reef corals: interspecific and spatial variation. *Coral Reefs*, *24*(1), 129-137.

Kristensen, E., Andersen, F. Ø., Holmboe, N., Holmer, M., & Thongtham, N. (2000). Carbon and nitrogen mineralization in sediments of the Bangrong mangrove area, Phuket, Thailand. *Aquatic Microbial Ecology*, *22*(2), 199-213.

LaValley KJ, Jones LA, Gomez-Chiarri M, Dealteris J & Rice M (2009) Bacterial community profiling of the eastern oyster (*Crassostrea virginica*): comparison of culture-dependent and culture-independent outcomes. *Journal of Shellfish Research*, 28 (4): 827–835.

Legrand, T. P., Catalano, S. R., Wos-Oxley, M. L., Stephens, F., Landos, M., Bansemer, M. S., Stone, D. A. J., Qin, J. G., & Oxley, A. (2018). The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. *Frontiers in Microbiology*, 8, 2664.

Li, T., Long, M., Ji, C., Shen, Z., Gatesoupe, F. J., Zhang, X., ... & Li, A. (2016). Alterations of the gut microbiome of largemouth bronze gudgeon (*Coreius guichenoti*) suffering from furunculosis. *Scientific Reports*, *6*(1), 1-9.

Li, Y. F., Yang, N., Liang, X., Yoshida, A., Osatomi, K., Power, D., Batista, F. M., & Yang, J. L. (2018). Elevated seawater temperatures decrease microbial diversity in the gut of *Mytilus coruscus*. *Frontiers in Physiology*, *9*, 839.

Lu, G., Wang, F., Yu, Z., Lu, M., Wang, Y., Liu, C., Xue, Z., Wu, Y., Wang, L. & Song, L. (2017). Bacterial communities in gills and intestines of yesso scallop (*Patinopecten yessoensis*) and its habitat waters in Changhai (Dalian, China). *Invertebrate Survival Journal*, *14*(1), 340-351.

Luo, P., Hu, C. Q., Zhang, L. P., & Ren, C. H. (2009). PCR-DGGE analysis of bacterial communities in marine *Litopenaeus vannamei* culture system. *Journal of Fishery Sciences of China*, *16*(1), 31-38.

Meisterhans, G., Raymond, N., Girault, E., Lambert, C., Bourrasseau, L., De Montaudouin, X., Garabetian, F. & Jude-Lemeilleur, F. (2016). Structure of Manila clam (*Ruditapes philippinarum*) microbiota at the organ scale in contrasting sets of individuals. *Microbial Ecology*, *71*(1), 194-206.

Milan, M., Carraro, L., Fariselli, P., Martino, M. E., Cavalieri, D., Vitali, F., Boffo, L., Patarnello, T., Bargelloni, L. & Cardazzo, B. (2018). Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. *Aquatic Toxicology*, *194*, 195-207.

Milan, M., Smits, M., Dalla Rovere, G., Iori, S., Zampieri, A., Carraro, L., Martino, C., Papetti, C., Ianni, A., Ferri, N. & Martino, G. (2019). Host-microbiota interactions shed light on mortality events in the striped venus clam *Chamelea gallina*. *Molecular Ecology*, *28*(19), 4486-4499.

Moriarty, D. J. (1997). The role of microorganisms in aquaculture ponds. *Aquaculture*, *151*(1-4), 333-349.

Moriarty, D. J. W. (1990). Interactions of microorganisms and aquatic animals, particularly the nutritional role of the gut flora. *Microbiology in Poecilotherms*, 217-222.

Morris, R. M., Vergin, K. L., Cho, J. C., Rappé, M. S., Carlson, C. A., & Giovannoni, S. J. (2005). Temporal and spatial response of bacterioplankton lineages to annual convective overturn at the Bermuda Atlantic Time-series Study site. *Limnology and Oceanography*, *50*(5), 1687-1696.

Mouchka, M. E., Hewson, I., & Harvell, C. D. (2010). Coral-associated bacterial assemblages: current knowledge and the potential for climate-driven impacts. *Integrative and Comparative Biology*, *50*(4), pp.662-674.

Nishimura, Y., & Nagata, T. (2007). Alphaproteobacterial dominance in a large mesotrophic lake (Lake Biwa, Japan). *Aquatic Microbial Ecology*, *48*(3), 231-240.

Paillard, C., Le Roux, F., & Borrego, J. J. (2004). Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. *Aquatic Living Resources*, 17, 477-498.

Parveen, S., Hettiarachchi, K. A., Bowers, J. C., Jones, J. L., Tamplin, M. L., McKay, R., Beatty, W., Brohawn, K., DaSilva, L. V. & DePaola, A. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology*, *128*(2), 354-361.

Patwardhan, S., Foustoukos, D. I., Giovannelli, D., Yücel, M., & Vetriani, C. (2018). Ecological succession of sulfur-oxidizing epsilon-and gammaproteobacteria during colonization of a shallow-water gas vent. *Frontiers in Microbiology*, *9*, 2970.

Pernthaler, J., Zöllner, E., Warnecke, F., & Jürgens, K. (2004). Bloom of filamentous bacteria in a mesotrophic lake: identity and potential controlling mechanism. *Applied and Environmental Microbiology*, *70*(10), 6272-6281.

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., & Le Roux, F. (2015). *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Frontiers in Microbiology*, *6*, 686.

Pierce, M. L., Ward, J. E., Holohan, B. A., Zhao, X., & Hicks, R. E. (2016). The influence of site and season on the gut and pallial fluid microbial communities of the eastern oyster, *Crassostrea virginica* (Bivalvia, Ostreidae): community-level physiological profiling and genetic structure. *Hydrobiologia*, *765*(1), 97-113.

Pinhassi, J., & Berman, T. (2003). Differential growth response of colony-forming α- and γ-proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. *Applied and Environmental Microbiology*, *69*(1), 199-211.

Pinhassi, J., & Hagström, Å. (2000). Seasonal succession in marine bacterioplankton. *Aquatic Microbial Ecology*, *21*(3), 245-256.

Pinhassi, J., Sala, M. M., Havskum, H., Peters, F., Guadayol, O., Malits, A., & Marrasé, C. (2004). Changes in bacterioplankton composition under different phytoplankton regimens. *Applied and Environmental Microbiology*, 70, 6753-6766.

Prieur, D., Mevel, G., Nicolas, J. L., Plusquellec, A., & Vigneulle, M. (1990). Interactions between bivalve molluscs and bacteria in the marine

environment. *Oceanography and Marine Biology: Annual Review*, *28*, 277-352.

Pujalte, M. J., Ortigosa, M., Macián, M. C., & Garay, E. (1999). Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. *International Microbiology*, *2*(4), 259-266.

Ravikumar, S., Kathiresan, K., Ignatiammal, S. T. M., Selvam, M. B., & Shanthy, S. (2004). Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers. *Journal of Experimental Marine Biology and Ecology*, *312*(1), 5-17.

Roterman, Y. R., Benayahu, Y., Reshef, L., & Gophna, U. (2015). The gill microbiota of invasive and indigenous *Spondylus* oysters from the Mediterranean Sea and northern Red Sea. *Environmental Microbiology Reports*, *7*(6), 860-867.

Sakami, T., Fujioka, Y., & Shimoda, T. (2008). Comparison of microbial community structures in intensive and extensive shrimp culture ponds and a mangrove area in Thailand. *Fisheries Science*, *74*(4), 889-898.

Samain, J. F., & McCombie, H. (2008). *Summer mortality of Pacific oyster Crassostrea gigas: the Morest project*. Editions Quae.

Sweet, M. J., Croquer, A., & Bythell, J. C. (2011). Bacterial assemblages differ between compartments within the coral holobiont. *Coral Reefs*, *30*(1), 39-52.

Tanaka, R., Ootsubo, M., Sawabe, T., Ezura, Y., & Tajima, K. (2004). Biodiversity and in situ abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture*, *241*(1-4), 453-463.

The draft aquaculture (zones – Franklin Harbor) policy 2015. 63p. https://pir.sa.gov.au/_data/assets/pdf_file/0004/247234/Franklin_Harbor_Draft_Policy_Report.pdf

Thompson, F. L., & Swings, J. (2006). Taxonomy of the vibrios. *The Biology of Vibrios*, 27-43.

Thompson, F. L., Iida, T., & Swings, J. (2004). Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews*, *68*(3), 403-431.

Trabal Fernández, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Ascencio-Valle, F., & Romero, J. (2014). Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiology Ecology*, *88*(1), 69-83.

Van der Valk, A. G., & Attiwill, P. M. (1984). Decomposition of leaf and root litter of *Avicennia marina* at Westernport Bay, Victoria, Australia. *Aquatic Botany*, *18*(3), 205-221.

Vethanayagam, R. R. (1991). Purple photosynthetic bacteria from a tropical mangrove environment. *Marine Biology*, *110*(1), 161-163.

Vezzulli, L., Previati, M., Pruzzo, C., Marchese, A., Bourne, D. G., Cerrano, C., & VibrioSea Consortium. (2010). *Vibrio* infections triggering mass mortality events in a warming Mediterranean Sea. *Environmental Microbiology*, *12*(7), 2007-2019.

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L., & Pruzzo, C. (2018). Comparative 16SrDNA gene-based microbiota profiles of the Pacific oyster

(*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microbial Ecology*, 75(2), 495-504.

Vovides, A. G., Bashan, Y., López-Portillo, J. A., & Guevara, R. (2011). Nitrogen fixation in preserved, reforested, naturally regenerated and impaired mangroves as an indicator of functional restoration in mangroves in an arid region of Mexico. *Restoration Ecology*, *19*(201), 236-244.

Wang, L., Zhao, X., Xu, H., Bao, X., Liu, X., Chang, Y., & Ding, J. (2018). Characterization of the bacterial community in different parts of the gut of sea cucumber (*Apostichopus Japonicus*) and its variation during gut regeneration. *Aquaculture Research, 49*(5), 1987-1996.

Ward, T. M., Mcleay, L. J., Dimmlich, W. F., Rogers, P. J., McClatchie, S. A. M., Matthews, R., Kaempf, J. & Van Ruth, P. D. (2006). Pelagic ecology of a northern boundary current system: effects of upwelling on the production and distribution of sardine (*Sardinops sagax*), anchovy (*Engraulis australis*) and southern bluefin tuna (*Thunnus maccoyii*) in the Great Australian Bight. *Fisheries Oceanography, 15*(3), 191-207.

Webster, N. S., Cobb, R. E., & Negri, A. P. (2008). Temperature thresholds for bacterial symbiosis with a sponge. *The ISME Journal*, 2, 830-842.

Yamamoto, M., & Takai, K. (2011). Sulfur metabolisms in epsilon-and gamma-Proteobacteria in deep-sea hydrothermal fields. *Frontiers in Microbiology, 2*, 192.

Yin, Q., Fu, B., Li, B., Shi, X., Inagaki, F., & Zhang, X. H. (2013). Spatial variations in microbial community composition in surface seawater from the ultra-oligotrophic center to rim of the South Pacific Gyre. *PLoS One, 8*(2), e55148.

Yuan, X., He, L., Yin, K., Pan, G., & Harrison, P. J. (2011). Bacterial distribution and nutrient limitation in relation to different water masses in the coastal and northwestern South China Sea in late summer. *Continental Shelf Research*, *31*(11), 1214-1223.

Yuan, X., Yin, K., Harrison, P. J., Cai, W. J., He, L., & Xu, J. (2010). Bacterial production and respiration in subtropical Hong Kong waters: influence of the Pearl River discharge and sewage effluent. *Aquatic Microbial Ecology*, *58*(2), 167-179.

Yuan, X., Yin, K., Harrison, P. J., Cai, W. J., He, L., & Xu, J. (2010). Bacterial production and respiration in subtropical Hong Kong waters: influence of the Pearl River discharge and sewage effluent. *Aquatic Microbial Ecology*, *58*(2), 167-179.

Yuan, X., Yin, K., Harrison, P. J., He, L., & Xu, J. (2011). Variations in apparent oxygen utilization and effects of P addition on bacterial respiration in subtropical Hong Kong waters. *Estuaries and Coasts*, *34*(3), 536-543.

Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*, 30(5), 614-620.

Zhang, M., Sun, Y., Chen, L., Cai, C., Qiao, F., Du, Z., & Li, E. (2016). Symbiotic bacteria in gills and guts of Chinese mitten crab (*Eriocheir sinensis*) differ from the free-living bacteria in water. *PloS One*, *11*(1), e0148135.

Zhou, Z., Liu, Y., Shi, P., He, S., Yao, B., & Ringø, E. (2009). Molecular characterization of the autochthonous microbiota in the gastrointestinal tract of adult yellow grouper (*Epinephelus awoara*) cultured in cages. *Aquaculture*, *286*(3-4), 184-189.

Table 3.1. Experimental design and characteristics of sampling habitats Coffin Bay and Franklin Harbor.

Habitat	Coffin Bay		Franklin Harbor	
Season	Summer (Feb)	Winter (Aug)	Summer (Feb)	Winter (Aug)
Oysters (No.)	30	30	30	30
Water samples (2 L)	3	3	3	3
Temperature (°C)	22	12.6	23.2	12.8
Salinity (‰)	38.7	36.4	39	36.9
DO (mg/L)	6.27	7.83	6.08	8.1
pH	8.68	8.33	8.7	8.49

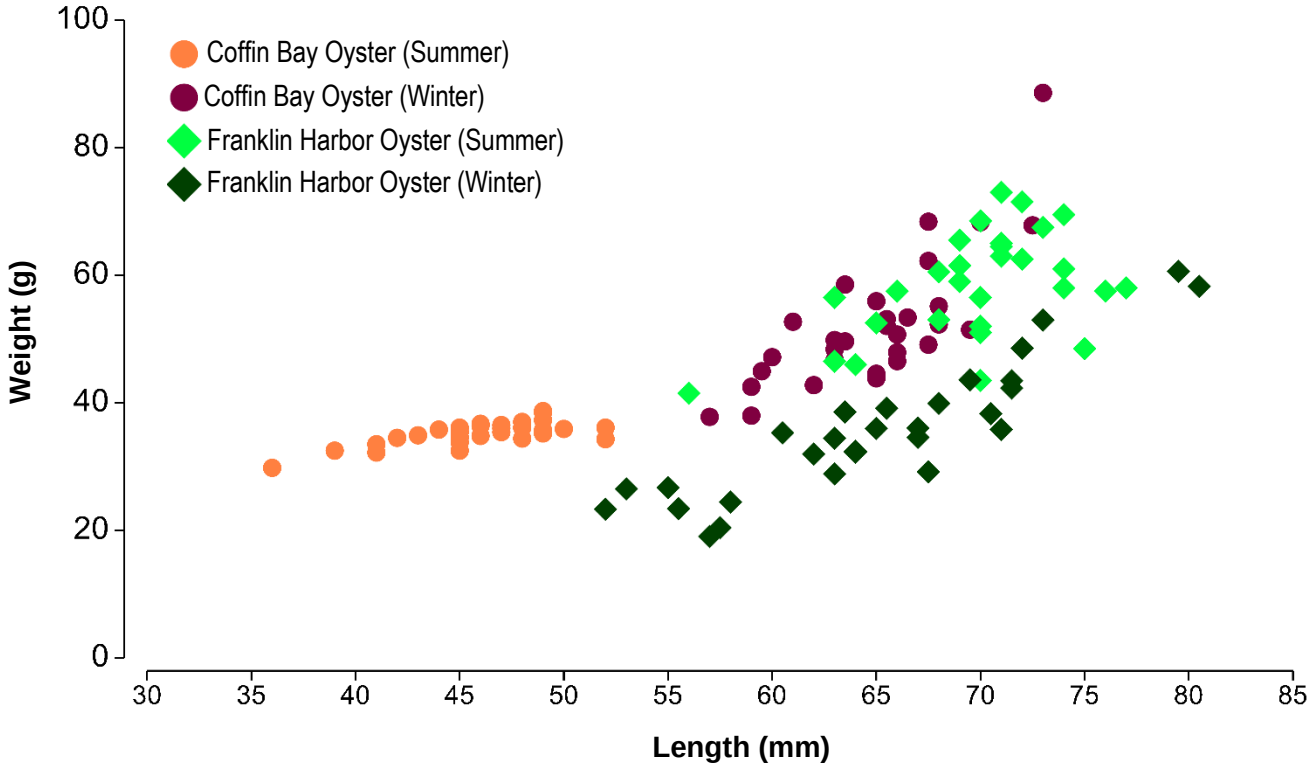


Figure 3.1. Scatter plot displaying the length-weight ordination of oysters collected from Coffin Bay and Franklin Harbor in summer and winter.

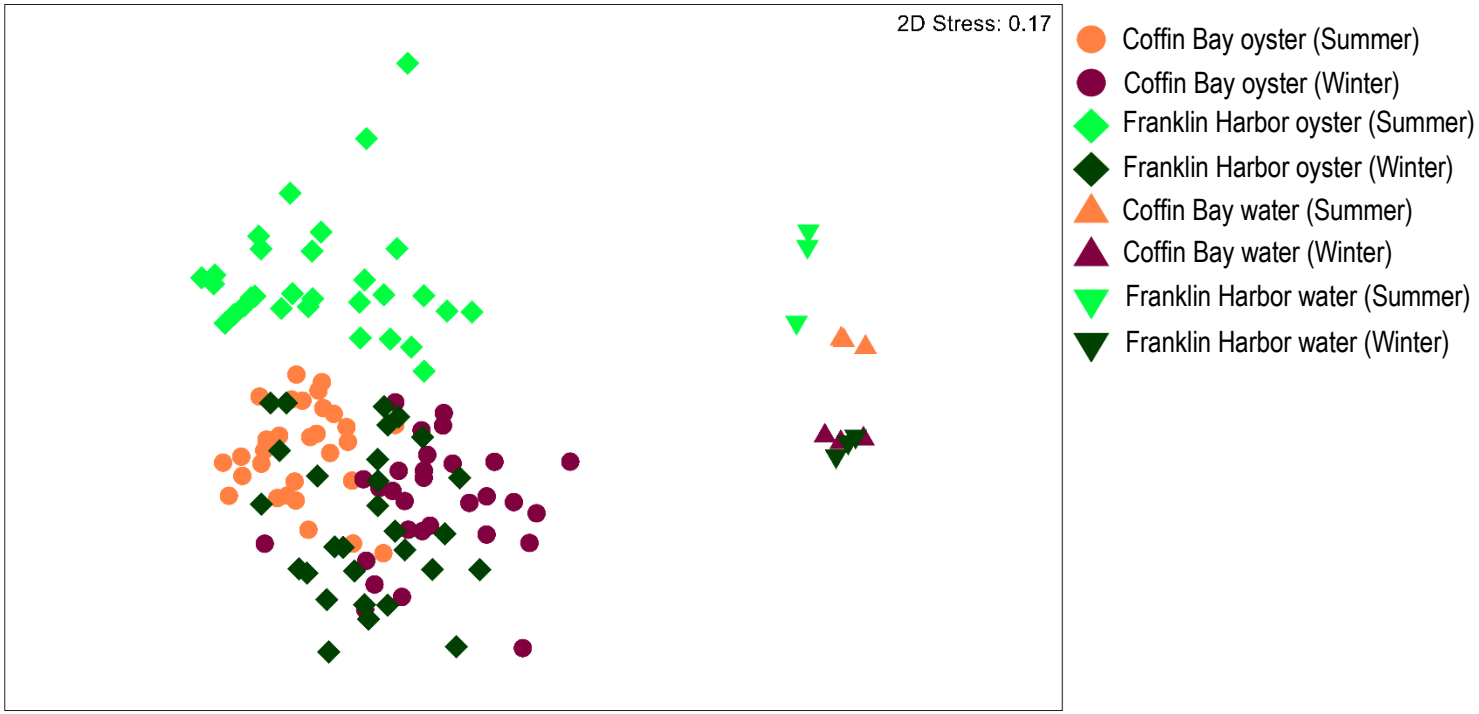


Figure 3.2. Ordination plot displaying season-wise global bacterial communities of the oyster gut and water samples collected from Coffin Bay and Franklin Harbor in summer and winter, as assessed by non-metric multi-dimensional scaling (nMDS) using Bray-Curtis Similarity.

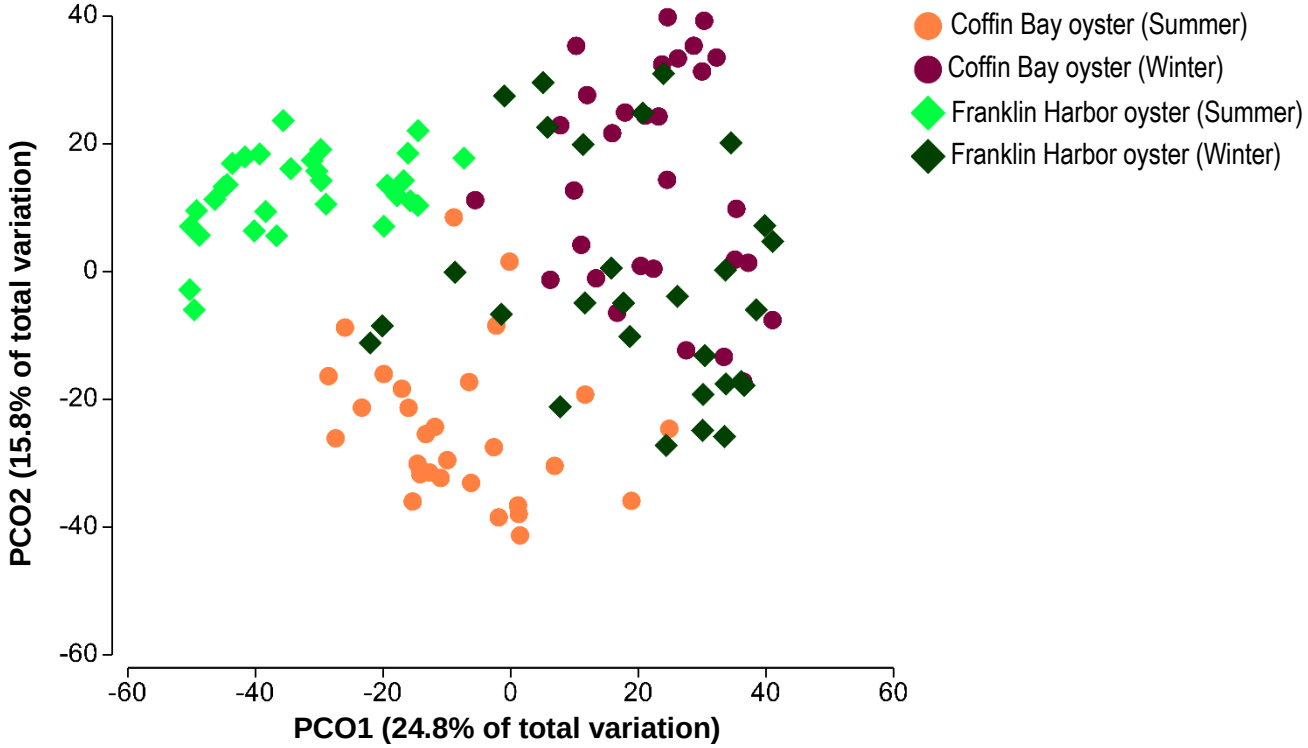


Figure 3.3. Ordination plot showing the similarities in the gut bacterial communities of oysters collected from different habitats in summer and winter, as assessed by Principal Co-ordinates Analysis (PCoA).

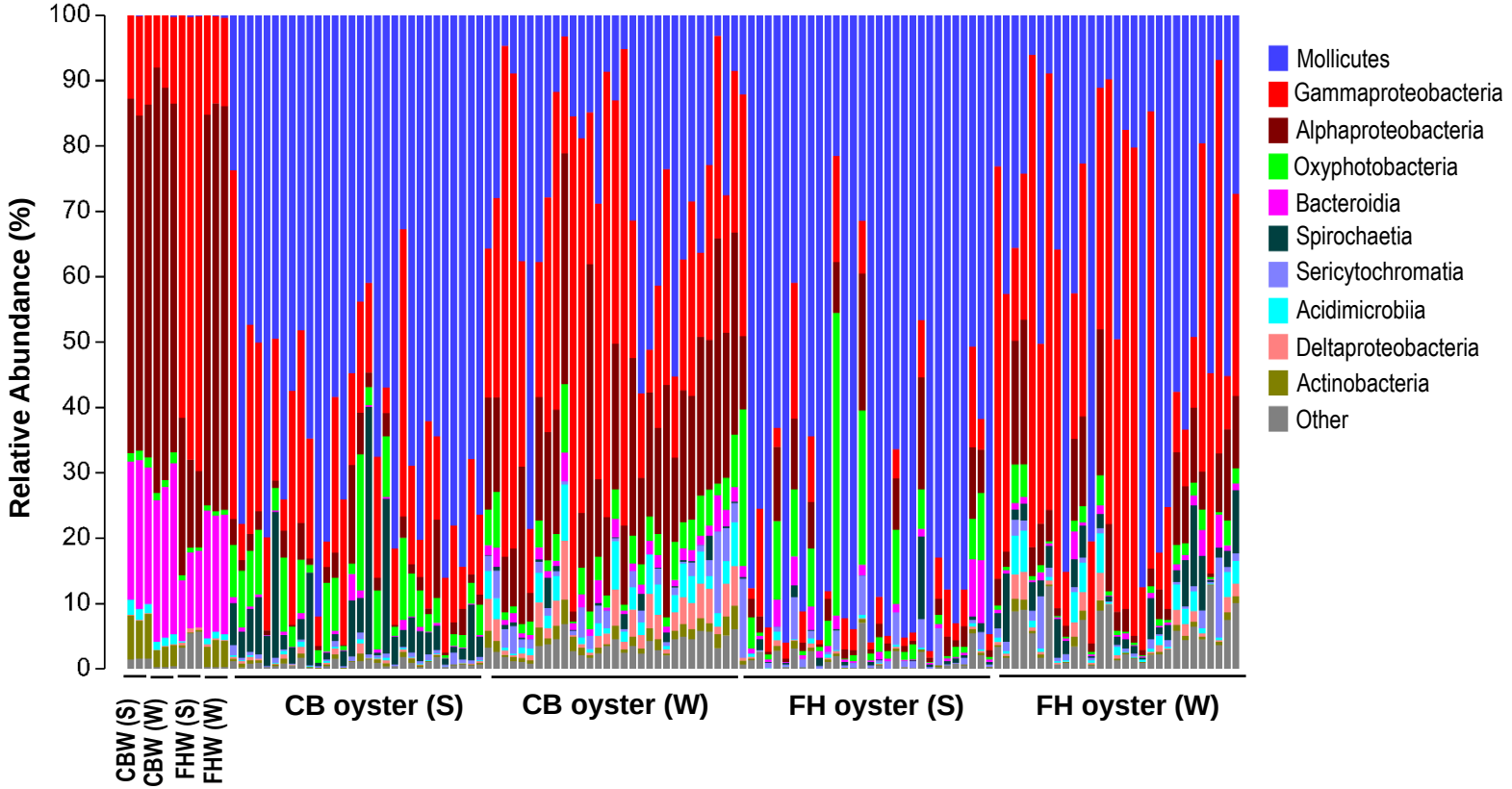


Figure 3.4. Relative abundance (%) of bacterial classes in oyster gut and water samples in Coffin Bay and Franklin Harbor. CWW(S) = Coffin Bay Water Summer, CBW(W) = Coffin Bay Water Winter, FHW(S) = Franklin Harbor Water Summer, FHW(W) = Franklin Harbor Water Winter, CB = Coffin Bay, FH = Franklin Harbor, S = Summer and W = Winter.

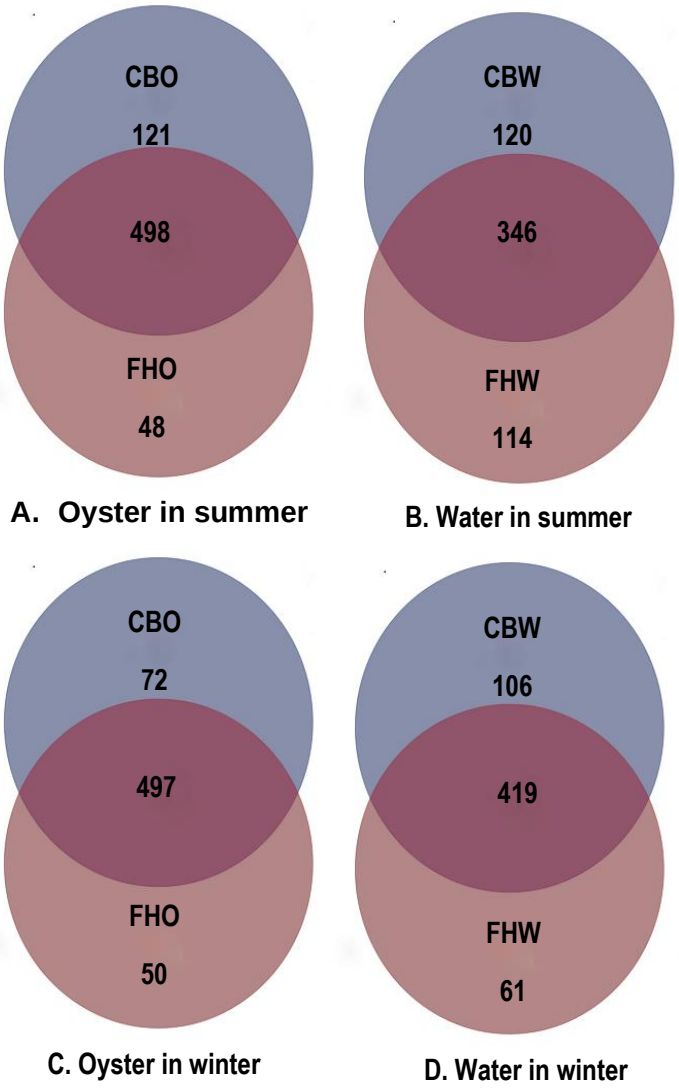


Figure 3.5. Venn diagrams show the distribution of unique and common OTUs in the oyster gut and water samples of both habitats in summer and winter. **A.** Coffin Bay oyster (CBO) vs Franklin Harbor oyster (FHO) in summer, **B.** Coffin Bay water (CBW) vs Franklin Harbor water (FHW) in summer, **C.** Coffin Bay oyster (CBO) vs Franklin Harbor oyster (FHO) in winter, **D.** Coffin Bay water (CBW) vs Franklin Harbor water (FHW) in winter.

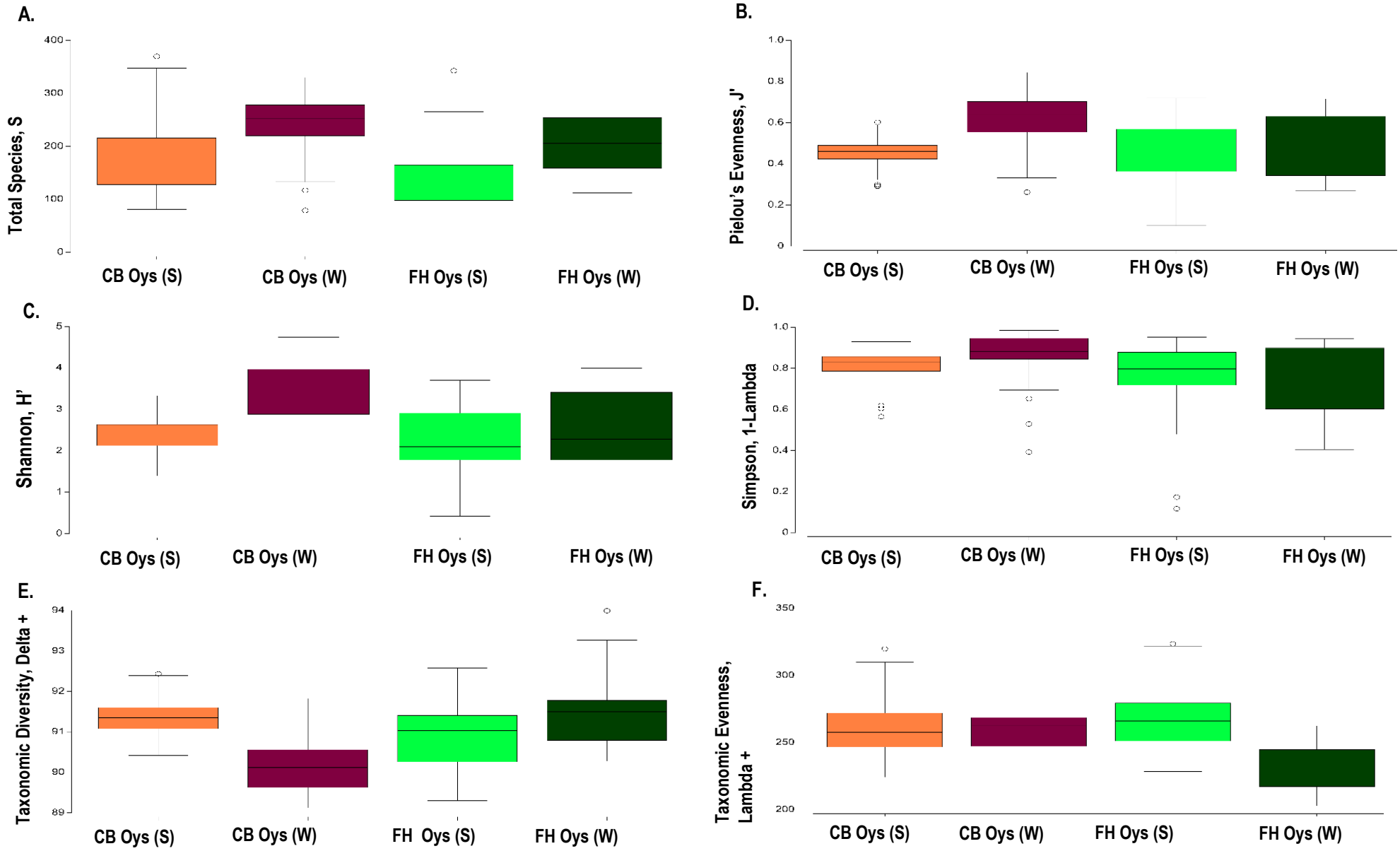

A.
Total Species, S
400
300
200
100
0
CB Oys (S)
CB Oys (W)
FH Oys (S)
FH Oys (W)
B.
Pielou's Evenness, J'
1.0
0.8
0.6
0.4
0.2
0
CB Oys (S)
CB Oys (W)
FH Oys (S)
FH Oys (W)
C.
Shannon, H'
5
4
3
2
1
0
CB Oys (S)
CB Oys (W)
FH Oys (S)
FH Oys (W)
D.
Simpson, 1-Lambda
1.0
0.8
0.6
0.4
0.2
0
CB Oys (S)
CB Oys (W)
FH Oys (S)
FH Oys (W)
E.
Taxonomic Diversity, Delta +
94
93
92
91
90
89
CB Oys (S)
CB Oys (W)
FH Oys (S)
FH Oys (W)
F.
Taxonomic Evenness, Lambda +
350
300
250
200
CB Oys (S)
CB Oys (W)
FH Oys (S)
FH Oys (W)

Figure 3.6. Season-wise diversity indices in oyster guts, collected from Coffin Bay and Franklin Harbor, **A.** Species richness (S), **B.** Pielou's evenness (J), **C.** Shannon diversity (H), **D.** Simpson diversity (1-Lambda), **E.** Taxonomic diversity (Delta+) and **F.** Taxonomic evenness (Lambda+). CB Oys (S)=Coffin Bay oyster in summer, CB Oys (W)=Coffin Bay oyster in winter, FH Oys (S)=Franklin Harbor oyster in summer and FH Oys (W)=Franklin Harbor oyster in winter. The significance level across different seasons was verified by Ordinary one-way ANOVA where Alpha set at 0.05. The box plot displayed the five-number summary data values such as the minimum, first quartile, median, third quartile, and maximum, with some biological outliers (°).

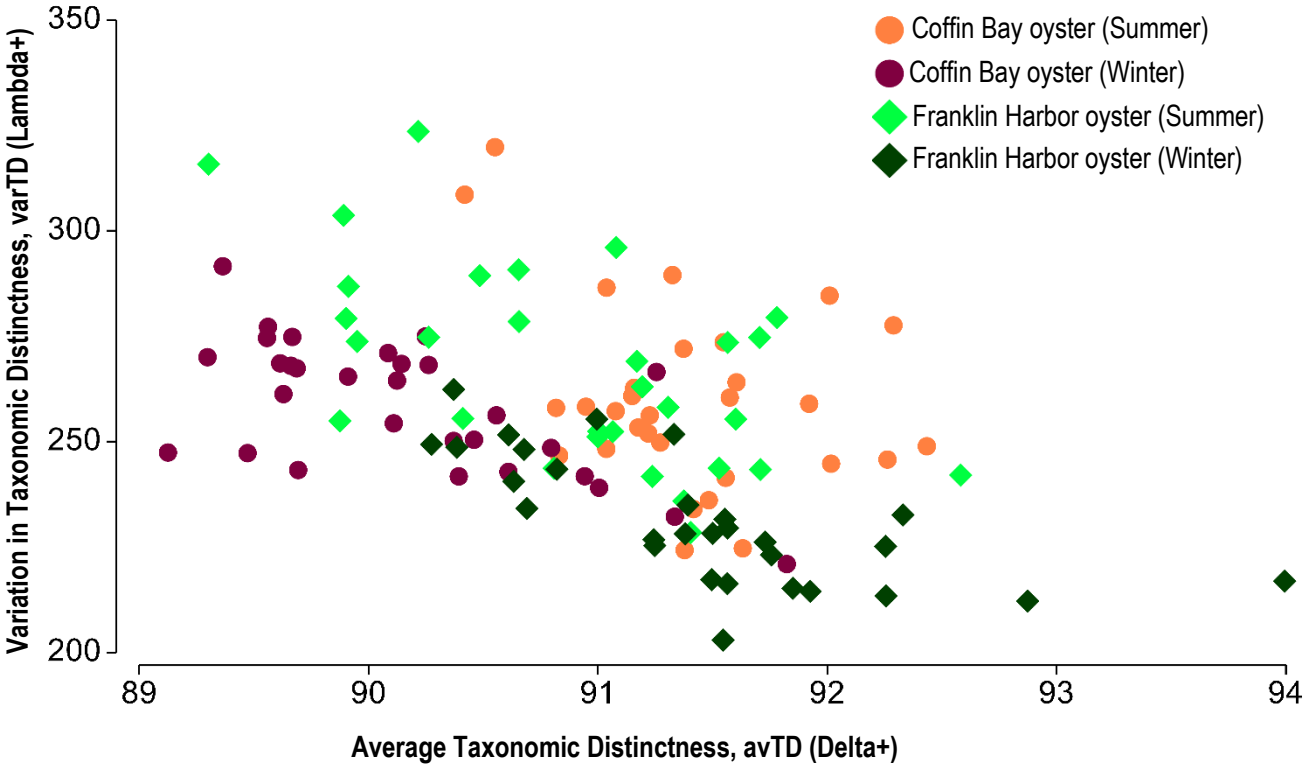
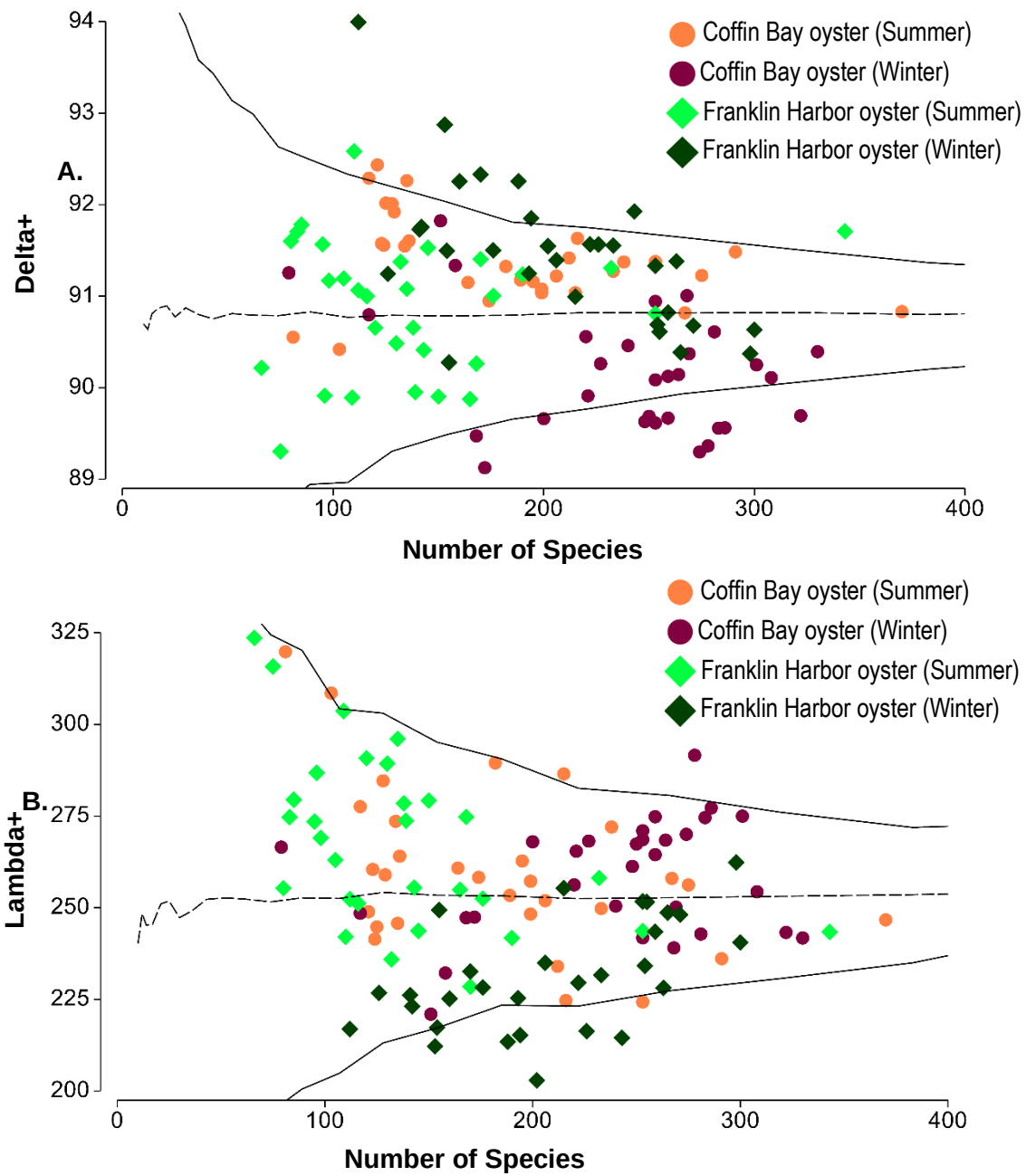


Figure 3.7. Scatter plot representing the Taxonomic distinctness (TD) measures such as the average taxonomic distinctness (avTD, delta+) and variation in taxonomic distinctness (varTD, lambda+) in the gut bacterial communities



in oyster, collected from Coffin Bay and Franklin Harbor.

Figure 3.8. Funnel plots displaying the pattern of seasonal changes in the gut bacterial Species diversity with **A.** Taxonomic diversity (Delta+), and **B.** Taxonomic evenness (Lambda+) in oysters of both habitats. Average taxonomic distinctness (avTD, delta+) and variation in taxonomic distinctness (varTD, lambda+) were plotted against species (OTU) richness.

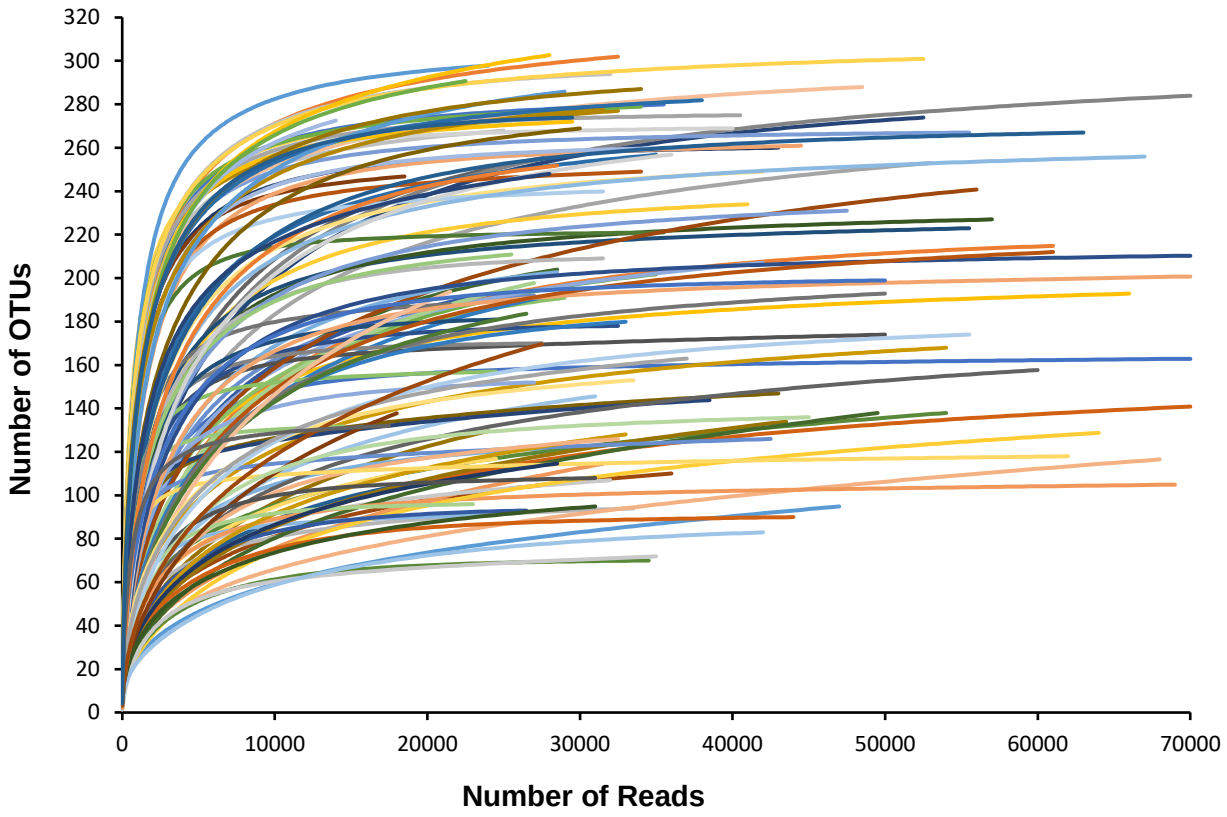


Figure 3.9. Pattern of OTU reads of oyster samples collected from Coffin Bay and Franklin Harbor.

CHAPTER 4

Seasonal pattern of bacterial community composition in Pacific oyster *Crassostrea gigas* gut and the environment

Highlight

1. The bacterial composition and abundance change with season in oyster gut and environment.
2. The bacterial diversity differed between one winter and the next.
3. Tenericutes were prevalent in the oyster gut in all seasons, except for winter, whereas Proteobacteria were prevalent in seawater all year around.
4. *Vibrio* abundance was very low in the oyster gut and seawater throughout the year.
5. Spirochaetes abundance was high in the oyster gut in summer.

4.1. Abstract

Microbial symbiosis between a bivalve and the marine environment depends on the dynamics of intrinsic factors in the host and extrinsic factors in the environment. However, little is known about the long-term change over seasons in the microbial community between marine bivalves and the environment. This study explores seasonal dynamics in oyster gut microbiota and surrounding environments. The oysters and the ambient seawater in a commercial oyster culture region in Coffin Bay, South Australia, were seasonally collected from one winter to the next. The microbial community was characterized using 16S rDNA sequencing of the V1–V2 region through Illumina platform. The seasonal change significantly impacted microbial community in oyster gut and environment, and revealed differences in the bacterial composition and relative abundance of oyster gut and seawater. Tenericutes were prevalent in oyster gut in all seasons except for winter, whereas Proteobacteria were prevalent in seawater all year round. Interestingly, microbial diversity differed between one winter and the next. Mollicutes (Phylum: Tenericutes) was the prevalent bacterial phylum in oyster gut throughout the year, indicating the persistency as permanent residential microbes in the host regardless of seasonal change. The abundance of other bacterial groups such as Proteobacteria, Cyanobacteria, Actinobacteria, Bacteroidetes, Spirochaetes was low and acted as transient microbes in oyster gut. As a result, Tenericutes could out-compete with other microbial taxa and become dominant in oyster gut. This study characterizes resident and transient microbiota communities in oyster gut. Understanding microbial community in the oyster gut and environment will help identify the occurrence of opportunistic pathogens in the ecosystem.

Keywords: Seasonal variation, gut microbiota, Pacific oyster *Crassostrea gigas*.

4.2. Introduction

Multiple factors are involved in shaping microbial communities in any environment. Variability of physical and chemical parameters represents habitat heterogeneity and influences the microbial community structure of an ecosystem. The host bacteria symbiosis in the marine environment found dependent on multiple factors that influence the establishment of microbial communities (Cavanaugh, 1994). However, it is not easy to consider all the factors involved concurrently to conclude due to the robustness of the information and intricate ways of their interactions in nature. Even though the effect of separate drivers cannot be isolated from each other, the relative influence of a couple of drivers can be pooled to explain such interactions. However, there is still a lack of information on microbiota in different physiological and environmental conditions. By investigating seasonal variation, this study identifies the bacterial community structure in the environment and the Pacific oyster gut and explains microbial symbiosis in marine bivalves.

Marine bivalves, including Pacific oyster *Crassostrea gigas*, are important in commercial aquaculture. The global aquaculture production of bivalves has increased over ten-fold in the past 40 years, from 1.2 million tonnes in 1974 to 13.5 million tonnes in 2015, and this increasing trend is likely to continue in the coming decade (Wijsman *et al*., 2019). Oysters are an economically important species that contributes to 33% of global bivalve production. In addition to the critical contribution to aquaculture, bivalves are ecologically important to maintain ecosystem functions such as nutrient cycling, trophic interactions in food webs, and reef habitat for other marine animals (Vaughn and Hoellein, 2018). As suspension feeders, bivalves can filter various seston particles and select food particles from their surrounding environment.

The selection efficiency depends on the particle size and its availability in the environment across seasons (Ward and Shumway, 2004). In a recent study, Rahman *et al.* (2020) reported the seasonal differences in the filtering capacity of oyster *Crassostrea gigas*, mussel *Mytilus galloprovincialis*, and cockle *Katelysia rhytiphora*. These authors found that oysters and mussels share large food particles (>8 µm), but mussels can filter even smaller food particles (<5 µm). Filtration rate and the filtration capacity in bivalves vary with seasonal temperature (Specht, J.A., and Fuchs, H.L., 2018), seston size (Rosa *et al.*, 2015), food particle density (Joyce *et al*., 2019) and structure of the filtering apparatus in bivalves (Hawkins *et al*., 1998). The typical pH of the bivalve gut is acidic (Owens, 1974), e.g., 5.5-6.9 in oyster guts (Morton, 1983). Microbes have specific functions related to survival in marine invertebrates in a specific environment. For example, chemoautotrophic bacteria and marine invertebrates have a mutualistic relation to access nutrition in deep-sea hydrothermal vents (Cavanaugh, 1983; Stein *et al.*, 1988). Herbivorous gastropods have polymer-degrading gut bacteria to support their nutritional requirement from wood materials (Zbinden *et al*., 2010).

The surrounding water can influence the bacterial composition in the oyster gut. The bacterial population of the marine environment fluctuates in different seasons (Gilbert *et al*., 2012). The pathogenicity of some bacteria is linked to season, particularly with the seasonal fluctuations of temperature. Microbes in bivalves have received considerable attention due to disease outbreaks of pathogenic microbes (Paillard *et al*., 2004; Travers *et al.*, 2015). A seasonal trend of proliferation and transmission of the population exists in many bacteria genera. For example, bacteria of the genus *Vibrio* can provoke severe mortality in many bivalves, including Pacific oysters, and have higher pathogenicity during summer (Wendling *et al*., 2014).

Pacific oysters can be found in different habitats globally due to translocation for aquaculture and understanding the seasonality of oyster gut microbes is important to reveal the role of microbes in other bivalves.

The Eyre Peninsula is the premium oyster growing area in South Australia. Among the Eyre oyster communities, Coffin Bay is well-known for its premium quality taste of oysters. It has contributed to local revenue through oyster farming in the Southern Australian economy. Coffin Bay is an inverse estuary of inter-connected bays with long, narrow marine inlets and tidally flushed year-round (Kämpf, J., 2014; and Kämpf, J., and Ellis, H., 2015). There is a shift in the ecosystem process depending on seasonal variation, and the seasonal succession of phytoplankton is a typical episode in marine environment. It is hypothesized that bacterial community composition differs seasonally in oyster gut and environment. Based on this hypothesis, this study aims to determine bacterial community composition and identify relative abundance of bacteria in oyster gut based on seasonal sampling year-round. Our main questions are to understand microbiomes residing in the gut and environment and track the changes in bacterial composition with season. Another question is to understand if there are any opportunistic pathogenic bacteria in the studied environment such as *Vibrio aestuarianus*, the causative agent of Pacific Oyster Mortality Syndrome (POMS).

4.3. Materials and Methods

4.3.1. Experimental Design, Sampling and Sample preparation

To characterize the seasonal variations, Pacific oysters and seawaters were sampled in an annual cycle in the Southern hemisphere from June 2016 to August 2017 (the first winter, labelled as “winter_trial” to the other winter, labelled as “winter”) from a commercial oyster farm in Coffin Bay, South Australia. The collected oysters were

likely to represent a mix of cohorts, as the commercial farmers were usually just graded market size oysters and kept together in a basket for sale, from where we collected samples for the present study. The effect of size class and cohort info was not considered in the study. A total of 30 Pacific oysters and three replicates of seawater samples (2L each) were collected in each season (Table 4.1). Upon collection, samples were immediately stored at 4^{o}C and then brought to the laboratory of Lincoln Marine Science Centre, Coffin Bay, for further processing. Immediately after arrival at the lab, oysters were cleaned and dried with a paper towel for length-weight measurement and oysters of 40–120 mm shell length were used for the study. Before collecting the gut contents, oyster shells were rinsed with 70% ethanol to minimize surface bacteria. Then the oyster knife was used to open oyster shells, and then gut contents were sucked gently through the mouth using a rubber-head fitted sterile glass pipette (Wheaton, DWK). The sucked gut contents (~200µl) were kept in individual sterile cryovial labelled with sampling code and stored in liquid nitrogen. On the following day, gut samples (stored in liquid nitrogen jar) and water samples (stored in 4^{o}C) were transported under temperature-controlled conditions to the South Australian Research and Development Institute (SARDI) laboratory, West Beach, South Australia. The gut samples containing cryovials were then transferred from liquid nitrogen to -80^{o}C for further processing. Moreover, the water samples kept at 4^{o}C refrigerator were then filtered using individual Nalgene™ Rapid-Flow™ filter unit (pore size 0.2µm, filter capacity 500mL, Sigma®) for each seawater sample. The filter paper was then cut into small pieces using a scalpel blade and stored into individual cryovials at -80^{o}C for further analysis.

4.3.2. Extraction of DNA from Oyster Gut and Seawater Samples

DNA was extracted from gut and seawater samples using the FastDNATM spin kit for soil (MP Biomedicals) following the manufacturer's instructions. Briefly, the samples were taken into a Lysing Matrix E tube with sodium phosphate buffer and MT buffer and then homogenized in the FastPrep® instrument for 40sec followed by several centrifugation steps at 14,000×g according to manufacturer's instructions. Finally, the extracted DNA was resuspended into DES (DNase/ Pyrogen Free Water). After extraction, the DNA quantification (ng/µl) was measured at $A_{260/280\ nm}$ and $A_{260/230\ nm}$ wavelengths absorbance using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). Then the extracted DNA was further purified by ethanol precipitation, and the precipitated DNA concentration (ng/µl) was measured again using the NanoDrop spectrophotometer. The precipitated DNA was then stored at -20^{o}C for further downstream analysis.

4.3.3. PCR amplification, Library Preparation and Illumina Amplicon Sequencing

According to Camarinha-Silva *et al*. (2014) and Legrand *et al*. (2018), the hypervariable conserved region (V1-V2) of the 16S rDNA gene was further amplified from the extracted DNA following the multi-step procedure, using universal eubacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 338R (GCTGCCTCCCGTAGGAGT). In brief, the targeted 16S rDNA amplicon libraries were produced by accompanying successive circles of PCR comprising a master-mix of 2.5mM De-oxynucleoside triphosphates, 2.5U/µl PrimeSTAR® HS DNA Polymerase (Takara), 5×PrimeSTAR® Buffer (Takara) and 10µm of each primer. In the first round of DNA synthesis, 20 PCR cycles were subjected to an initial denaturation step for 3min at 95°C with further denaturation for 10s at 98°C, flowed by elongation for 10s at 55°C, for 45s at 72°C, and a final elongation for 2min at

72°C in the Master cycler (Eppendorf, Germany). Using the similar PCR conditions, 1-µl of the resultant PCR product (round-1) was used as a template in a second 15-cycle round of PCR for incorporating sample-specific 6-nt barcodes and Illumina platform adaptors (10µm IlluminaFBC 27F, 10µm 338R adapter), further followed by a final 10-cycle round of PCR using a 1-µl of the template (round-2) with 10µm Illumina Multiplex and 10µm Illumina Index for incorporating Illumina multiplexing and indexing primers. Alongside the samples, the *Lactobacillus reuteri* and water were also sequenced as a positive and negative control, respectively. The final resultant PCR amplicons were visualized in 1% pre-stain gel electrophoresis to cross-check all samples (expected size ~438 bp). In the subsequent step, Agencourt PCR Clean-up protocol (Promega, Madison, WI) was followed for bead purification of the PCR products. The purified PCR amplicons were then quantified by Quant-iT™ Picogreen® dsDNA kit (Life Technologies). The library amplicon was prepared by pooling in equimolar ratios of 20ng of each sample with a unique barcode, and subsequently, the pooled library was quantified in PicoPlate. The science primer website was used to calculate the template concentration (nM), template volume to add and EB buffer volume. The concentration (ng/µl) of the final library was calculated according to the Australian Genome Research Facility (AGRF) recommendation (5-10nM in 20-30µl EB buffer). The library size was again confirmed on 1% gel electrophoresis. Finally, the cDNA library was sent to AGRF (North Melbourne, VIC, Australia) for Illumina MiSeq sequencing using 250bp paired-end sequencing.

4.3.4. Bioinformatics

A total of ~15.5 million raw sequence reads were obtained finally from 139 oysters and 11 seawater samples (n=139/150 oysters, with 11 oysters failing along the way;

n=11/12 seawater, with one water sample failed along the way). Paired-end reads were assembled by aligning the forward and reverse sequence reads, and the primers were identified and removed using PEAR version 0.9.5 (Zhang *et al*., 2014). The trimmed sequence were subsequently quality filtered to remove low-quality reads, full-length duplicate sequences (after counting), and singletons using Quantitative Insights into Microbial Ecology, QIIME (version 1.8; Caporaso *et al.*, 2010), USEARCH (version 8.0.1623; Edgar, 2010), and UPARSE software (Edgar, 2013). Finally, sequences were clustered into operational taxonomic units (OTUs) at a minimum identity of 97%, with putative chimeras removed using the RDP-gold database as a reference (Cole *et al*., 2014).

The resultant OTUs were taxonomically assigned through Seqmatch function in RDP and SILVA. Then each OTU was named according to the SILVA taxonomy and best match from RDP linages. A total of 6,934,257 paired-end reads (mean= 46,228 ± 17,675 reads/sample; min=18,256; max=112,618) with high quality were grouped into 22402 OTUs where average oyster gut (total 139 oysters) reads were 45,932 and seawater only (11 water samples) reads were 49,964. The OTUs were standardized and filtered (Zhang *et al*., 2016 and Legrand *et al*., 2018), and the host-associated datasets with the contribution >0.01% were finally used. According to the RDP and SILVA database, OTUs containing fungi and chloroplasts were then removed, clustered into 662 OTUs for further downstream analysis. Finally, rarefaction curves for all samples were constructed from raw data to estimate sequencing depth for each sample which showed a plateau, suggesting that all samples comprised adequate sequencing depth (Figure 4.1).

4.3.5. Statistical analysis

The filtered datasets of 662 OTUs from 150 samples (139 oysters and 11 seawater samples) were finally used for statistical analysis using Primer-E version 7.0.11 (Clarke *et al*., 2014). A length-weight scatterplot was constructed to see the size classes of the studied groups. Non-metric multidimensional scaling (nMDS) ordination plots were generated to visualize the global bacterial community structures using Bray-Curtis similarity resemblance (Bray and Curtis, 1957; Clarke *et al*., 2014). Main test (one-way) permutational multivariate analysis of variance (PERMANOVA) was used to assess differences between groups of samples, and the p-values were generated using unrestricted permutations of raw data (Anderson, 2001; Clarke *et al*., 2014). Season-wise classic diversity measures were generated as box plots in Primer-E for oyster and mussel gut samples such as Species richness (S), Pielou's evenness (J′), Shannon index (H′), Simpson index (1-λ), Average Taxonomic distinctness (Delta+) and Variation in Taxonomic Distinctness (Lambda+). Moreover, Average taxonomic distinctness (avTD, delta+) and variation in taxonomic distinctness (varTD, lambda+) were plotted against species (OTU) richness as funnel plots through TAXDTEST to display the seasonal changes of bacterial species diversity with taxonomic diversity and evenness. Subsequently, one-way ANOVA of these diversity indices (S, J′, H′, 1-λ, Delta+ and Lambda+) were generated in Graphpad prism (version 8.1.1). The phyla and class plots were used as a stacked bar chart in Primer-E. Furthermore, Differential abundant analyses were plotted at the phylum level using Microbiome analysis. For all statistical tests, differences were considered statistically significant at $p<0.05$.

4.4. Result

The V1-V2 region of 16S rDNA gene was profiled from a total of 139 oysters out of 150 oysters and 11 seawater samples out of 12 to determine the seasonal variation in

bacterial community composition of Pacific oyster gut and its surrounding environment across the year-round, including two consecutive winter samples labelled as winter_trial and winter from June 2016 to August 2017 (Table 4.1). When the oysters were collected from the commercial farm, they were pooled from a mix of oyster cohorts in oyster cages. Initially, the size class was checked to get preliminary cohort information of oysters sampled in the present study, though the size effect was not considered in the study. The length-weight scatter plot revealed that each separate group of samples was at a similar age. Remarkably, the Autumn group and two consecutive winter groups belonged to a similar size group (Figure 4.2).

4.4.1. Beta Diversity: Non-metric Multi-dimensional Scaling (nMDS)

The Beta diversity of Global bacterial community structure through nMDS ordinations of gut and water samples showed that oyster samples clustered quite independently to water samples across all seasons. Besides, despite some shared OTUs in the studied groups, oyster groups from five seasons (winter_trial, spring, summer, autumn, and winter) revealed a different pattern. Interestingly, the winter group presented a different pattern from one winter to the following winter (Figure 4.3). The clustering pattern was then supported by one-factor main test PERMANOVA (pseudo-F=15.014 and *p*-value=0.0001), showing significant differences among five seasonal groups. Besides, pairwise PERMANOVA tests was also confirmed significant differences in the global bacterial communities between all *priori groups* (*p*-value=0.0001) such as winter_trial vs spring, winter_trial vs summer, winter_trial vs winter, spring vs summer, spring vs autumn, spring vs winter, summer vs autumn, summer vs winter, autumn vs winter were found all (*p*-value=0.0001) significantly different patterns, and winter_trial vs autumn (*p*-value=0.0006).

4.4.2. Bacterial Taxonomic Information

In total, the studied microbial community composition was composed of 28 classes belonging to 17 bacterial phyla. The relative abundance revealed that Mollicutes (Phylum: Tenericutes) and gamma-proteobacteria were the most dominant bacterial classes in oyster gut in all seasons, whereas alpha-proteobacteria, gamma-proteobacteria and Bacteroidia (Phylum: Bacteroidetes) were dominant classes in the seawater samples (Figure 4.4). Despite seasonal differences in relative abundance, there was a similarity in the gut bacterial composition in oysters across different seasons. Though Mollicutes was almost absent in seawater (0.06-0.11%), it was an abundant bacterial class in oyster gut (about 65%). On the other hand, the abundances of alpha-proteobacteria (36-60%) and Bacteroidia (17-23%) was higher in seawater, and their abundance was very low in the oyster guts (1.5 – 2% and 0.5-1.75% respectively), indicating that oysters can accumulate host-specific bacteria in their gut, different from their surrounding water. In oyster gut, pattern of the bacterial community in winter samples was found different from the other four *priori groups* (winter_trial, spring, summer, autumn), and a noticeable difference was found between the two consecutive winter samples. Remarkedly, Mollicutes was the most dominant class (78%) in oyster guts in the first winter (winter_trial), whereas gamma-proteobacteria (34%), Mollicutes (26%) and alpha-proteobacteria (20%) were found in winter of the following year. Moreover, in seawater, the microbial composition was similar in different seasons, and the only difference was found in autumn, where gamma-proteobacteria became relatively higher in autumn than the other seasons (Figure 4.4). In addition, the abundance of *Vibrio*, a species of interest that is a causative agent of the Pacific Oyster Mortality Syndrome (POMS) in summer, was very low in the oyster gut (0.03 - 0.37%) and seawater (0.10 - 5.5%) in different

seasons. Our result showed the presence of ubiquitous common *Vibrio* species in healthy oysters or seawater (Figure 4.4).

4.4.3. Alpha Diversity: Classic Species Diversity and Taxonomic Diversity

The season-wise Alpha-diversity measures showed that total number of bacterial species (species richness) in oyster gut, was comparatively higher among spring and both winter samples (mean OTUs/ Species of around 250), but lower in summer and autumn (mean OTUs/ species of around 180). Though the mean OTUs of both winter samples of consecutive years were similar (around 250), the individuals of winter_trial oysters were more consistent compared to the consecutive winter samples and from other seasons (Figure 4.5A). In addition, the contribution of species abundance (species evenness) in the bacterial community, was more consistent in all oyster gut samples collected in different seasons. However, the winter samples were comparatively more even (0.6), but comparatively less consistent than other four *priori* groups (winter_trial, spring, summer, autumn) (Figure 4.5B). The species richness and evenness in gut samples were significantly different (p-value<0.0001). The other classic diversity indices, such as Shannon (p-value<0.0001) and Simpson index (p-value=0.0008) of species diversity, were comparatively higher in winter samples (3.5 and 0.9, respectively) (Figure 4.5C and 4.5D). Thus, it can conclude that despite some similarities in the season-wise classic species diversity measures, there were also some seasonal differences, and found comparatively higher in the following winter samples than winter_trial, spring, summer and autumn samples.

On the other hand, the taxonomic diversity (Delta+) was similar among winter_trial (first winter), spring, summer and autumn, and lower in the second winter than other four groups (Figure 4.6). In comparison between two significantly different

(*p*-value <0.0001) consecutive winter samples, the winter_trial samples were more taxonomic diverse (Delta+) with less taxonomic even (Lambda+) than the following winter samples (Figure 4.6). The other three seasonal groups (spring, summer, and autumn) were more or less similar in taxonomic distinctness (Delta+) values with taxonomic evenness (Figure 4.6. Scatter lambda+ vs Delta+). However, the pattern of seasonal variation in species diversity and taxonomic diversity (Delta+) were different (Figure 4.7). Nevertheless, both winter samples were similar in species diversity which was not significantly different (*p*-value=0.9787). However, the following winter oysters were less taxonomically rich, but more evenly distributed in the gut bacterial community than the winter_trial oysters. (Figure 4.7). Nevertheless, the taxonomic evenness was not significantly different across the seasonal groups (*p*-value=0.9998).

4.5. Discussion

The perennial pattern of bacterial community composition in the gut of Pacific oysters and its surrounding environment of Coffin Bay revealed some degrees of differences in the composition and its relative abundance in oyster gut and its surrounding seawater samples across seasons. The Beta-diversity of global bacterial community in oyster gut and its surrounding environment reveals different bacterial clusters between oyster guts and seawater communities. This finding signifies that oyster gut can establish different microbial communities from the surrounding environment. This result coincides with previous studies where the bacterial community of surrounding water was different from Eastern oyster *Crassostrea virginica* tissue-associated microbiota (La Valley *et al*., 2009); and Pacific oyster *C. gigas* larvae (Asmani *et al*., 2016), indicates that species-specific gut residential microbes.

4.5.1. Permanent Residential Microbes

The present study clarifies that oyster gut can harbor host-specific residential bacterial composition. For instance, Mollicutes (Phylum: Tenericutes) was the most prevalent bacterial class in oyster gut throughout the year, although it was almost absent in seawater. Mollicutes was also found dominant and host-specific to gut or digestive gland in many bivalves such as eastern oyster *Crassostrea virginica* (Pimentel *et al*., 2021), Sydney rock oysters *Saccostrea glomerata* (Green and Barnes, 2010), abalone *Haliotis discus hannai* (Tanaka *et al*., 2004), sea urchin *Lytechinus variegatus* (Hakim *et al*., 2016) and freshwater mussel *Villosa nebulosa* (Aceves *et al*., 2017). This result also indicates that the specific function of Mollicutes in bivalve gut needs to be addressed through functional metagenomics in future studies. In a very recent metagenomic study in eastern oyster, *Crassostrea virginica*, it is established that the oyster associated Mollicutes help in carbon and energy acquisition in oyster gut (Pimentel *et al.,* 2021). Moreover, further study is recommended to recognize these microbes' transmission dynamics, such as vertically from parents to offspring or horizontally from the surroundings.

4.5.2. Transient Microbes

On the contrary, alpha-proteobacteria (Phylum: Proteobacteria) and Bacteroidia (Phylum: Bacteroidetes) were relatively abundant in seawater but was very low in the oyster gut, suggesting horizontal transmission of transient microbes from the environment to oyster gut tissues. In the present study, alpha-proteobacteria were prevalent in seawater across all seasons except the autumn when gamma-proteobacteria (Phylum: Proteobacteria) was also found relatively higher with alpha-proteobacteria. The coastal and marine microbial communities are quite complex to maintain their ecological succession. However, the phylum Proteobacteria and Bacteroidetes are ubiquitous in marine and coastal surface waters (Eilers *et al*., 2000;

Kirchman. 2002; Nishimura & Nagata, 2007; Yin *et al*., 2013;). Proteobacteria help in sulfur oxidization (Sakami *et al.,* 2008; Yamamoto and Takai, 2011; Patwardhan *et al.,* 2018), and in hydro-carbon degradation (Palleroni *et al*., 2004; Singleton *et al*., 2006), whereas Bacteroidetes help in polysaccharide degradation (Thomas *et al*., 2011). On the other hand, oyster gut was enriched with gamma-proteobacteria (34%) in winter but was comparatively low in other seasons. Likewise, abundance of gamma-proteobacteria was increased in winter in the gill of Northern Red Sea *Spondylus spinosus* oysters and declined in summer (Roterman *et al*., 2015).

4.5.3. Seasonal Variation in Gut Bacterial Composition and its Abundance

Despite the differences in global bacterial community, the composition showed taxonomic similarity, which is consistent with some previous studies on the microbial community of digestive glands and shells of eastern oyster, *Crassostrea virginica* (Arfken *et al.,* 2017) and in the gut microbiome of eastern oyster (*Crassostrea virginica*) and blue mussel (*Mytilus edulis*) (Pierce and Ward, 2019). Though the bacterial composition was taxonomically similar, its relative abundance in oyster gut and seawater was rehabilitated across seasons. The bacterial abundance and pattern of seasonal differences were also reported in a comparative study between *Crassostrea virginica* and *Mytilus edulis* (Pierce and Ward, 2019), and in coral mucus associated with predominant bacterial communities produced by *Acropora* spp. and *Porites* spp. (McKew *et al*., 2012), which are similar to the results of our present study. Seasonal differences in the intestinal microflora due to change in seasonal temperature were also found in hybrid tilapia (*Oreochromis niloticus*×*Oreochromis aureus*) (Al-Harbi and Uddin N., 2004); and in a study of four freshwater fish species such as silver carp (*Hypophthalmichthys molitrix*), common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*) and deep-bodied crucian carp (*Carassius cuvieri*) (Hagi *et al*.,

2004). On another note, Spirochetes increased in oyster gut in summer than in other seasons, also indicating a transient bacterial group. Likewise, Roterman *et al*. (2015) reported a similar increase in Spirochaetes in summer in the gill of *Spondylus* oyster species. The host-associated Spirochaetes are commensal organisms in some bivalve tissues, such as in the gills of lucinid clams *Lucinoma kazani* (Duperron *et al.*, 2007); and in the gut and digestive glands in *Cerastoderma edule*, *Laternula elliptica*, *Crassostrea gigas* (Husman *et al*., 2010). Some Spirochaetes species were also found as a causative agent in Akyoya oyster disease pearl oyster, *Pinctada fucata martensii* (Matsuyama *et al*., 2017). Spirochaetes was absent in seawater samples but only found in spring water. However, further research is required to clarify whether host-associated Spirochaetes is an opportunistic symbiont or pathogen, particularly in summer and to understand the respective functionality of Spirochaetes in bivalve gut through functional metagenomics.

4.5.4. Alpha-Diversity of Gut Bacteria

The Alpha-diversity of different classic diversity indices also reported seasonal differences in species richness, evenness, Shannon and Simpson index across different seasons, and found comparatively more diverse in the following winter than other seasons. Notwithstanding, the similarity in the consecutive winter samples in species richness, the following winter samples of the second year were more evenly distributed, and the Shannon and Simpson diversity were comparatively higher than the previous year's winter_trial samples. This result confirms that there are some other confounding extrinsic environmental factors such as temperature, salinity, pH and dissolved oxygen across seasons, which were not considered in the present study. The Coffin Bay region faces regular high-water exchange phenomenon through upwelling system (McClatchie *et al.*, 2006; Kämpf and Ellis, 2015), which might have

some definite effects on its water quality and microbial community, in turn. Moreover, the previous studies define that microbial community changes with salinity (Song *et al*., 2021; Pinnell and Turner, 2020; Dupont *et al*., 2014), dissolved oxygen (Cao *et al*., 2018; Nocker *et al*., 2007), seawater pH (Meron *et al*., 2012; Meron *et al*., 2011; Lei and VanderGheynst, 2000), ocean acidification (Crummett, 2020; Nelson, 2020) and upwelling water (Paterson *et al*., 2012; Wilson *et al*., 2018). Noticeably, along with four different seasonal comparisons, the microbial diversity of two consecutive winters differed. Interestingly, the winter_trial samples of previous year were more taxonomic diverse with less taxonomic evenness, compared with the following winter samples, which were less taxonomic diverse and showed more taxonomically even distribution of OTUs in oyster gut bacterial community. This result might be due to the collection time of the year. We collected the first winter samples on 16 June (early winter), and the next winter samples were collected on 17 August (late winter), which might affect other environmental parameters and successively its microbial association.

4.5.5. Season-wise Abundance of *Vibrio, Species of Interest*

Our precise curiosity about the abundance of *Vibrio*, a causative agent of summer mass mortality in oysters worldwide, was found very low in oyster gut (0.03 to 0.37%) and in surrounding environment (0.1 to 5.56%) in different seasons across the year. Though the occurrence of *Vibrio* in oyster gut could cause diseases and summer mass mortality in Pacific oysters (Samain and McCombie, 2008; Garnier *et al*., 2008; Petton *et al*., 2015), an insignificant number of *Vibrio* was described in healthy oysters, for instance, in the haemolymph and digestive glands of *Crassostrea gigas* and *Mytilus galloprovincialis* (Vezzulli *et al*., 2018), Sydney rock oysters *Saccostrea*

glomerata (Green and Bernes, 2010), and in the digestive tract of healthy oysters, *Crassostrea gigas* (Hernández-Zárate & Olmos-Soto, 2006).

4.6. Concluding Remarks

Overall, our result shows a significant effect of seasonal temperature on the microbial community in oyster gut and its surrounding natural environment year-round and reveals differences in bacterial community of oyster gut and seawater. The gut bacterial diversity and its relative abundance changed across the seasons in a year. Tenericutes was prevalent in oyster gut across the year except for winter, whereas Proteobacteria was prevalent in seawater all year round. Interestingly, microbial diversity was different to some extent in two-consecutive winter. Overall, some extrinsic factors other than seasonal temperatures such as other water quality parameters, the time of the year, some intrinsic factors of the host, and the functional role of different microbiomes in different host tissues could shape the bacterial community in healthy oysters. Our present study recommends the need to study functional metagenomics with the taxonomic characterization of microbial community and its transmission dynamics (e.g., vertical, or horizontal) in different tissues together on a monthly basis to get a clear representation of healthy organism's microbiome. Throughout the year, Mollicutes as a prevalent bacterial group in oyster gut suggests permanent residential microbes; and other relatively low bacterial groups such as Proteobacteria, Cyanobacteria, Actinobacteria Bacteroidetes, Spirochaetes suggests as transient microbes that might come from environment through the horizontal transmission of these transient microbiomes. Our findings of characterizing the resident and transient microbiota in a healthy ecosystem will help improve the

health of oyster management. The diversity and proliferation of opportunistic pathogens may serve as an indicator of oyster health.

4.7. References

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26(1), 32-46.

Aceves, A. K., Johnson, P., Bullard, S. A., Lafrentz, S., & Arias, C. R. (2018). Description and characterization of the digestive gland microbiome in the freshwater mussel *Villosa nebulosa* (Bivalvia: Unionidae). *Journal of Molluscan Studies*, *84*(3), 240-246.

Al-Harbi, A. H., & Uddin, M. N. (2004). Seasonal variation in the intestinal bacterial flora of hybrid tilapia (*Oreochromis niloticus*×*Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia. *Aquaculture*, *229*(1-4), 37-44.

Arfken, A., Song, B., Bowman, J. S., & Piehler, M. (2017). Denitrification potential of the eastern oyster microbiome using a 16S rRNA gene based metabolic inference approach. *PloS One*, 12(9).

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., & Nicolas, J. L. (2016). Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture*, *464*, 434-444.

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, 27(4), 325-349.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A. P., Schaumburg, F., Becker, K., Wos-Oxley, M. L., & Pieper, D. H. (2014). Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environmental Microbiology*, 16(9), 2939-2952.

Cao, Y., Kwok, B. H., van Loosdrecht, M., Daigger, G., Png, H. Y., Long, W. Y., & Eng, O. K. (2018). The influence of dissolved oxygen on partial nitritation/ anammox performance and microbial community of the 200,000 m3/d activated sludge process at the Changi water reclamation plant (2011 to 2016). *Water Science and Technology*, *78*(3), 634-643.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., & Huttley, G. A. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5), 335.

Cavanaugh, C. M. (1994). Microbial symbiosis: patterns of diversity in the marine environment. *American Zoologist*, 34, 79-89.

Clarke, K. R., Gorley, R. N., Somerfield, P. J., & Warwick, R. M. (2014). Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd.

Cole J. R., Wang Q., Fish J. A., Chai B., McGarrell D. M., and Sun Y. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42 D633–D642.

Crummett, L. T. (2020). Acidification decreases microbial community diversity in the Salish Sea, a region with naturally high pCO2. *Plos One*, *15*(10), e0241183.

Duperron, S., Fiala-Medioni, A., Caprais, J. C., Olu, K., & Sibuet, M. (2007). Evidence for chemoautotrophic symbiosis in a Mediterranean cold seep clam (Bivalvia: Lucinidae): comparative sequence analysis of bacterial 16S rRNA, APS reductase and RubisCO genes. *FEMS Microbiology Ecology*, *59*(1), 64-70.

Dupont, C. L., Larsson, J., Yooseph, S., Ininbergs, K., Goll, J., Asplund-Samuelsson, J., McCrow, J.P., Celepli, N., Allen, L.Z., Ekman, M. and Lucas, A.J., (2014). Functional tradeoffs underpin salinity-driven divergence in microbial community composition. *PloS One*, *9*(2), e89549.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

Edgar R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10 996–998. 10.1038/nmeth.2604.

Eilers, H., Pernthaler, J., Glöckner, F. O., & Amann, R. (2000). Culturability and in situ abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology*, *66*(7), 3044-3051.

Garnier, M., Labreuche, Y., & Nicolas, J. L. (2008). Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. *Systematic and Applied Microbiology*, *31*(5), 358-365.

Gilbert, J. A., Steele, J. A., Caporaso, J. G., Steinbrück, L., Reeder, J., Temperton, B., Huse, S., McHardy, A.C., Knight, R., Joint, I. & Sommerffield, P. (2012).

Defining seasonal marine microbial community dynamics. *The ISME Journal*, 6, 298-308.

Green, T. J., & Barnes, A. C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology*, 109(2), 613-622.

Hagi, T., Tanaka, D., Iwamura, Y., & Hoshino, T. (2004). Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture*, *234*(1-4), 335-346.

Hakim, J. A., Koo, H., Kumar, R., Lefkowitz, E. J., Morrow, C. D., Powell, M. L., Watts, S. A., & Bej, A. K. (2016). The gut microbiome of the sea urchin, *Lytechinus variegatus*, from its natural habitat demonstrates selective attributes of microbial taxa and predictive metabolic profiles. *FEMS Microbiology Ecology*, 92(9), fiw146.

Hawkins, A. J. S., Bayne, B. L., Bougrier, S., Héral, M., Iglesias, J. I. P., Navarro, E., Smith, R.F.M. & Urrutia, M. B. (1998). Some general relationships in comparing the feeding physiology of suspension-feeding bivalve molluscs. *Journal of Experimental Marine Biology and Ecology*, *219*(1-2), 87-103.

Hernández-Zárate, G., & Olmos-Soto, J. (2006). Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. *Journal of Applied Microbiology*, *100*(4), 664-672.

Husmann, G., Gerdts, G., & Wichels, A. (2010). Spirochetes in crystalline styles of marine bivalves: Group-specific PCR detection and 16S rRNA sequence analysis. *Journal of Shellfish Research*, *29*(4), 1069-1075.

Joyce, P. W., Kregting, L., & Dick, J. T. (2019). Relative impacts of the invasive Pacific oyster, *Crassostrea gigas*, over the native blue mussel, *Mytilus edulis*, are mediated by flow velocity and food concentration. *NeoBiota*, *45*, 19.

Kämpf, J. (2014). South Australia's large inverse estuaries: On the road to ruin. In *Estuaries of Australia in 2050 and beyond* (pp. 153-166). *Springer*, Dordrecht.

Kämpf, J., & Ellis, H. (2015). Hydrodynamics and flushing of Coffin Bay, South Australia: A small tidal inverse estuary of interconnected bays. *Journal of Coastal Research*, *31*(2), 447-456.

Kirchman, D. L. (2002). The ecology of Cytophaga–Flavobacteria in aquatic environments. *FEMS Microbiology Ecology*, *39*(2), 91-100.

La Valley, K. J., Jones, S., Gomez-Chiarri, M., Dealteris, J., & Rice, M. (2009). Bacterial community profiling of the eastern oyster (*Crassostrea virginica*): comparison of culture-dependent and culture-independent outcomes. *Journal of Shellfish Research*, *28*(4), 827-835.

Legrand, T. P., Catalano, S. R., Wos-Oxley, M. L., Stephens, F., Landos, M., Bansemer, M. S., Stone, D. A. J., Qin, J. G., & Oxley, A. (2018). The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. *Frontiers in Microbiology*, 8, 2664.

Lei, F., & VanderGheynst, J. S. (2000). The effect of microbial inoculation and pH on microbial community structure changes during composting. *Process Biochemistry*, *35*(9), 923-929.

Matsuyama, T., Yasuike, M., Fujiwara, A., Nakamura, Y., Takano, T., Takeuchi, T., ... & Nakayasu, C. (2017). A Spirochaete is suggested as the causative agent of Akoya oyster disease by metagenomic analysis. *PloS One*, *12*(8), e0182280.

McClatchie, S., Middleton, J. F., & Ward, T. M. (2006). Water mass analysis and alongshore variation in upwelling intensity in the eastern Great Australian Bight. *Journal of Geophysical Research: Oceans*, *111*(C8).

McKew, B. A., Dumbrell, A. J., Daud, S. D., Hepburn, L., Thorpe, E., Mogensen, L., & Whitby, C. (2012). Characterization of geographically distinct bacterial communities associated with coral mucus produced by *Acropora* spp. and *Porites* spp. *Applied and Environmental Microbiology*, *78*(15), 5229-5237.

Meron, D., Atias, E., Kruh, L. I., Elifantz, H., Minz, D., Fine, M., & Banin, E. (2011). The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*. *The ISME Journal*, *5*(1), 51-60.

Meron, D., Rodolfo-Metalpa, R., Cunning, R., Baker, A. C., Fine, M., & Banin, E. (2012). Changes in coral microbial communities in response to a natural pH gradient. *The ISME Journal*, *6*(9), 1775-1785.

Morton, B. R. I. A. N. (1983). Feeding and digestion in Bivalvia. *The Mollusca. Physiology Part*, *2*, 65-147.

Nelson, K. S., Baltar, F., Lamare, M. D., & Morales, S. E. (2020). Ocean acidification affects microbial community and invertebrate settlement on biofilms. *Scientific Reports*, *10*(1), 1-9.

Nishimura, Y., & Nagata, T. (2007). Alphaproteobacterial dominance in a large mesotrophic lake (Lake Biwa, Japan). *Aquatic Microbial Ecology, 48*(3), 231-240.

Nocker, A., Lepo, J. E., Martin, L. L., & Snyder, R. A. (2007). Response of estuarine biofilm microbial community development to changes in dissolved oxygen and nutrient concentrations. *Microbial Ecology, 54*(3), 532-542.

Owen, G. (1974). Feeding and digestion in the Bivalvia. *Advances in Comparative Physiology and Biochemistry, 5*, 1-35.

Paillard, C., Le Roux, F., & Borrego, J. J. (2004). Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. *Aquatic Living Resources*, 17, 477-498.

Palleroni, N. J., Port, A. M., Chang, H. K., & Zylstra, G. J. (2004). *Hydrocarboniphaga effusa* gen. nov., sp. nov., a novel member of the γ-Proteobacteria active in alkane and aromatic hydrocarbon degradation. *International Journal of Systematic and Evolutionary microbiology, 54*(4), 1203-1207.

Paterson, J. S., Nayar, S., Mitchell, J. G., & Seuront, L. (2012). A local upwelling controls viral and microbial community structure in South Australian continental shelf waters. *Estuarine, Coastal and Shelf Science, 96,* 197-208.

Patwardhan, S., Foustoukos, D. I., Giovannelli, D., Yücel, M., & Vetriani, C. (2018). Ecological succession of sulfur-oxidizing Epsilon-and Gammaproteobacteria during colonization of a shallow-water gas vent. *Frontiers in Microbiology, 9,* 2970.

Pierce, M. L., & Ward, J. E. (2019). Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) and the Blue Mussel (*Mytilus edulis*): Temporal Variation and the Influence of Marine Aggregate-Associated Microbial Communities. *Msphere*, 4(6).

Pimentel, Z. T., Dufault-Thompson, K., Russo, K. T., Scro, A. K., Smolowitz, R. M., Gomez-Chiarri, M., & Zhang, Y. (2021). Microbiome Analysis Reveals Diversity and Function of Mollicutes Associated with the Eastern Oyster, *Crassostrea virginica*. *Msphere*, *6*(3), e00227-21.

Pinnell, L. J., & Turner, J. W. (2020). Temporal changes in water temperature and salinity drive the formation of a reversible plastic-specific microbial community. *FEMS Microbiology Ecology*, *96*(12), fiaa230.

Rahman, M. A., Henderson, S., Miller-Ezzy, P. A., Li, X. X., & Qin, J. G. (2020). Analysis of the seasonal impact of three marine bivalves on seston particles in water column. *Journal of Experimental Marine Biology and Ecology*, *522*, 151251.

Rosa, M., Ward, J. E., Ouvrard, M., Holohan, B. A., Espinosa, E. P., Shumway, S. E., & Allam, B. (2015). Examining the physiological plasticity of particle capture by the blue mussel, *Mytilus edulis* (L.): Confounding factors and potential artifacts with studies utilizing natural seston. *Journal of Experimental Marine Biology and Ecology*, *473*, 207-217.

Roterman, Y. R., Benayahu, Y., Reshef, L., & Gophna, U. (2015). The gill microbiota of invasive and indigenous *Spondylus* oysters from the Mediterranean Sea and northern Red Sea. *Environmental Microbiology Reports*, *7*(6), 860-867.

Sakami, T., Fujioka, Y., & Shimoda, T. (2008). Comparison of microbial community structures in intensive and extensive shrimp culture ponds and a mangrove area in Thailand. *Fisheries Science, 74*(4), 889-898.

Singleton, D. R., Sangaiah, R., Gold, A., Ball, L. M., & Aitken, M. D. (2006). Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. *Environmental Microbiology, 8*(10), 1736-1745.

Song, Y., Gilleaudeau, G. J., Algeo, T. J., Over, D. J., Lyons, T. W., Anbar, A. D., & Xie, S. (2021). Biomarker evidence of algal-microbial community changes linked to redox and salinity variation, Upper Devonian Chattanooga Shale (Tennessee, USA). *Bulletin*, *133*(1-2), 409-424.

Specht, J. A., & Fuchs, H. L. (2018). Thermal and viscous effects of temperature on Mercenaria mercenaria suspension feeding. *Marine Ecology Progress Series*, *589*, 129-140.

Stein, J. L., Cary, S. C., Hessler, R. R., Vetter, R. D., Felbeck, H., Ohta, S., & Childress, J. J. (1988). Chemoautotrophic symbiosis in a hydrothermal vent gastropod. *The Biological Bulletin*, *174*(3), 373-378.

Stevens, H., Stübner, M., Simon, M., & Brinkhoff, T. (2005). Phylogeny of Proteobacteria and Bacteroidetes from oxic habitats of a tidal flat ecosystem. *FEMS Microbiology Ecology*, *54*(3), 351-365.

Tanaka, R., Ootsubo, M., Sawabe, T., Ezura, Y., & Tajima, K. (2004). Biodiversity and in situ abundance of gut microflora of abalone (*Haliotis discus hannai*)

determined by culture-independent techniques. *Aquaculture*, *241*(1-4), 453-463.

Thomas, F., Hehemann, J. H., Rebuffet, E., Czjzek, M., & Michel, G. (2011). Environmental and gut bacteroidetes: the food connection. *Frontiers in Microbiology*, *2*, 93.

Travers, M. A., Miller, K. B., Roque, A., & Friedman, C. S. (2015). Bacterial diseases in marine bivalves. *Journal of Invertebrate Pathology*, 131, 11-31.

Vaughn, C. C., & Hoellein, T. J. (2018). Bivalve impacts in freshwater and marine ecosystems. *Annual Review of Ecology, Evolution, and Systematics*, 49, 183-208.

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L., & Pruzzo, C. (2018). Comparative 16S rDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microbial Ecology*, 75(2), 495-504.

Ward, J. E., & Shumway, S. E. (2004). Separating the grain from the chaff: particle selection in suspension-and deposit-feeding bivalves. *Journal of Experimental Marine Biology and Ecology*, *300*(1-2), 83-130.

Wendling, C. C., Batista, F. M., & Wegner, K. M. (2014). Persistence, seasonal dynamics and pathogenic potential of *Vibrio* communities from Pacific oyster hemolymph. *PloS One*, 9, e94256.

Wijsman, J. W. M., Troost, K., Fang, J., & Roncarati, A. (2019). Global production of marine bivalves. Trends and challenges. In Goods and Services of Marine Bivalves (pp. 7-26). *Springer*, Cham.

Wilson, J. M., Litvin, S. Y., & Beman, J. M. (2018). Microbial community networks associated with variations in community respiration rates during upwelling in nearshore Monterey Bay, California. *Environmental Microbiology Reports*, *10*(3), 272-282.

Yamamoto, M., & Takai, K. (2011). Sulfur metabolisms in epsilon-and gamma-proteobacteria in deep-sea hydrothermal fields. *Frontiers in Microbiology*, *2*, 192.

Yin, Q., Fu, B., Li, B., Shi, X., Inagaki, F., & Zhang, X. H. (2013). Spatial variations in microbial community composition in surface seawater from the ultra-oligotrophic center to rim of the South Pacific Gyre. *PLoS One*, *8*(2), e55148.

Zaccone, R., Caroppo, C., La Ferla, R., Zampino, D., Caruso, G., Leonardi, M., ... & Sitran, R. (2004). Deep-chlorophyll maximum time series in the Augusta Gulf (Ionian Sea): microbial community structures and functions. *Chemistry and Ecology*, *20*(sup1), 267-284.

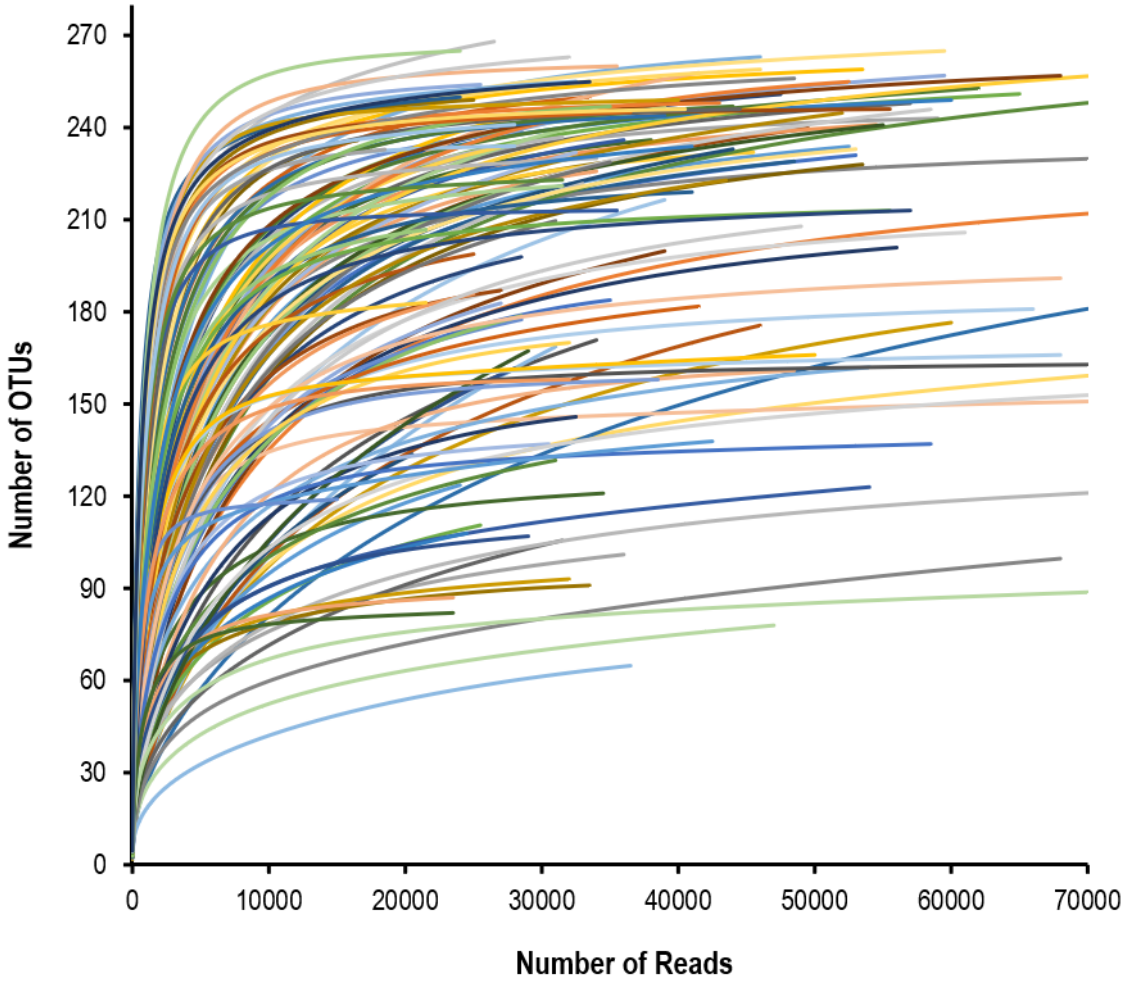
Zbinden, M., Pailleret, M., Ravaux, J., Gaudron, S. M., Hoyoux, C., Lambourdière, J., Warén, A., Lorion, J., Halary, S., & Duperron, S. (2010). Bacterial communities associated with the wood-feeding gastropod *Pectinodonta* sp. (Patellogastropoda, Mollusca). *FEMS Microbiology Ecology*, 74(2), 450-463.

Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*, 30(5), 614-620.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W. T., Du, Z & Li, E. (2016). Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. *Aquaculture*, *454*, 72-80.

Table 4.1. Experimental design and characteristics of sampling habitats comparing the oyster gut microbiota collected from Coffin Bay, Southern Australia in 1-year period from June 2016 to August 2017.

Habitat	Coffin bay				
Season	Winter_trial (June 16)	Spring (Nov 16)	Summer (Feb 17)	Autumn (May 17)	Winter (Aug 17)
Oysters (No.)	30	30	30	30	30
Water samples (2 L)	-	3	3	3	3
Collection Date	06 Jun 2016	15 Nov 2016	07 Feb 2017	15 May 2017	07 Aug 2017
Temperature (°C)	13.85	16.17	18.02	14.90	12.55


Number of OTUs
270
240
210
180
150
120
90
60
30
0
0
10000
20000
30000
40000
50000
60000
70000
Number of Reads

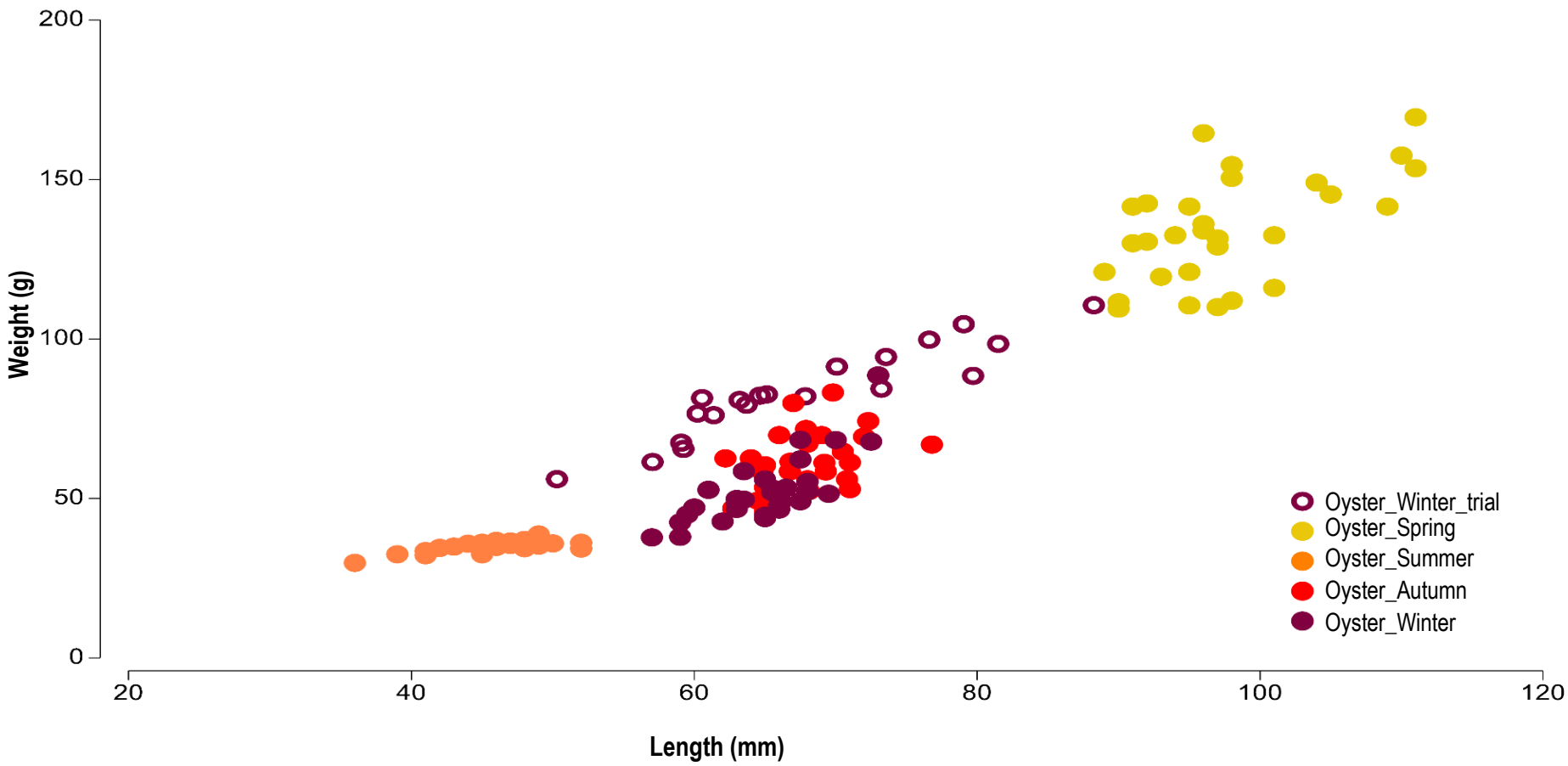

200
150
100
50
0
Weight (g)
20
40
60
80
100
120
Length (mm)
Oyster_Winter_trial
Oyster_Spring
Oyster_Summer
Oyster_Autumn
Oyster_Winter

Figure 4.2. Scatter plot displaying the length vs weight ordination of oyster samples, collected year-round from Coffin Bay, South Australia. The collected oysters were of different size group, and individuals of each group of oysters were in similar size group. The size

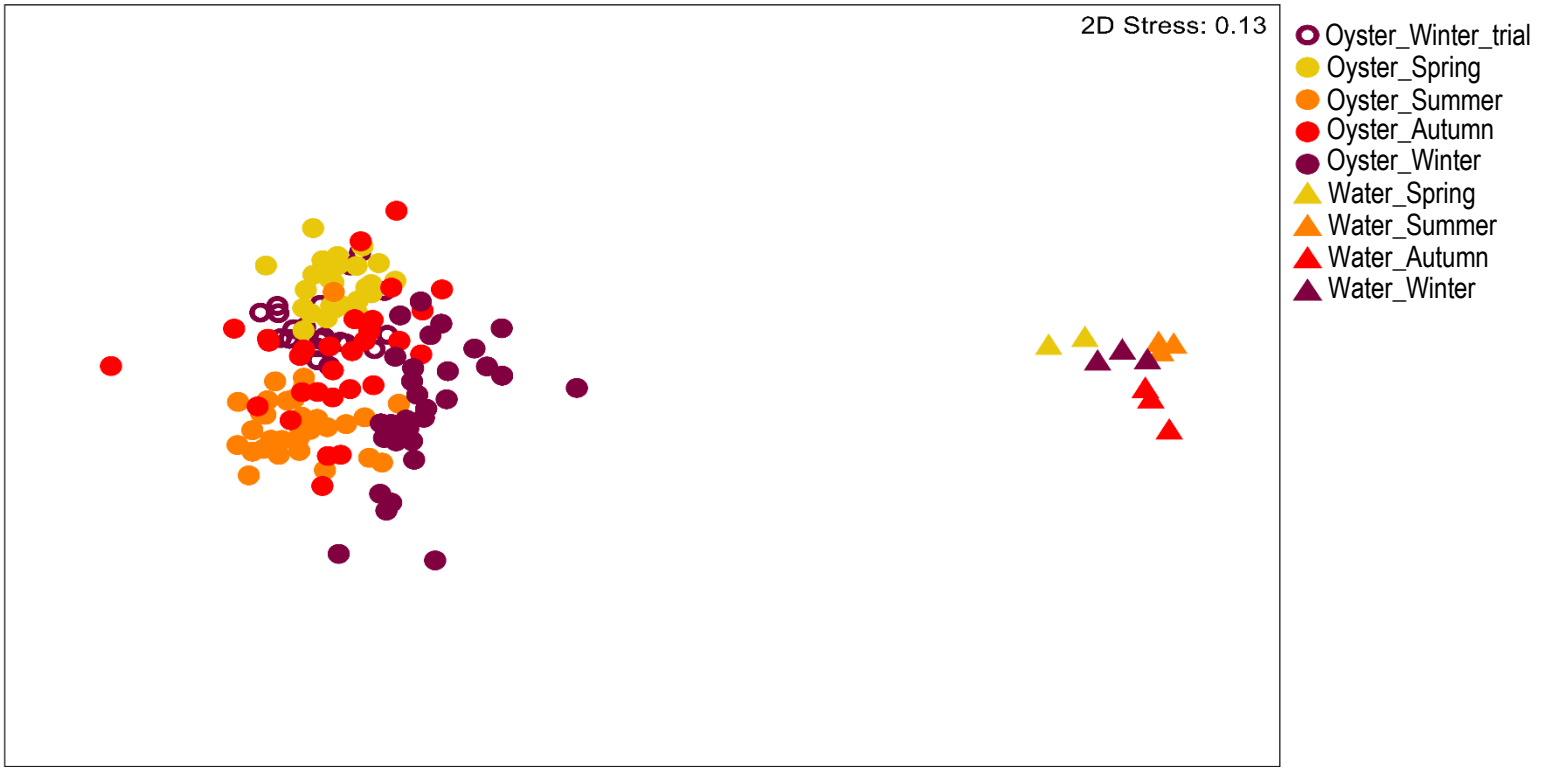


Figure 4.3. Global bacterial community structure of the oyster gut, and its surrounding seawater samples in year-round from one winter to the following winter in four different seasons, visualized by non-metric multidimensional scaling (nMDS) ordination using Bray-Curtis similarity. Main-test PERMANOVA was found significantly different (*p*-value=0.0001), and pairwise PERMANOVA were all significantly different among all different seasonal comparisons (*p*-value=0.0001), and winter_trial vs autumn (*p*-value=0.0006).

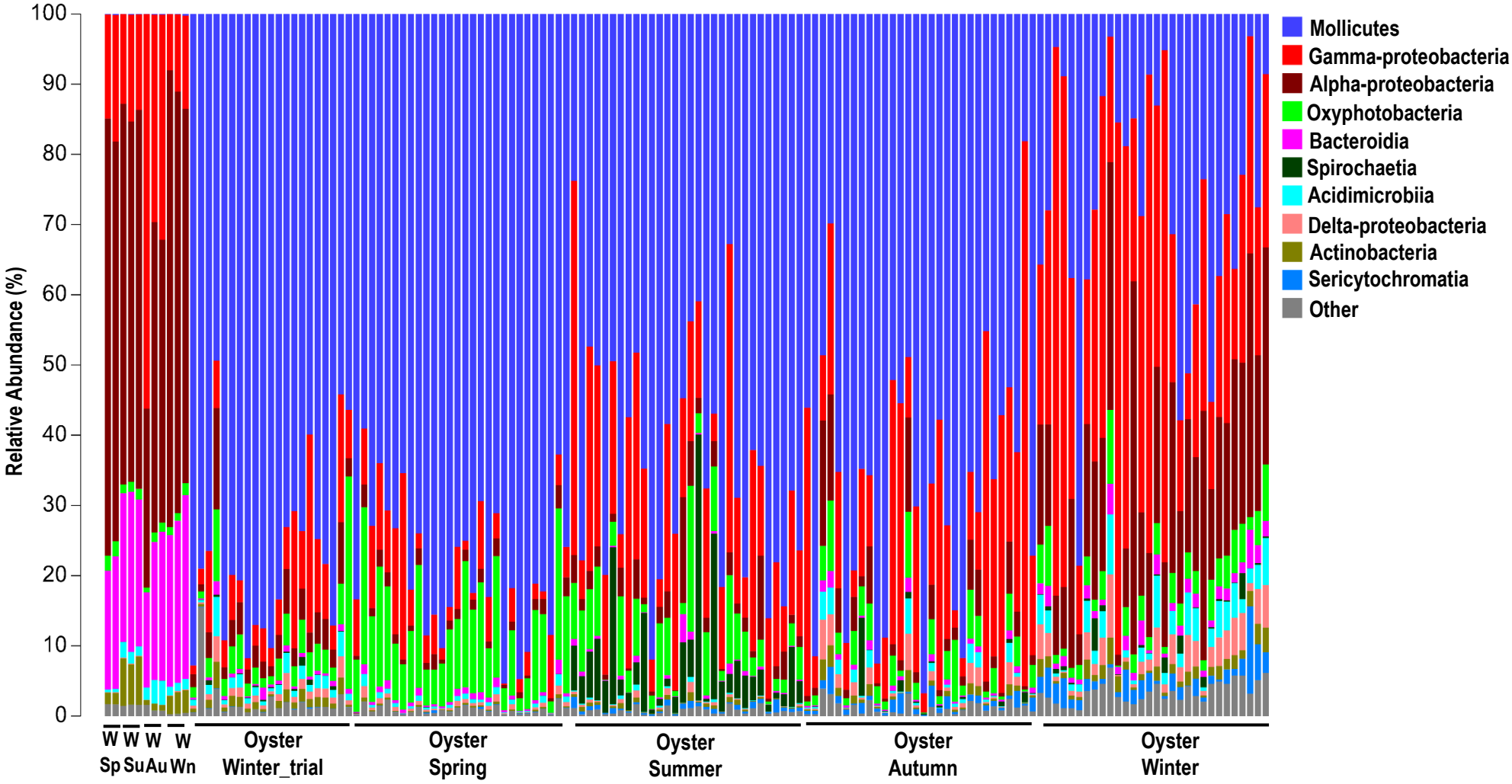


Figure 4.4. Relative abundance (%) of different bacterial classes in oyster and seawater in different seasons in Coffin Bay, South Australia.

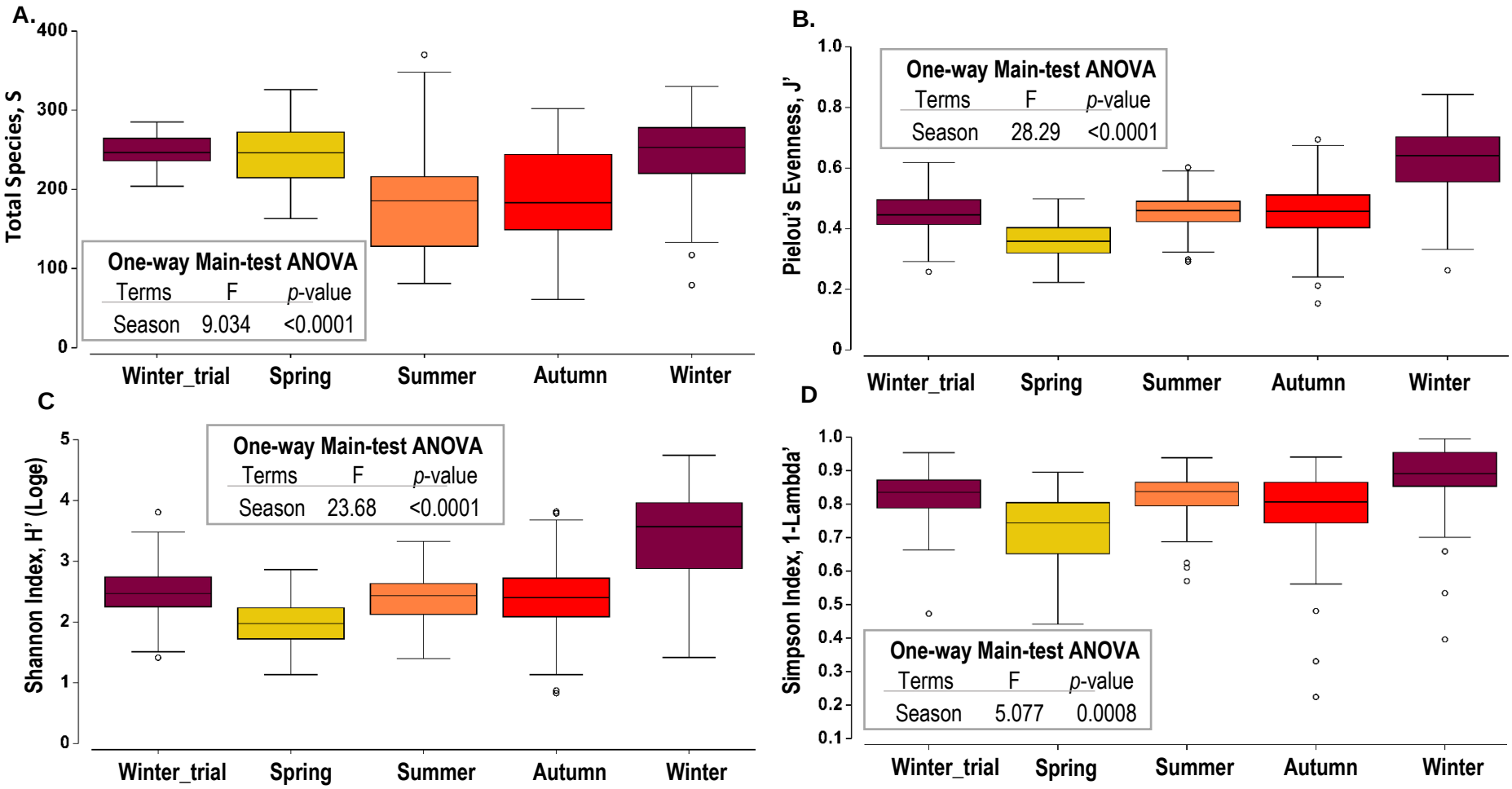
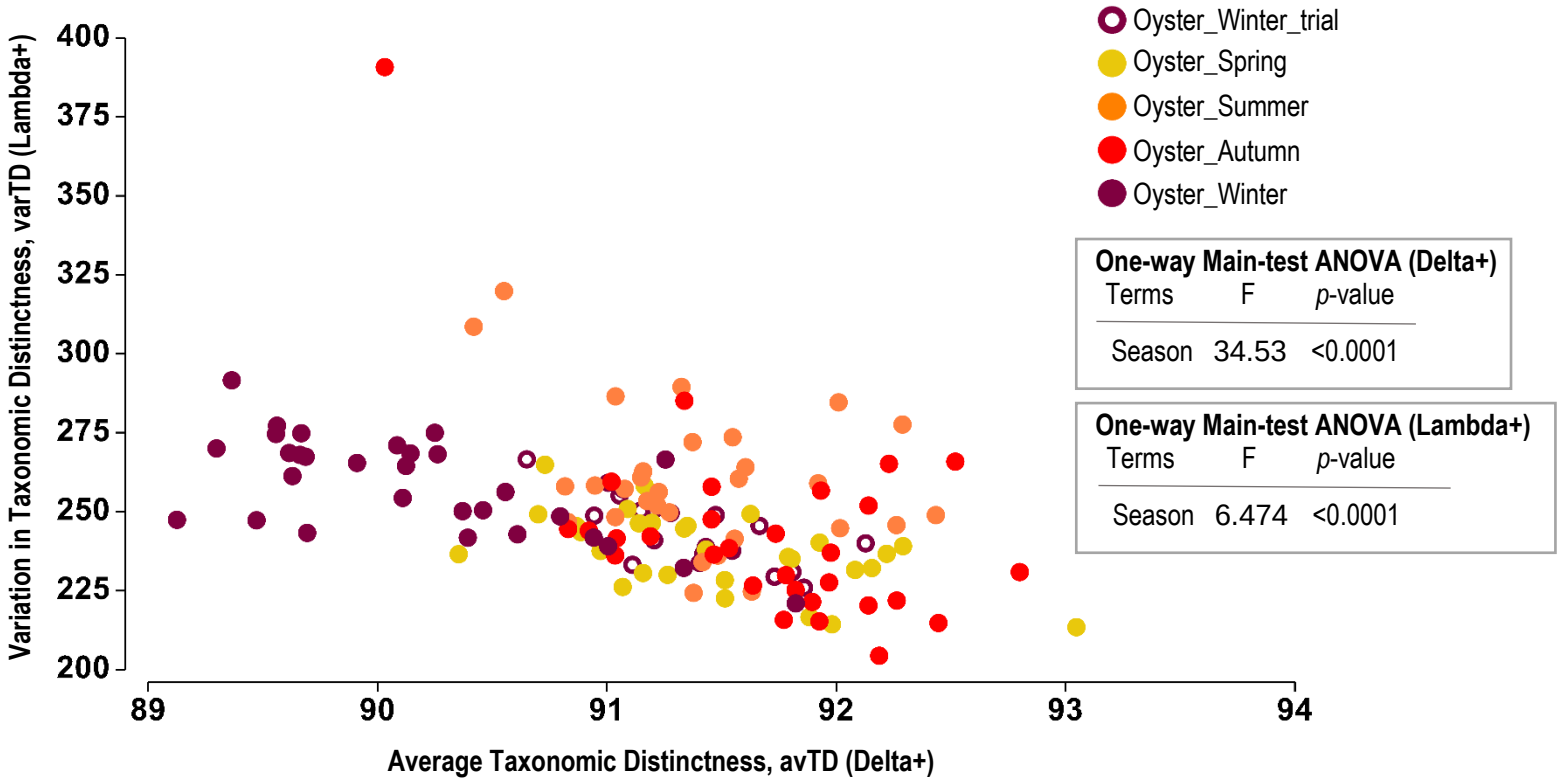


Figure 4.5. Season-wise alpha-diversity indices (Classic species diversity) in oyster guts, collected year-round from Coffin Bay.

A. Species richness (S), **B.** Pielou's evenness (J), **C.** Shannon diversity (H), **D.** Simpson diversity (1-Lambda'). The significance level across different seasons was verified by Ordinary one-way ANOVA where Alpha set at 0.05. The box plot displayed the five-number summary data values such as the minimum, first quartile, median, third quartile, and maximum, with some biological outliers (°).

Figure 4.6. Scatter plot representing measures of Taxonomic distinctness (TD) such as the average taxonomic distinctness (avTD,



delta+) as a function of variation in taxonomic distinctness (varTD, lambda+) in the gut bacterial communities in oyster, collected year-round from Coffin Bay, South Australia.

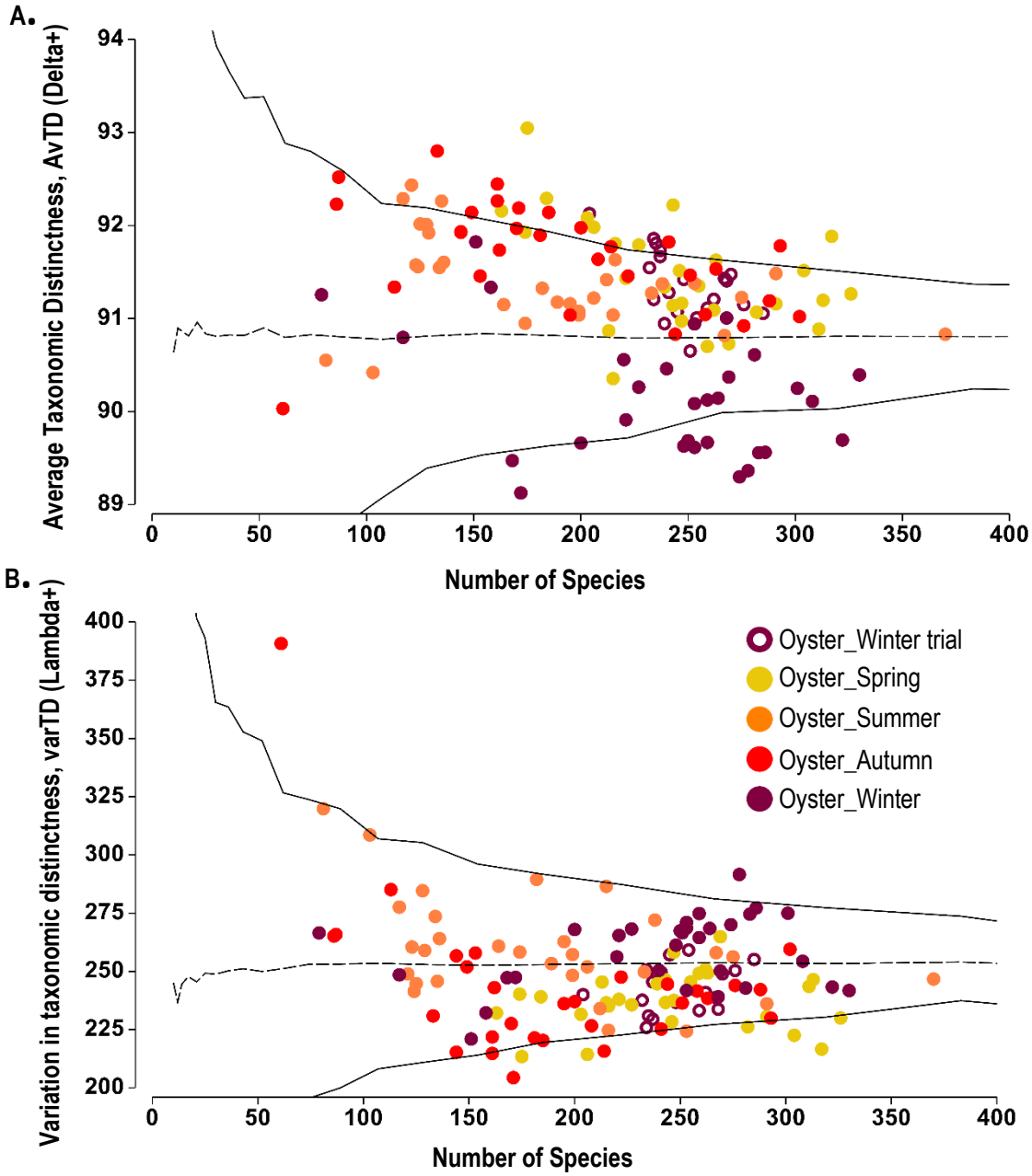


Figure 4.7. Funnel plots demonstrate a pattern of seasonal variation in the gut bacterial Species diversity with **A.** Taxonomic diversity (Delta+), and **B.** Taxonomic evenness (Lambda+) in oysters. Average taxonomic distinctness (avTD, Delta+) and Variation in taxonomic distinctness (varTD, Lambda+) were plotted against species (OTU) richness.

CHAPTER 5

Impact of feed composition on the gut bacterial composition in Pacific oyster *Crassostrea gigas* and Mediterranean mussel *Mytilus galloprovincialis*

Highlights

1. Feed composition and host phylogeny directly affected the gut bacterial community.
2. In oysters, Mollicutes, Gamma-proteobacteria and Bacteroidia were the most dominant bacterial class in pre-trial, micro-algae fed, and macro-algae fed oysters, respectively.
3. In mussels, Gamma-proteobacteria, Bacteroidia and Fusobacteria were dominant in pre-trial, micro-algae fed mussels, and macro-algae fed mussels, respectively.
4. Spirochaeta was commonly present in oyster guts but absent in mussel guts.
5. Species diversity and taxonomic diversity were high in micro-algae fed oysters and macro-algae fed mussels.

5.1. Abstract

Feed composition and food digestion can affect the gut bacterial community in fish, but little is known on how dietary type impacts gut microbiota in molluscan bivalve. This study investigates impact of feed composition on the gut bacterial composition in two commercially important bivalves: Pacific oyster *Crassostrea gigas* and Mediterranean mussel *Mytilus galloprovincialis*. For two months, these two bivalve species were fed with two types of marine algae, live *Isochrysis galbana* (micro-algae) and the powder of dried seaweed *Ulva* sp. (macro-algae) under a laboratory condition. The 16S rDNA of the V1–V2 region was sequenced through an Illumina MiSeq platform to compare gut bacterial community at the start and the end of the feeding trial in oysters and mussels. Both alpha and beta diversities directly impact of feed composition on gut microbiota of both bivalves, but the degree of impact differed between bivalve species. The difference in the gut bacterial assemblages in pre-trial, micro-algae and macro-algae fed oysters and mussels indicates that the diet type and host species can shape composition of gut bacteria. Spirochaeta was absent in mussel guts at start and end of the trial despite its prevalence in oysters, revealing host specificity in colonizing gut bacteria. However, diet type was the main driver in shaping the gut bacterial community, followed by the bivalve species. The pattern of microbial dynamics related to dietary manipulation can predict the symbiotic relationship between the host and gut microbe community. The modulation of gut microbes might be achieved using beneficial probiotics associated with different foods in marine bivalve aquaculture.

Keywords: Gut bacteria, *Crassostrea gigas*, *Mytilus galloprovincialis*, microalgae, macroalgae.

5.2. Introduction

Diet type and feeding mode may influence gut bacterial community of filter feeders due to the interaction between microorganisms and nutrients in gut (Vaughn & Hakenkamp, 2001; Pierce & Ward, 2019). The bivalve gut consists of digestive diverticula, gut tubules with blind sacs for phagocytosis and intracellular digestion to form an ideal inhabitant for resident microbiota (Griffin *et al*., 2021). The establishment of a microbial community in gut largely depends on gut micro-environment governed by the type of ingested food. Therefore, food type has a selective advantage for bacterial groups (Woodcock *et al*., 2007). The co-evolution of host and microbes favours a long-term inter-dependence. The host provides habitat, and microbes, in turn, provide nutrition to host. The ingested food can affect multifaceted physiological processes in animals, including gut bacteria. The microbial community in the host gut is expected to adapt to a changing environment, particularly with a dietary shift. The gut microbes of aquatic vertebrates change with the diet succession in the environment (Baldo *et al*., 2015). In abalone, dietary administration of beneficial bacteria can modulate the gut microbial community (Iehata *et al*., 2014). The change in the microbial community is a rapid adaptation and critical for host fitness.

Pacific oyster *Crassostrea gigas* and Mediterranean mussel *Mytilus galloprovincialis* are filter feeders and consume microbes in environment. These two species are ideal candidates for a comparative study to understand the impact of marine microbes through analysis of gut microbial communities because they are

suspension feeders with a similar feeding mode. In addition, Pacific oysters and Mediterranean mussels can be found in a similar region due to translocation for aquaculture. Therefore, understanding the impact of feed composition on the gut microbes in these two species is important to reveal the role of microbes in bivalves. Previous studies on oysters and mussels have reported the link between gut microbial community and pathogen susceptibility in Pacific oyster *Crassostrea gigas* and Mediterranean mussel *Mytilus galloprovincialis* (Vezzulli *et al.*, 2018), and the influence of marine seston on the microbial community in eastern oyster *Crassostrea virginica* and blue mussel *Mytilus edulis* (Pierce and Ward, 2019). The host and the environment can synergistically shape the gut microbiome in freshwater mussels (Weingarten *et al*. 2019).

As suspension feeders, bivalves can filter a wide variety of seston particles and select different sized food particles from the surrounding environment. The selection efficiency depends on particle size and availability in the environment across seasons (Ward and Shumway, 2004). Besides, the gut of bivalves can encounter various microbes (Griffin *et al.*, 2021) because of filtering a massive amount of water in a capacity of 3–5 L/h/g dry mass (Cranford *et al.*, 2011). In a recent study, Rahman *et al.* (2020) reported the seasonal differences in filtering capacity of oyster *Crassostrea gigas*, mussel *Mytilus galloprovincialis* and cockle *Katelysia rhytiphora* and found that oysters and mussels select similar food sources (large food particles >8µm), but mussels can filter even smaller food particles (<5µm) compared to oysters. Besides, filtration rate and filtration capacity in bivalves vary with seasonal temperature (Specht and Fuchs, 2018), seston size (Rosa *et al.*, 2015), food particle density (Joyce *et al*., 2019) and structure of the filtering apparatus in bivalves (Hawkins *et al*., 1998). We thus hypothesize that marine bivalve with

similar filtering ability and food preference may harbour a host-specific microbial community, but the community composition of microbes is feed-dependent, varying with the type of feed composition.

Pacific oysters and Mediterranean mussels are important species in aquaculture in a large spectrum of marine environments worldwide. Diet manipulation and commercial diets have been introduced for many important species in aquaculture, and such knowledge is important for sustainable aquaculture management. However, our knowledge is limited on the gut microbial response of these filter feeders to feed microalgae and grounded macroalgae as diets. Therefore, this study aims to understand the impact of diet type on the microbial community in oyster and mussel. Understanding microbial dynamics in the gut of Pacific oysters and Mediterranean mussels has a significant implication for formulating a suitable diet using marine microalgae and green seaweed, macroalgae in molluscan aquaculture.

5.3. Material and Methods

5.3.1. Experimental Design and Maintenance of Animal

Pacific oyster *C. gigas* and Mediterranean mussel *M. galloprovincialis* were collected from Coffin Bay, South Australia. A total of 120 oysters and 120 mussels (shell length 50-70 mm; weight 30-70 g) were collected and acclimatized for 2 weeks prior to the feeding trial in a flow-through seawater system at 20^{o}C. The bivalves were starved for 2 days before starting the feeding experiment. Besides, the bivalves were fed *ad libitum* with a mixed microalgae diet every day consisting of *Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros muelleri* throughout the acclimatization period. Moreover, different water quality parameters (temperature, salinity, pH and DO) were recorded daily using multiple probes. The seawater was changed every 2 days, and the dead animals were rapidly removed from the system and replaced.

5.3.2. Feeding Trial

The trial was carried out for 56 days in the marine aquarium laboratory at Flinders University. The acclimatized animals were transferred into glass aquaria (50×40×40cm). Before starting feeding trial, 10 oysters and 10 mussels were sampled to collect their gut content as pre-trial samples. Then, a total of 12 glass aquaria were finally divided into two feeding groups: six micro-algae fed, and six macro-algae fed, with three replicates for each bivalve species. There were 20 animals in each aquarium. Moreover, each food type contained a single type of algae to minimize the confounding effect from other feed. Dried macroalgae were crushed and filtered through a 150mm mesh, slightly watered, stirred and then extruded into algal powder using a feed processor. According to the experimental design, both bivalves were fed two different types of food: microalgae (*Isochrysis galbana)* at 0.45 L of 2×10^6 cells/ml, or macroalgae (*Ulva* sp.) at 5% of flesh weight every second day at 11:00 h for 56 days.

5.3.3. Oyster, Mussel and Water Sampling

At the end of the feeding trial on day 56, fifteen animals (five individuals in each tank, three replicates) for each bivalve species were taken to collect digestive glands as post-trial samples. Finally, 30 Pacific oysters (15-microalgae fed + 15-macroalage fed) and 30 Mediterranean mussels (15-microalgae fed + 15-macroalgae fed) were used. Two liters of water were collected in three replicates (Table 5.1), and water samples were stored immediately at 4^oC. Upon collection, oysters and mussels were cleaned and dried with a blotting paper towel for recording shell length and weight.

To minimize possible contamination, the oysters and mussels were cleaned using 70% ethanol, and then the gut content (~200µl) of each individual was collected and stored into individual sterile cryovials in liquid nitrogen for further downstream analysis. Finally, the cryopreserved guts and refrigerated water samples were transported to the Molecular Science Laboratory at South Australian Research and Development Institute (West Beach, South Australia, Australia) for further analysis.

5.3.4. DNA Extraction from Gut and Water Samples

DNA was extracted from gut samples using the FastDNA™ spin kit for soil (MP Biomedicals) following the manufacturer's manual. Besides, DNA was also extracted from seawater samples using the same kit, following the water filtration through 0.22µM Nalgene™ Rapid-Flow™ filters (Sigma), and then the filter discs were placed into the associated lysing matrix tubes. Then the ethanol precipitation of all DNA samples was carried out to concentrate the DNA, and then quantified the DNA concentration (ng/µl) using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and stored at -20°C until downstream library preparation.

5.3.5. PCR Amplification, Library Preparation and Sequencing

The hypervariable region (V1-V2) of the 16S rDNA gene was amplified from extracted DNA using a multi-step approach, using universal eubacterial primers 27F and 338R according to Camarinha-Silva *et al*. (2014) and Legrand *et al*. (2018). Briefly, 16S rDNA gene amplicon libraries were generated by conducting consecutive rounds of PCR comprising 2.5 mM deoxynucleoside triphosphates, 2.5 U/µl PrimeSTAR® HS DNA Polymerase (Takara Bio), 5× PrimeSTAR® Buffer (Takara Bio) and 10µm of each primer. In the first round, 25ng of the sample DNA was subjected to initial denaturation of 95°C for 3min, followed by 20 cycles of 98°C,

55°C for 10s, and 72°C for 45s. One microliter template from round 1 was used in a 2nd 15-cycle round of PCR with the same cycling parameters to incorporate sample-specific 6nt barcodes and Illumina platform adaptors, followed by further 10 cycles using 1µl of template from round 2 to incorporate Illumina multiplexing and indexing primers. PCR products were then visualized by gel electrophoresis, and those of the expected size (~438 bp) were subsequently purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using Quant-iT™ Picogreen® dsDNA kit (Life Technologies). Amplicons were pooled in equimolar ratios (20ng) and sequenced on the Illumina MiSeq platform (Illumina) using 250nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF, North Melbourne, VIC, Australia). Amplicons obtained from gDNA extracts of *Lactobacillus reuteri* were sequenced alongside the samples as a control.

5.3.6. Bioinformatics

A total of ~11.3 million raw sequence reads were obtained from a total of 79 samples (n=39/40 oyster; n=34/40 mussel; n=6/6 seawater). Reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang *et al*., 2014), and the primers were identified and trimmed. Then, the trimmed sequences were processed using Quantitative Insights into Microbial Ecology - QIIME (version 1.8; Caporaso *et al*., 2010), USEARCH (version 8.0.1623; Edgar, 2010), and UPARSE software (Edgar, 2013). Using USEARCH tools, sequences were quality filtered to remove low-quality reads, full-length duplicate sequences and singletons. Sequences were clustered into operational taxonomic units (OTUs) at a minimum identity of 97%, with putative chimeras removed using the RDP-gold database as a reference (Cole *et al*. 2014).

A total of 7,814,725 high quality, paired-end reads (mean=98920 ± 42,636 reads/sample; min=1716, max=199,584) were clustered into 8,311 OTUs. These OTUs were further filtered as conducted previously (Legrand *et al*., 2018) where only those contributing to >0.01% of the bivalve-associated (n=73) or >0.01% of the seawater dataset (n=6) were retained. The resultant OTUs were interrogated using the Seqmatch function of RDP database (Wang *et al*., 2007) and SILVA (Quast *et al*., 2012). The taxonomic lineages based on the SILVA taxonomy and the best hit from RDP were assigned for each OTU. The OTUs representing chloroplast or fungi were removed from the dataset, leaving a total of 699 OTUs for downstream analysis. Rarefaction curves were generated to assess (retrospectively) the sequencing depth of each sample (Figure 5.1).

5.3.7. Statistical analysis

The final datasets, including 699 OTUs from 79 samples (39 oyster guts, 34 mussel guts and six water samples), were used for statistical analysis using Primer-E version 7.0.11 (Clarke *et al*., 2014). Non-metric multidimensional scaling (nMDS) ordination plots were generated to visualize the global bacterial community structure using Bray-Curtis similarity resemblance (Bray and Curtis, 1957; Clarke *et al*., 2014). Besides, the principal coordinate analysis (PCoA) without water samples was also generated to verify the ordination of gut bacterial community in oysters and mussels. Main test (one-way) permutational multivariate analysis of variance (PERMANOVA) was used to assess differences between groups of samples (species vs diet type). The p-values were generated using unrestricted permutations of raw data (Anderson, 2001; Clarke *et al*., 2014). When the main test detected significant difference, pairwise two-way PERMANOVA was then performed among different *priori groups* of samples (Oyster_Pre-trial vs Mussel_Pre-trial, Oyster_*Iso* vs Mussel_*Iso*, Oyster_*Ulva* vs

Mussel_*Ulva*, Oyster_*Iso* vs Oyster_*Ulva*, and Mussel_*Iso* vs Mussel_*Ulva*) (Anderson, 2001). Different univariate diversity measures were generated in Graphpad prism version 8.1.2 for oyster and mussel gut samples considering diet (algae) type as a factor, such as Species richness (S), Pielou's evenness (J′), Shannon index (H′), Simpson index (1-λ), Average Taxonomic distinctness (Delta+) and Variation in Taxonomic Distinctness (Lambda+). Subsequently, an unpaired Welch t-test of each index was performed in Graphpad prism version 8.1.2. Multivariate measures of these diversity indices (S, J′, H′, 1-λ, Delta+ and Lambda+) were generated among the *priori groups* of oyster vs mussel vs micro-algae (*Iso*) vs macro-algae (*Ulva*), and they were analyzed using ordinary one-way ANOVA in Graphpad Prism (version 8.4.2). As ANOVA showed the differences in group means, Tukey's multiple comparisons test was performed to visualize the groups with different means. The phylum and class plots were used as a stacked bar chart in Primer-E. Differences were considered statistically significant for all statistical tests at $p<0.05$.

5.4. Results

5.4.1. Beta Diversity: Global Bacterial Community

To determine the impact of feed composition on gut microbial community, the V1-V2 region of 16S rDNA gene was profiled from the gut samples of 40 oysters and 40 mussels (10 pre-trial + 15 micro-algae post-feeding trial + 15 macro-algae fed post-feeding trial samples) and 6 water samples (3 micro-algae fed system + 3 macro-algae fed system) (Table 5.1). Due to some procedural errors in water samples preparation, we finally discarded all six water samples from the experimental datasheet. The pattern of OTU reads of all gut samples showed a plateau for each sample, indicating that the maximum sequencing depth read was reached for all gut samples used (Figure 5.1). Moreover, the non-metric multidimensional scaling (nMDS) plot highlighted a

distinct global community structure of oyster and mussel gut samples. The independent clustering pattern from each group showed the differences among pre-trial, micro-algae fed and macro-algae fed global gut bacterial communities between oysters and mussels (Figure 5.2). Both bivalves showed different clusters of pre-trial, micro-algae fed and macro-algae fed bacterial communities. This observation was further verified with the main test PERMANOVA, which significantly differed between species and diet type (*p*-value=0.0001, pseudo-F=17.647). Besides, the pair-wise PERMANOVA among different *priori groups* (Oyster_Pre-trial vs Mussel_Pre-trial, Oyster_*Iso* vs Mussel_*Iso*, Oyster_*Ulva* vs Mussel_*Ulva*, Oyster_*Iso* vs Oyster_*Ulva*, Mussel_*Iso* vs Mussel_*Ulva*) were significantly different (*p*-value=0.0001). Moreover, the gut bacterial communities of oysters and mussels are quite different but more similar within the individuals of each species, and the diet type is a major driver rather than the bivalve species in regulating bacterial communities in gut (Figure 5.3). The principal coordinates analysis (PCoA) showed that the type of algae in diet had a more significant influence on gut bacterial community structure, followed by bivalve species.

5.4.2. Relative Abundance of Different Bacterial Taxa

Among the filtered OTUs of 699, the gut bacterial taxa revealed 42 classes belonging to 21 bacterial Phyla in total. The mean relative abundance notably changed in both bivalves before the start and the end of both trials (micro-algae and macro-algae treatment). In oysters, Mollicutes (Phylum: Tenericutes) was the most dominant bacterial taxon (39.13%) in the pre-trial samples but became less abundant at the end of the feeding trial in both micro-algae fed oysters (3.17%) and macro-algae fed oysters (8.22%). The other abundant bacterial class was alpha-proteobacteria which was similar in abundance among three *priori* groups of oysters, such as pre-trial

(17.84%), micro-algae fed trial (18.37%) and macro-algae fed trial (18.15%) (Figure 5.4). Besides, Bacteroidia and gamma-proteobacteria were less abundant in the gut of pre-trial oysters (6.98% and 8.86%, respectively) and became more abundant at the end of the feeding trial such as micro-algae fed oysters (22.04% and 25.01%), and macro-algae fed oysters (19.40% and 18.93%). The other group of Proteobacteria (Class: Campylobacteria) was relatively low in pre-trial oysters but was in a high abundance in micro-algae fed oysters (20.12%) and low in macro-algae fed oysters (7.67%).

On the other hand, in mussel guts, the dominant bacterial classes in pre-trial mussel gut were gamma-proteobacteria (25.32%) and alpha-proteobacteria (23.25%). Bacteroidia (Phylum: Bacteroidetes) was found high (69.75%) in micro-algae fed mussels, which was comparatively lower than pre-trial mussels (16.67%) and macro-algae fed mussels (17.05%) (Figure 5.4). In contrast, Fusobacteriia was the most abundant bacteria in macro-algae fed mussels (33.17%), which was found lower in pre-treated mussels (6.18%) and micro-algae fed mussels (6.53%). Surprisingly, Spirochaetia (Phylum: Spirochetes) was absent in mussel guts. However, this bacterial class was found in oyster guts at both the feeding trial's start and end. Besides, Oxyphotobacteria (Phylum: Cyanobacteria) was relatively low in micro-algae fed oysters (0.53%) and mussels (0.03%) compared to pre-trial and macro-algae fed oysters and mussels. Moreover, Mollicutes (Phylum: Tenericutes) was almost absent in micro-algae fed mussels (0.03%) but was low in the start trial (7.76%) and macro-algae fed mussels (2.09%). However, it was the most dominant bacterial taxa in pre-treated oyster guts (39.13%), though its abundance became relatively low after the feeding trial in micro-algae fed (3.17%) and macro-algae fed oysters (8.22%) (Figure 5.4). The relative abundance of different bacterial taxa among three *priori* groups in

both oysters and mussels indicates the impact of food type in gut bacterial community, and the change of bacterial abundance was species-specific.

5.4.3 Alpha Diversity: Diversity Indices

The Alpha-diversity measures revealed that species richness was higher in micro-algae, *Isochrysis galbana* fed oysters (mean OTUs of 325) than in macro-algae, *Ulva* fed (mean OTUs of 318) oysters and pre-trial oysters (mean OTUs of 239). Likewise, in mussel guts, the species richness was higher in macro-algae fed oysters (mean OTUs of 368) than pre-trial mussels (mean OTUs of 356) and micro-algae fed mussels (mean OTUs of 277). The species richness of micro-algae fed mussels was significantly lower than macro-algae fed mussels (Figure 5.5A). The gut bacterial species of micro-algae fed oysters was more evenly distributed (0.65) in the microbial community than in pre-trial (0.52) and macro-algae fed oysters (0.52). There was a significant difference in the Pielou's species evenness between two types of algae-fed oysters with significantly higher in micro-algae fed oysters. On the other hand, the pre-trial mussels were more evenly distributed in Pielou's species evenness (0.65) in the gut bacterial community than both algae-treated mussels (0.45 and 0.55, respectively). The macroalgae fed mussels were significantly higher in evenness than micro-algae fed mussels (Figure 5.5B).

The Shannon diversity of micro-algae fed oysters (3.8) was significantly different from pre-trial oysters (2.9), and macro-algae fed oysters (3.0). In mussels, the Shannon index was higher in the pre-trial mussels (3.8) than algae-fed mussels such as micro-algae fed (2.5) and macro-algae (3.2) mussels, and it was significantly different between pre-treated mussels and micro-algae fed mussels (Figure 5.5C). Besides, the micro-algae fed oysters (0.95) showed a significantly higher Simpson

index than pre-trial fed oysters (0.76), but not significantly different from macro-algae fed oysters (0.87) and. In contrast, Simpson index was similar among three *priori groups* of mussels (Figure 5.5D). Pre-trial oysters were less taxonomically diverse (78.38) than micro-algae (79.03), and macro-algae (79.02) fed oysters, but more taxonomically even (196.5) compared with micro-algae fed oysters (182.8) and macro-algae fed oysters (174.0). In contrast, micro-algae fed mussels were less taxonomic diverse (77.80) but more taxonomically even (204.1) than macro-algae fed (78.77 and 175.3 respectively) and pre-trial fed mussels (78.49 and 197.9 respectively) (Figure 5.5E and 5.5F). Thus, pre-trial oysters were significantly lower in taxonomic diversity than both algae-fed oysters, whereas micro-algae fed mussels were significantly lower than macro-algae fed mussels and pre-trial mussels. Likewise, macro-algae fed oysters were significantly lower in taxonomic evenness than pre-treated ones but not significantly different from micro-algae fed oysters. On the other hand, micro-algae treated mussels were significantly higher in taxonomic evenness than pre-trial, and macro-algae fed mussels. The pattern of alpha diversity measures among pre-trial and two types of algae fed oysters and mussels clearly revealed the effect of diet type in gut bacterial diversity. However, the changes in diversity indices were both species and algae type-specific, and they changed differently among pre-trial, micro-algae, and macro-algae fed oysters and mussels.

5.5. Discussion

This study reveals that the feed composition could directly affect gut microbial composition, and this impact varied with type of feed ingested and host species. The difference of gut bacterial community prior and at the end of the 56-day feeding trial clearly shows species-dependent impact on the global gut bacterial composition between Pacific oysters and Mediterranean mussels. These two molluscan species are

phylogenetically related inter-generic bivalves. Both oysters (Ostreida) and mussels (Mytilida) are in a sister group of a monophyly clade, Pterimorphia, with some phylogenetic similarity. For example, they have compound lateral and frontal cilia in gills, and the shell of the calcific crystal outer layer has an aragonitic inner layer (Lemer *et al*., 2016).

The Beta diversity of the present study demonstrates the distinct gut bacterial assemblages in the pre-trial, micro-algae (*Isochrysis galbana*) and macro-algae fed (*Ulva* sp.) oysters and mussels, indicating the impact of diet type and host genetics in shaping gut bacterial composition. Similar results have been reported in some surgeonfish (Family: Acanthuridae) species and three other coral reef fish species of the central Red sea (Miyake *et al*., 2015), where distinct gut bacterial patterns of micro and macro-algavores were found, and host phylogeny and diet type were both driving the changes of the gut bacteria. The principal coordinates in the present study suggest that the diet (algae) type has a more significant influence in structuring the gut bacterial community than the host species. The gut bacteria of mammalian species also show a similar result of clustering the bacterial community along with diet rather than host phylogeny (Muegge *et al.,* 2011). The influence of different types of food is widely established in human gut microbiota (Scott *et al*., 2013; Wu *et al*., 2013; Rothe & Blaut, 2013; Graf *et al*., 2015; and Doré, J., & Blottière, 2015). However, there is still a lack of information in bivalve gut microbiota response to dietary manipulation. Our present study has provided evidence to help the bivalve industry produce healthy and fit bivalves through modulating gut bacteria via diet manipulation.

Among the significant changes, in particular, Mollicutes (Phylum: Tenericutes) was the most dominant bacterial class (39.13%) in pre-trial oysters. It became less abundant in both micro-algae fed oysters (3.17%) and macro-algae fed

oysters (8.22%), indicating the direct impact of diet type in oysters. Besides, this bacterial class was also decreased in mussels in the present study after feeding trial (0.03% in micro-algae fed and 2.09% in macro-algae fed mussels), and its abundance was relatively high in pre-trial (7.76%), indicating that the change of mussel gut microbiota depends on the type of dietary algae. Mollicutes can be abundant in the gut of other oyster species, such as Sydney rock oysters *Saccostrea glomerata* (Green and Barnes, 2010), eastern oyster *Crassostrea virginica* gut (King et al., 2012; Pimentel *et al.*, 2021), and mussel species such as *Brachidontes* sp. in an Indonesian lake (Cleary *et al*., 2015), and freshwater mussel *Villosa nebulosa* (Aceves *et al*., 2017). Mollicutes was also decreased after feeding CARB-R and FAT-R in mice (Clarke *et al*., 2012).

In the present study, Bacteroidia was found extremely higher (69.75%) in micro-algae fed mussels than in pre-treated (16.67%) and macro-algae fed mussels (17.05%). Clarke *et al*. (2012) also reported high Bacteroidetes levels after a feeding trial in mice. Bacteroidetes occurs in the digestive tract of mussel *Mytilus coruscus* (Yang *et al.,* 2021) and the gut of snail *Achatina fulica* (Cardoso *et al*., 2012). Bacteroidetes can help nutrient cycling in the redox process in ark shell bivalve *Scapharca subcrenata* (Lukwambe *et al.,* 2020). In contrast, Fusobacteriia was the most abundant bacterial taxon in macro-algae fed mussels (33.17%) but was similar in abundance in pre-treated mussels (6.18%) and micro-algae fed mussels (6.53%). Our results clearly direct the effect of diet on gut bacteria in mussels, and it varies differently with the type of algal food in treatment. Fusobacteria has also been found in the digestive gland of freshwater mussel *Villosa nebulosa* (Aceves *et al*., 2018) and in the gut of Chilean marine mussel *Mytilus chilensis* (Santibáñez *et al*., 2022). This type of bacteria might have some specific functional role in the bivalve gut. For example, some Fusobacteria can produce H_2 and degrade nitramine (Zhao *et al*.,

2009). Moreover, the compositional overlap was noticed in the gut of oysters and mussels in the recent study, and relative abundance of bacterial taxa differed among feeding treatments - pre-trial, micro-algae or macro-algae fed bivalves. Though the bacterial composition was similar in oysters and mussels, its abundance differed, indicating some degrees of host specificity across bivalve species. In addition, bivalve-associated microbes are similar in different bivalve species (King *et al*., 2012). For example, a similar bacterial composition exists in the gut of eastern oyster *Crassostrea virginica* and blue mussel *Mytilus edulis* (Pierce and Ward, 2019). Besides, bivalve microbial symbiosis and the host specificity were found different in both molluscan species. For instance, Spirocheata was completely absent in the mussel gut before the start and end of feeding trial, but it was found in the oyster gut. The host-specificity in bacterial colonization is demonstrated in the gut of oysters and mussels, and these bacteria might have some specific functions in gut health. Spirochaetes is symbionts in some other oyster spp., such as Northern Red Sea oyster, *Spondylus spinosus* (Roterman *et al*., 2015) and pearl oyster *Pinctada fucata* (Matsuyama *et al*., 2019), and helps digestion in *Saccostrea glomerata* (Green and Barnes, 2010). The host-specificity and the associated bacterial colonization might depend on the difference in selecting food particles and filtration for suspended particles in bivalves. Significant differences have been found in selecting food particles among oysters, mussels, and cockles (Rahman *et al*., 2020). Besides, the filtering capacity is different among bivalve species and varies over temperatures (Fuchs and Specht, 2018; Rahman *et al*., 2020). However, the direct impact of selecting and filtering different food types on gut bacterial composition has not been studied in bivalves yet. In the present study, taxonomic composition of gut bacteria was profiled in host fed micro-algae or macro-algae. Further studies need to correlate

with food selection and filtration rate and use metagenomics to characterize taxonomic composition with functions of gut microbiota.

Furthermore, both species diversity and taxonomic diversity were high in micro-algae fed oysters and macro-algae fed mussels, indicating that marine micro-algae fed oysters and macro-algae fed mussels were more fit in terms of bacterial diversity than other *priori* groups. In other studies, the bacterial diversity is low in the gut of *Mytilus coruscus* in high temperature (Li *et al.*, 2018). Likewise, several mortality events occurred in Pacific oysters *Crassostrea gigas* in summer (Costil *et al*., 2005, Dégremont *et al*., 2010, Fleury and Huvet, 2012) and blue mussel *Mytilus edulis* (Mallet *et al*., 1990; Myrand *et al*., 2000). These events suggest that low bacterial diversity and high temperature may out-balance the abundance of beneficial and pathogenic bacteria, making bivalves more susceptible to stress and diseases. This also indicates a definite interconnection with bacterial diversity and diseases susceptibility. Therefore, a high bacterial diversity would lead to health and fitness bivalves.

The innate immunity in marine bivalves with associated microbes has not been extensively explored, compared with mammals, but a similar microbial association for the development of immunity is expected. Gut bacteria act as a physical barrier inhibiting invasion of pathogens into the host gut to improve the host immunity. Colonization of beneficial bacteria in gut mucosal surface can exclude pathogens by limiting nutrients and space in the binding sites. In addition, certain microbes show antagonistic activity by preventing colonization of pathogens in gut. Thus, modulation of gut microbes using probiotics of beneficial bacteria might improve the health status of marine invertebrates in aquaculture. The probiotic is an approach to use the live microbial supplement and modulate the microbial community in the host or the

ambient environment (Verschuere *et al*., 2000). Similar to our study, micro-algae *Isochrysis galbana* was used to feed a mussel (*Mytilus chilensis*) and a clam (*Mulinia edulis*) to improve animal growth and health (Velasco & Navarro, 2005). In another study, Avendaño & Riquelme, (1999) fed bivalves with mixed-culture probiotic and micro-algae, *Isochrysis galbana* and improved bivalve growth. Besides, green macro-alga *Ulva lactuca* could suppress harmful algal bloom species (Tang & Gobler, 2011). Our study confirmed that micro-algae (*Isochrysis galbana*) fed oysters and macro-algae (*Ulva*) fed mussels were more diverse in species and taxonomic diversity.

5.6. Conclusion

The diet type strongly affected the gut microbial composition in both bivalve guts *Crassostrea gigas* and *Mytilus galloprovincialis*. Dietary manipulation could affect the gut microbial community, but the response of bacteria to diet change varied with feed types across two bivalve species. Gamma-proteobacteria and Bacteroidia became abundant in microalgae fed oysters and mussels, respectively. In contrast, Bacteroidia and Fusobacteria were abundant in macroalgae fed oysters and mussels. Besides, host species could directly impact the gut bacterial community. For example, Spirochaeta was absent in mussel guts before and at the end of the trial, whereas it was present in oyster guts during the entire period of study. The information about dietary manipulation related to microbial dynamics helps modulate microbial symbiosis in different molluscan species. Modulation of gut microbes could be used to favour the growth of beneficial bacteria in marine bivalves in aquaculture. Future research is recommended to further correlate with food selection and filtration rate in bivalve species and its functional metagenomics with taxonomic characterization.

5.7. References

Aceves, A. K., Johnson, P., Bullard, S. A., Lafrentz, S., & Arias, C. R. (2018). Description and characterization of the digestive gland microbiome in the freshwater mussel *Villosa nebulosa* (Bivalvia: Unionidae). *Journal of Molluscan Studies*, *84*(3), 240-246.

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26(1), 32-46.

Avendaño, R. E., & Riquelme, C. E. (1999). Establishment of mixed-culture probiotics and microalgae as food for bivalve larvae. *Aquaculture Research*, *30*(11-12), 893-900.

Baldo, L., Riera, J. L., Tooming-Klunderud, A., Albà, M. M., & Salzburger, W. (2015). Gut microbiota dynamics during dietary shift in eastern African cichlid fishes. *PLoS One* 10: e0127462.

Bray, J. R., & Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs*, 27(4), 325-349.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A. P., Schaumburg, F., Becker, K., Wos-Oxley, M. L., & Pieper, D. H. (2014). Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environmental Microbiology*, 16(9), 2939-2952.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., & Huttley, G. A. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5), 335.

Cardoso, A. M., Cavalcante, J. J., Vieira, R. P., Lima, J. L., Grieco, M. A. B., Clementino, M. M., Vasconcelos, A. T. R., Garcia, E.S., de Souza, W., Albano, R.M., & Martins, O. B. (2012). Gut bacterial communities in the giant land snail *Achatina fulica* and their modification by sugarcane-based diet. *PloS One*, *7*(3), e33440.

Clarke, K. R., Gorley, R. N., Somerfield, P. J., & Warwick, R. M. (2014). Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd.

Clarke, S. F., Murphy, E. F., Nilaweera, K., Ross, P. R., Shanahan, F., O'Toole, P. W., & Cotter, P. D. (2012). The gut microbiota and its relationship to diet and obesity: new insights. *Gut Microbes*, *3*(3), 186-202.

Cleary, D. F., Becking, L. E., Polónia, A. R., Freitas, R. M., & Gomes, N. C. (2015). Composition and predicted functional ecology of mussel-associated bacteria in Indonesian marine lakes. *Antonie van Leeuwenhoek*, *107*(3), 821-834.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C.R. & Tiedje, J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, *42*(D1), D633-D642.

Costil, K., Royer, J., Ropert, M., Soletchnik, P., & Mathieu, M. (2005). Spatio-temporal variations in biological performances and summer mortality of the Pacific oyster *Crassostrea gigas* in Normandy (France). *Helgoland Marine Research*, *59*(4), 286-300.

Cranford, P. J., Ward, J. E., & Shumway, S. E. (2011). Bivalve filter feeding: variability and limits of the aquaculture biofilter. *Shellfish Aquaculture and the Environment*, 81-124.

Dégremont, L., Soletchnik, P., & Boudry, P. (2010). Summer mortality of selected juvenile pacific oyster *Crassostrea gigas* under laboratory conditions and in comparison, with field performance. *Journal of Shellfish Research*, *29*(4), 847-856.

Doré, J., & Blottière, H. (2015). The influence of diet on the gut microbiota and its consequences for health. *Current Opinion in Biotechnology*, *32*, 195-199.

Edgar R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10 996–998. 10.1038/nmeth.2604.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

Fleury, E., & Huvet, A. (2012). Microarray analysis highlights immune response of Pacific oysters as a determinant of resistance to summer mortality. *Marine Biotechnology*, *14*(2), 203-217.

Fuchs, H. L., & Specht, J. A. (2018). Evidence for diverse responses to viscosity in suspension-feeding bivalves: Reply to Riisgård & Larsen (2018). *Marine Ecology Progress Series*, *596*, 267-270.

Graf, D., Di Cagno, R., Fåk, F., Flint, H. J., Nyman, M., Saarela, M., & Watzl, B. (2015). Contribution of diet to the composition of the human gut microbiota. *Microbial Ecology in Health and Disease*, *26*(1), 26164.

Green, T. J., & Barnes, A. C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the *paramyxean* parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology*, 109(2), 613-622.

Griffin, T. W., Baer, J. G., & Ward, J. E. (2021). Direct comparison of fecal and gut microbiota in the blue mussel (*Mytilus edulis*) discourages fecal sampling as a proxy for resident gut community. *Microbial Ecology*, *81*(1), 180-192.

Hawkins, A. J. S., Bayne, B. L., Bougrier, S., Héral, M., Iglesias, J. I. P., Navarro, E., Smith, R. M. F., & Urrutia, M. B. (1998). Some general relationships in comparing the feeding physiology of suspension-feeding bivalve molluscs. *Journal of Experimental Marine Biology and Ecology*, *219*(1-2), 87-103.

Iehata, S., Nakano, M., Tanaka, R., & Maeda, H. (2014). Modulation of gut microbiota associated with abalone *Haliotis gigantea* by dietary administration of host-derived *Pediococcus* sp. Ab1. *Fisheries Science*, 80, 323-331.

Joyce, P. W., Kregting, L., & Dick, J. T. (2019). Relative impacts of the invasive Pacific oyster, Crassostrea gigas, over the native blue mussel, *Mytilus edulis*, are mediated by flow velocity and food concentration. *NeoBiota*, *45*, 19.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One*, *7*(12), e51475.

Legrand, T. P., Catalano, S. R., Wos-Oxley, M. L., Stephens, F., Landos, M., Bansemer, M. S., Stone, D. A. J., Qin, J. G., & Oxley, A. (2018). The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. *Frontiers in Microbiology*, 8, 2664.

Lemer, S., González, V. L., Bieler, R., & Giribet, G. (2016). Cementing mussels to oysters in the pteriomorphian tree: a phylogenomic approach. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1833), 20160857.

Li, Y. F., Yang, N., Liang, X., Yoshida, A., Osatomi, K., Power, D., Batista, F. M. & Yang, J. L. (2018). Elevated seawater temperatures decrease microbial diversity in the gut of *Mytilus coruscus*. *Frontiers in Physiology*, *9*, 839.

Lukwambe, B., Nicholaus, R., Zhao, L., Yang, W., Zhu, J., & Zheng, Z. (2020). Microbial community and interspecies interaction during grazing of ark shell bivalve (*Scapharca subcrenata*) in a full-scale bioremediation system of mariculture effluents. *Marine Environmental Research*, *158*, 104956.

Mallet, A. L., Carver, C. E. A., & Freeman, K. R. (1990). Summer mortality of the blue mussel in eastern Canada: Spatial, temporal, stock and age variation. *Marine Ecology Progress Series. Oldendorf*, *67*(1), 35-41.

Matsuyama, T., Takano, T., Nakayasu, C., Odawara, K., Tsuchihashi, Y., Tanaka, S., Yasuike, M., Fujiwara, A., Nakamura, Y., & Masaoka, T. (2019). Spatiotemporal dynamics of *Spirochaeta*, the putative etiologic agent of Akoya oyster disease in pearl oysters, as determined by quantitative PCR. *Aquaculture*, *513*, 734433.

Miyake, S., Ngugi, D. K., & Stingl, U. (2015). Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology*, *24*(3), 656-672.

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., Henrissat, B., Knight, R. & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, *332*(6032), 970-974.

Myrand, B., Guderley, H., & Himmelman, J. H. (2000). Reproduction and summer mortality of blue mussels *Mytilus edulis* in the Magdalen Islands, southern Gulf of St. Lawrence. *Marine Ecology Progress Series*, *197*, 193-207.

Pierce, M. L., & Ward, J. E. (2018). Microbial ecology of the Bivalvia, with an emphasis on the family *Ostreidae*. *Journal of Shellfish Research*, *37*(4), 793-806.

Pierce, M. L., & Ward, J. E. (2019). Gut microbiomes of the eastern oyster (*Crassostrea virginica*) and the blue mussel (*Mytilus edulis*): Temporal variation and the influence of marine aggregate-associated microbial communities. *Msphere*, *4*(6), e00730-19.

Pimentel, Z. T., Dufault-Thompson, K., Russo, K. T., Scro, A. K., Smolowitz, R. M., Gomez-Chiarri, M., & Zhang, Y. (2021). Microbiome analysis reveals diversity and function of Mollicutes associated with the eastern oyster, *Crassostrea virginica*. *Msphere*, *6*(3), e00227-21.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590-D596.

Rahman, M. A., Henderson, S., Miller-Ezzy, P. A., Li, X. X., & Qin, J. G. (2020). Analysis of the seasonal impact of three marine bivalves on seston particles in water column. *Journal of Experimental Marine Biology and Ecology, 522*, 151251.

Rosa, M., Ward, J. E., Ouvrard, M., Holohan, B. A., Espinosa, E. P., Shumway, S. E., & Allam, B. (2015). Examining the physiological plasticity of particle capture by the blue mussel, *Mytilus edulis* (L.): Confounding factors and potential artifacts with studies utilizing natural seston. *Journal of Experimental Marine Biology and Ecology*, *473*, 207-217.

Roterman, Y. R., Benayahu, Y., Reshef, L., & Gophna, U. (2015). The gill microbiota of invasive and indigenous *Spondylus* oysters from the Mediterranean Sea and northern Red Sea. *Environmental Microbiology Reports*, *7*(6), 860-867.

Rothe, M., & Blaut, M. (2013). Evolution of the gut microbiota and the influence of diet. *Beneficial Microbes*, *4*(1), 31-37.

Santibáñez, P., Romalde, J., Maldonado, J., Fuentes, D., & Figueroa, J. (2022). First characterization of the gut microbiome associated with *Mytilus chilensis* collected at a mussel farm and from a natural environment in Chile. *Aquaculture*, *548*, 737644.

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. *Pharmacological Research*, *69*(1), 52-60.

Specht, J. A., & Fuchs, H. L. (2018). Thermal and viscous effects of temperature on *Mercenaria mercenaria* suspension feeding. *Marine Ecology Progress Series*, *589*, 129-140.

Tang, Y. Z., & Gobler, C. J. (2011). The green macroalga, *Ulva lactuca*, inhibits the growth of seven common harmful algal bloom species via allelopathy. *Harmful Algae*, *10*(5), 480-488.

Vaughn, C. C., & Hakenkamp, C. C. (2001). The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology*, *46*(11), 1431-1446.

Velasco, L. A., & Navarro, J. M. (2005). Feeding physiology of two bivalves under laboratory and field conditions in response to variable food concentrations. *Marine Ecology Progress Series*, *291*, 115-124.

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, 64(4), 655-671.

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L., & Pruzzo, C. (2018). Comparative 16S rDNA gene-based microbiota profiles of the Pacific oyster (*Crassostrea gigas*) and the Mediterranean mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microbial Ecology*, 75(2), 495-504.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261-5267.

Weingarten, E. A., Atkinson, C. L., & Jackson, C. R. (2019). The gut microbiome of freshwater Unionidae mussels is determined by host species and is selectively retained from filtered seston. *Plos One*, *14*(11), e0224796.

Woodcock, S., Van Der Gast, C. J., Bell, T., Lunn, M., Curtis, T. P., Head, I. M., & Sloan, W. T. (2007). Neutral assembly of bacterial communities. *FEMS Microbiology Ecology*, 62, 171-180.

Wu, G. D., Bushmanc, F. D., & Lewis, J. D. (2013). Diet, the human gut microbiota, and IBD. *Anaerobe*, *24*, 117-120.

Yang, L., Lv, L., Liu, H., Wang, M., Sui, Y., & Wang, Y. (2021). Effects of ocean acidification and microplastics on microflora community composition in the digestive tract of the thick shell mussel *Mytilus coruscus* through 16S RNA gene sequencing. *Bulletin of Environmental Contamination and Toxicology*, *107*(4), 616-625.

Zhang, J., Kobert, K., Flouri, T., & Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics*, 30(5), 614-620.

Zhao, J. S., Manno, D., & Hawari, J. (2009). *Psychrilyobacter atlanticus* gen. nov., sp. nov., a marine member of the phylum Fusobacteria that produces H2 and degrades nitramine explosives under low temperature conditions. *International Journal of Systematic and Evolutionary Microbiology*, *59*(3), 491-497.

Table 5.1. Experimental design listing samples collected and sequenced to compare the gut bacterial community after a 56-day feeding trial in Pacific oysters and Mediterranean mussels.

Species	Oyster			Mussel		
Trial	Pre-trial	Micro-algae* fed	Macro-algae* fed	Pre-trial	Micro-algae* fed	Macro-algae* fed
No. of bivalve samples	10	15	15	10	15	15
Water samples (2 L)	-	3	3	-	3	3
Time of collection	Before trial	After trial	After trial	Before trial	After trial	After trial

*****Micro-algae (*Isochrysis galbana*) and Macro-algae (*Ulva* sp.) were used in the feeding trial.

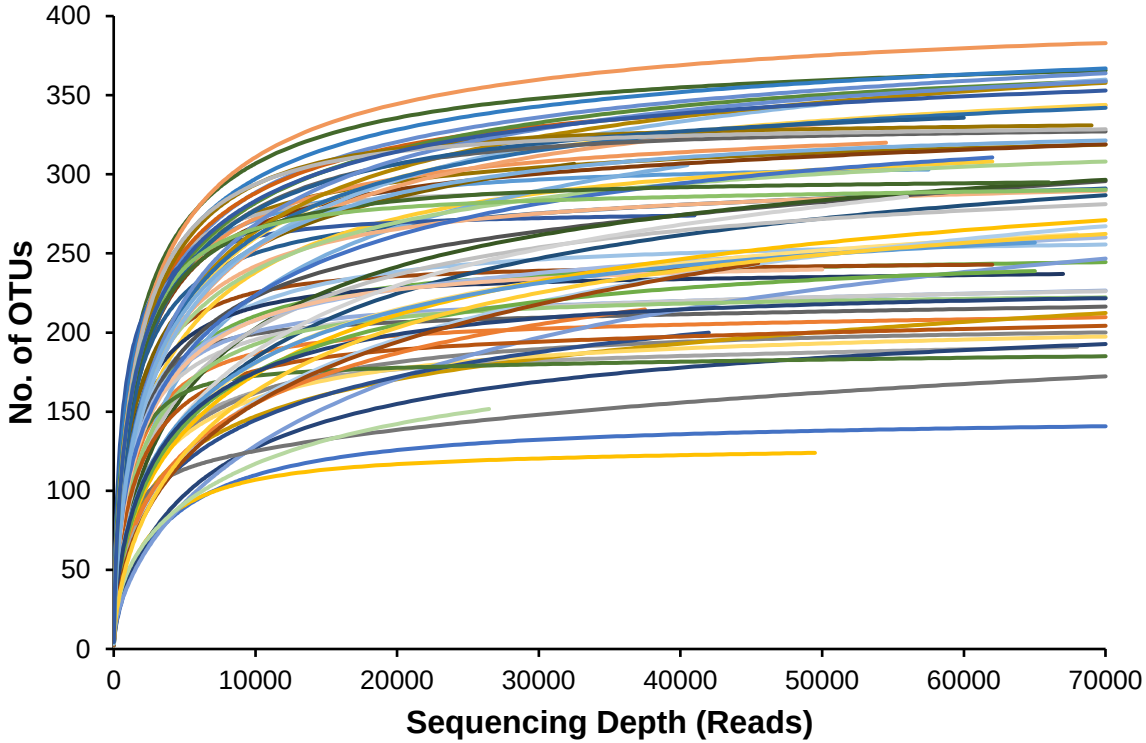


Figure 5.1. Rarefaction curves showing the number of resolved OTUs against sequencing depth of the final 73 gut samples analysed (39 oysters and 34 mussels).

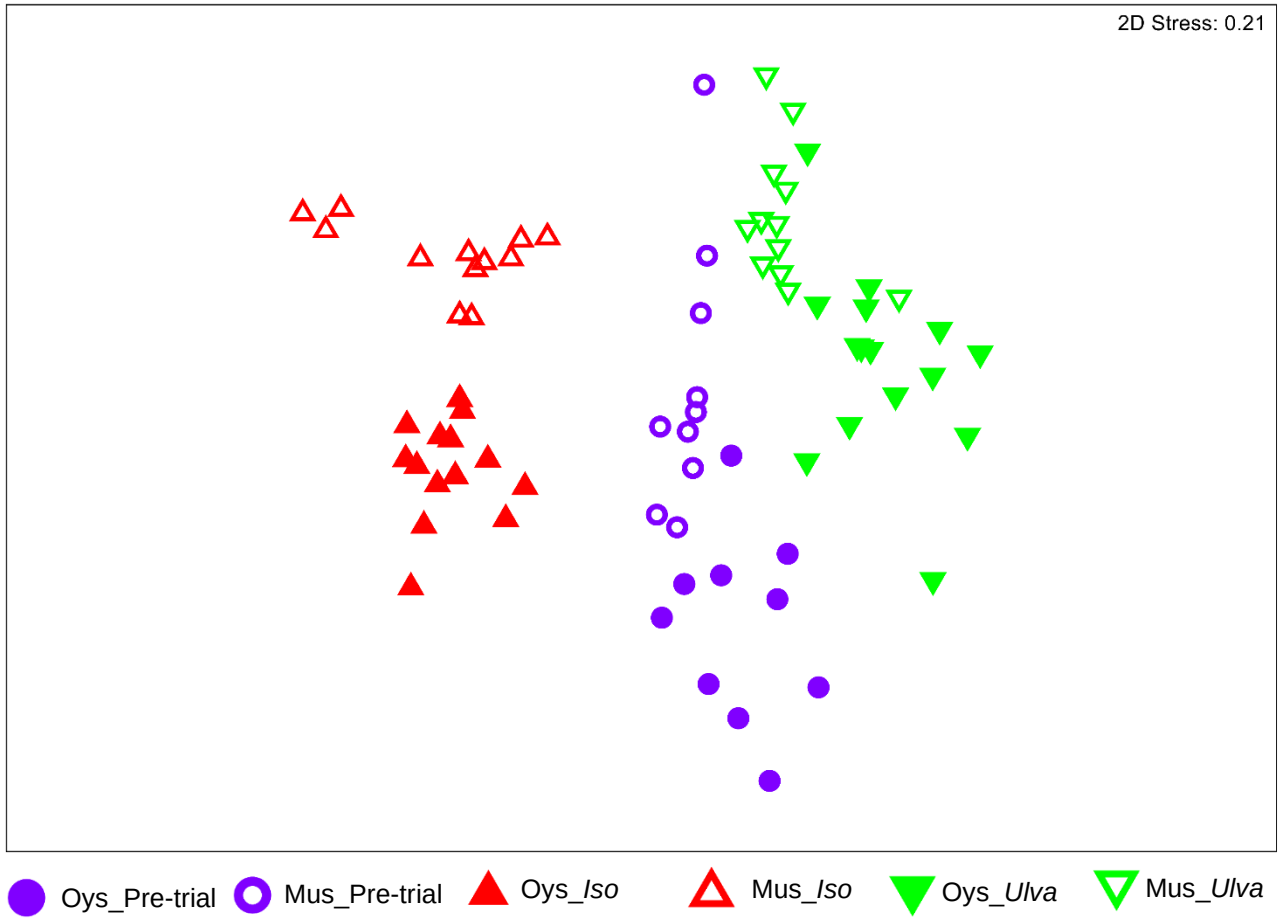


Figure 5.2. Ordination plot representing the differences in the global bacterial community composition among pre-trial, micro-algae (*Isochrysis galbana*) fed, and macro-algae (*Ulva*) fed oyster and mussel guts as assessed by non-metric

multidimensional scaling (nMDS) using Bray-Curtis dissimilarity.

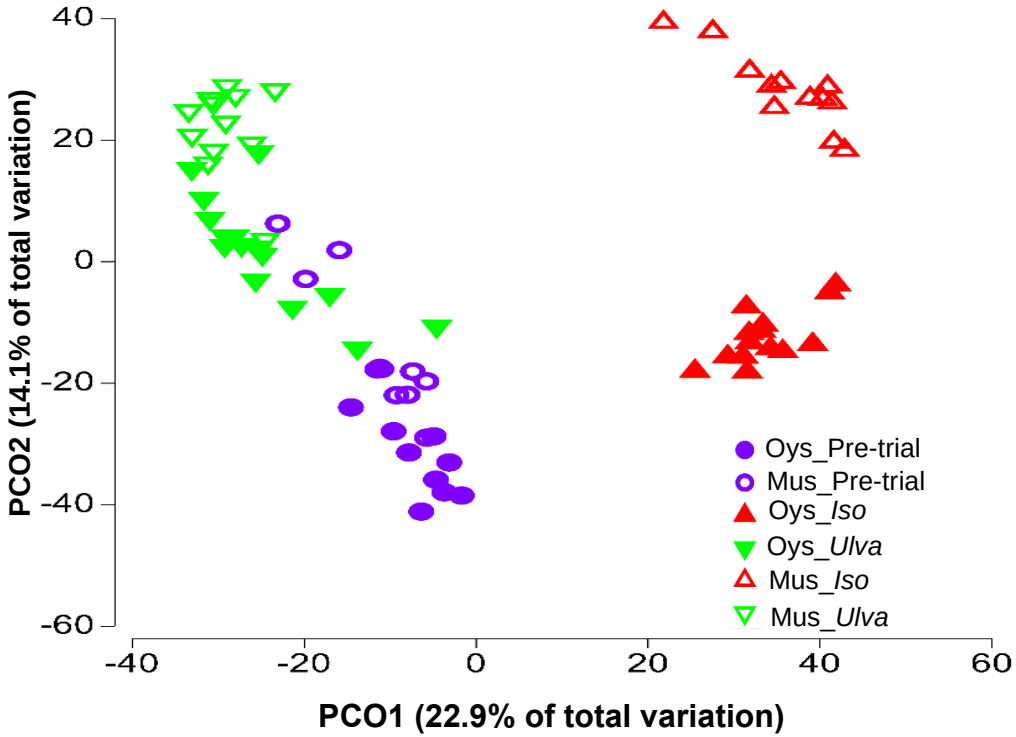


Figure 5.3. Ordination plot showing the similarities in the gut bacterial communities among pre-trial, micro-algae (*Isochrysis galbana*) fed and macro-algae (*Ulva*) fed oysters and mussels, as assessed by Principal Co-ordinates Analysis (PCoA).

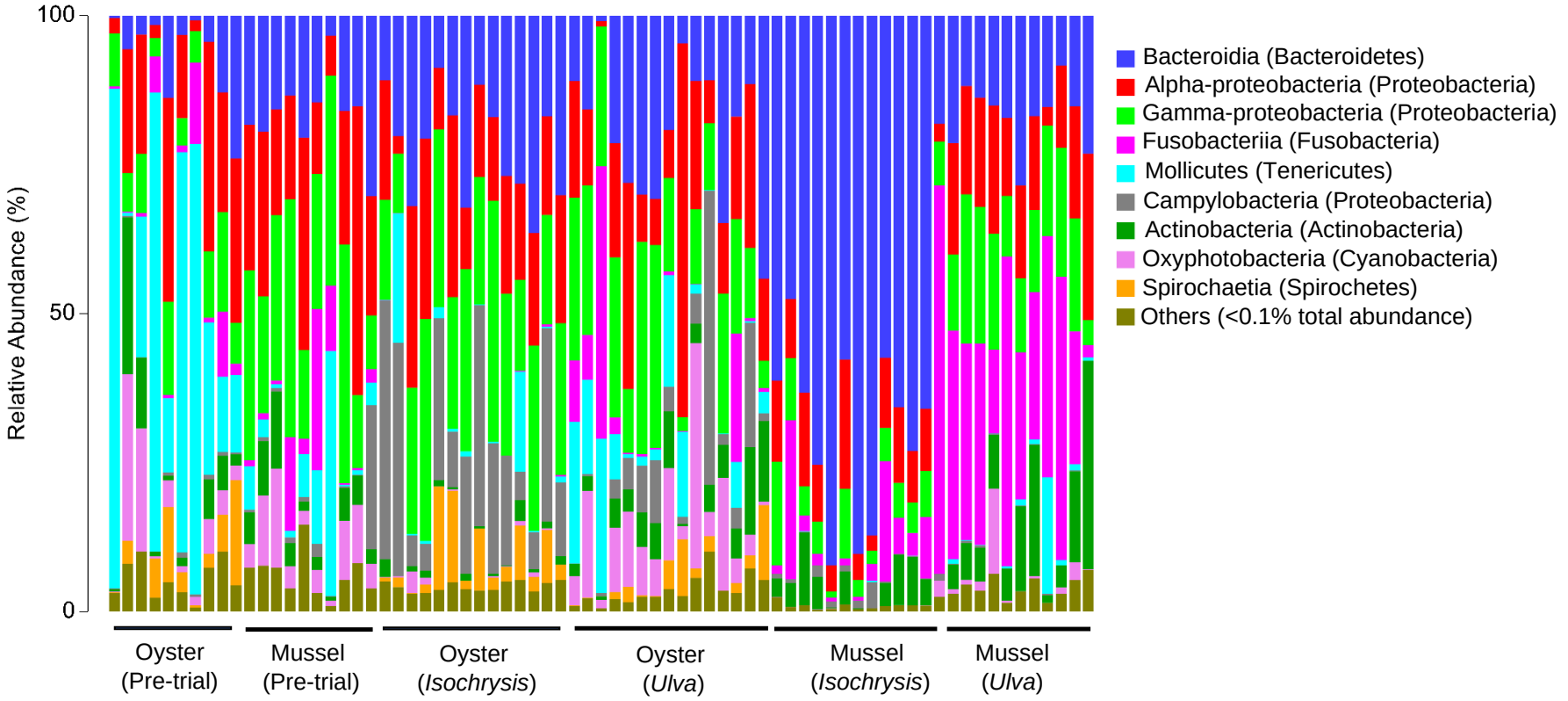
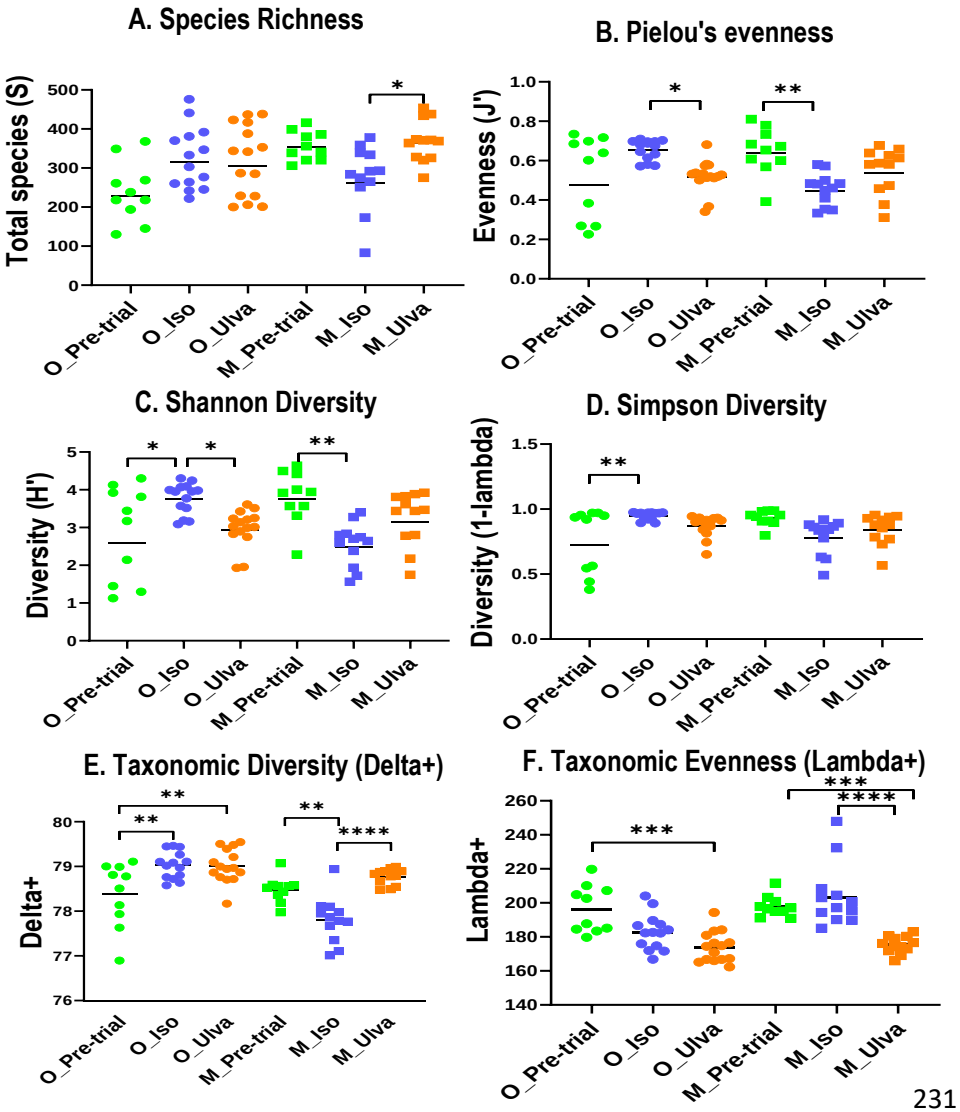


Figure 5.4. Mean relative abundances (%) of bacterial classes (phylum) associated with oyster and mussel guts before starting the feeding trial and post-feeding trial. Oyster (Pre-trial) = Pre-trial oyster gut samples, Mussel (Pre-trial) = Pre-trial mussel gut samples, Oyster (*Isochrysis*) =



Micro-algae, *Isochrysis galbana* fed oyster gut samples, Oyster (*Ulva*) = Macro-algae, *Ulva* sp. fed oyster gut samples, Mussel (*Isochrysis*) = Micro-algae, *Isochrysis galbana* fed mussel gut samples, Mussel (*Ulva*) = Macro-algae, *Ulva* sp. fed mussel gut samples.

Figure 5.5. Diversity indices between bivalve species and algae type used in the feeding trial.

A. Species richness (S), **B.** Pielou's evenness (J),

C. Shannon diversity (H), **D.** Simpson diversity,

E. Taxonomic diversity (Delta+), and

F. Taxonomic evenness (Lambda+) using Welch's t-test. Asterisks represent the level of significance ($p < 0.05$).

O=Oyster, M=Mussel, Pre-trial=Before start

feeding trial, Iso=*Isochrysis galbana*, micro-algae fed, Ulva=*Ulva* Sp, macro-algae fed.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1. Introduction

Microbes are vital for most aspects of animal life history, and the microbial association has drawn attention to better understand biological and ecological systems. Bacterial symbiosis is an ever-present feature of every biotic system and the base for the growth and evolution of eukaryotic organisms. Many studies have focused on teleosts gut bacteria (Cahill, 1990; Pérez *et al*., 2010), along with humans and mammals, including the gnotobiotic zebrafish model (Rawls *et al*., 2004; Roeselers *et al.,* 2011). However, gut bacteria in marine invertebrates are relatively less studied than mammals and fishes, but bacterial community structure of the bivalves (Tanaka *et al*., 2004; Hernández-Zárate *et al*., 2006) and crustaceans (Rungrassamee *et al*., 2014; Chen *et al*., 2015) and echinoderms (Amaro *et al*., 2009; Gao *et al*., 2014) are well characterized. Marine invertebrates living in the different ecological niches have diverse microbial associations, and the evolutionary forces drive the emergence and existence of these associations. The host-bacteria interaction is vital for metabolism, nutrition, and immune function to prevent pathogenic diseases and mortality. Mass mortality and infectious diseases are global concerns in marine invertebrate aquaculture. Generally, pathogenic diseases are manifested by the synergistic impacts of pathogens, environmental stress, and physiological imbalance of host. Invertebrates have a non-adaptive, innate immunity to protect pathogenic invaders (Cooper *et al*., 1992; Rinkevich, 1996; Frank *et al*., 1997). Therefore, it is intrinsically difficult to treat an infected population of marine invertebrates because (1) they cannot be treated with antigen as a preventive measure, (2) their enormous number and relatively smaller size so mass treatment can only be applied even if only a group of individuals are infected, (3) some species, sedentary bivalves, for example, are cultured in open sea which is not suitable for chemotherapeutic applications and

(4) the microbial association and susceptibility to disease varies at different life stages. Considering these limitations, I believe that understanding microbial dynamics is essential for the health management of marine invertebrates.

In a healthy ecosystem, animals maintain microbial equilibrium and protect themselves from pathogenic diseases. The microbes contribute to an animals' fitness, but their modulations impact the chance of survival. Therefore, the microbial diversity in marine invertebrates has become increasingly important to understand microbial symbiosis. In aquaculture (particularly semi-intensive and intensive), animals are exposed to various stressors that might cause an imbalance of microbial equilibrium. The high animal density, imbalanced nutrition, pollution and anthropogenic disturbances of aquatic systems favour the multiplication of pathogenic microbes. Physiological and environmental stresses induced modulations of gut bacteria may lead to the establishment of non-indigenous opportunists and predispose host to the potential pathogens. Thus, gut bacteria dynamics are important considerations for disease management in aquaculture. Characterization of gut bacteria will contribute to understanding host-microbe relations and the possibility of identifying potentially probiotic bacteria for aquaculture. The application of probiotics and prebiotics have already been proven useful for aquaculture. The gut bacteria community structure and their dynamics due to seasonal, environmental, and dietary change will improve gut microbial interaction, efficiently applying probiotics and prebiotics. As such, marine invertebrate health can be better managed by an advanced understanding of their gut bacteria. In this context, the modulation of gut bacteria through probiotics and prebiotics is becoming a health management tool in aquaculture (Ninawe and Selvin, 2009; Mohapatra *et al*., 2013).

This thesis summarises the gut bacteria community structure and its dynamics in marine invertebrates, particularly the species with aquaculture importance, such as Pacific oyster *Crassostrea gigas* and Mediterranean mussel *Mytilus galloprovincialis*.

6.2. Key Research Outcomes

The main research aim was to know the bacterial community composition and evaluate if the gut microbial change is related to species, habitat, season, or feed composition. The thesis is presented in four data chapters, each with specific objectives. The key research outcomes are summarized below chapter-wise.

6.2.1. Gut Bacteria between Two Inter-generic Bivalves

The gut bacterial composition differed in abundance between two phylogenetically related co-habiting farmed Pacific oyster *Crassostrea gigas* and wild Mediterranean mussel *Mytilus galloprovincialis.* Tenericutes was the main taxon driving change of relative abundances across two bivalve guts. Besides, Tenericutes and Spirochaetes were dominant in the gut of both bivalve species but almost absent in seawater. The peak abundance of *Mycoplasma* spp. belonging to Tenericutes occurred in summer in the gut of both bivalves. The influence of host phylogeny on bivalve gut bacterial communities was clarified by getting the majority (~90%) shared bacterial OTUs out of 644 OTUs, and only 35 were unique to oysters 28 to mussels (irrespective of the season). Some inter-generic differences across the bivalves were detected, and the top three most prevalent taxa were *Anaplasmataceae*, *Spirochaetaceae* and *Mycoplasmataceae* in oysters, while in mussels, the most prevalent were *Mycoplasmataceae* and *Spiroplasmataceae*. Furthermore, the abundance of bacterial families and OTUs also differed among gut samples, and certain taxa preferred one bivalve species to the other. Along with these inter-generic differences, intra-generic differences were also observed between size-based mussel cohorts. The phyla-level

shifts between summer and winter were noticed in the gut bacterial community composition of oysters and mussels. The species-level had a more significant influence on global gut bacterial community structure, followed by season. At the global bacterial community level, changes in species/OTU richness and diversity (Shannon and Simpson's diversity and Pielou's evenness) were observed in both oysters and mussels, with a marked increase in these indices' measures occurring in winter. When the season changes, the taxonomic bacterial diversity changes in oysters, whereas in mussels, the taxonomic diversity does not change, but species diversity does.

6.2.2. Gut Bacterial Composition Between Two Different Habitats

Comparatively, the gut bacterial community in nutrient-rich Coffin Bay oysters had higher species diversity than in nutrient-lean Franklin Harbor oysters, and the bacterial diversity was higher in winter than in summer. Oysters in both habitats accumulated a taxonomically similar group of bacteria, and the relative abundance of bacterial community varied with seasons. In particular, Mollicutes (Tenericutes) was the dominant phylum in summer, which was decreased in winter, whereas Proteobacteria was dominant in winter oysters. Moreover, in Coffin Bay water, alpha-proteobacteria was dominant in both seasons, but in Franklin Harbor water, the ambient alpha-proteobacteria were abundant in winter, and gamma-proteobacteria were dominant in summer. Noticeably, *Vibrio* belonging to gamma-proteobacteria was most dominant (24.25%) in the water of Franklin Harbor in summer. Finally, oysters colonized host-specific bacteria in their gut, different from the surrounding water, and the abundance and pattern of bacterial diversity were both season and habitat-dependent. This study provides valuable information on the difference in

bacterial community between oyster gut and ambient water in two nutrient-contrasting habitats in summer and winter.

6.2.3. Seasonal Pattern of Gut Bacterial Composition

The effect of seasonal temperature was clearly elucidated on the microbial community in oyster gut and the natural environment and revealed the differences in the bacterial composition and abundance year-round in oyster gut and seawater. Particularly, Tenericutes was prevalent in oyster gut across the year, excluding winter, whereas Proteobacteria was prevalent in seawater. Interestingly, microbial diversity was different in two successive winters. Mollicutes (Phylum: Tenericutes) was a prevalent bacterial group in oyster gut throughout the year and almost absent in seawater, suggesting that this phylum is permanent microbes and host-specificity in oysters. In contrast, other relatively low bacterial groups such as Proteobacteria, Cyanobacteria, Actinobacteria, Bacteroidetes, and Spirochaetes are transient microbes from the environment through horizontal transmission. In another note, some seasonal differences were observed despite some similarities in season-wise diversity measures. Comparatively, the second winter oysters were higher in species richness but lower in taxonomic diversity than other four *priori* groups.

6.2.4. Impact of Feed Composition on the Gut Bacteria

The present study indicates the direct impacts of food type and host species on gut bacterial community. The differences in the global gut bacterial assemblages in the pre-trial, micro-algae and macro-algae fed oysters, and mussels indicate that food type and host species can shape the composition of gut bacteria. The principal coordinates analysis (PCoA) also showed that type of algae had a comparatively more significant influence on gut bacterial community structure, followed by bivalve species. Furthermore, relative abundance of different bacterial taxa among three *priori* groups

in both oysters and mussels shows the impact of food type on gut bacterial community, and the change of bacterial abundance was species-specific. Though the gut bacterial communities between oysters and mussels were quite different, they were more similar within the individuals of same species.

In summary, Mollicutes, gamma-proteobacteria and Bacteroidia were the most dominant bacterial class in pre-trial, micro-algae fed and macro-algae fed oysters, respectively. On the other hand, gamma-proteobacteria, Bacteroidia and Fusobacteria were dominant in pre-trial, micro-algae fed mussels, and macro-algae fed mussels, respectively. In particular, Spirochaetia was present in oyster guts, but it was absent in mussel guts, revealing host-specificity in colonizing gut bacteria. Furthermore, species diversity and taxonomic diversity were high in micro-algae fed oysters and macro-algae fed mussels. The changes in diversity indices were both species-specific and algae type-specific and the changes varied among pre-trial, micro-algae and macro-algae fed oysters and mussels. However, food type was the main driver in regulating the gut bacterial community, followed by bivalve species.

6.3. Overall Discussion on the Dynamics of Gut Microbiota in Bivalves

6.3.1. Host Phylogeny and Season

Differences in the global bacterial community of each bivalve species to that of the surrounding environment in the present study suggest that marine bivalves living in the same habitat harbour different gut bacterial communities, and the composition of bacteria in gut and the environment changes with the season. Despite differences in the global community, both bivalves were colonized by similar bacterial taxa at the phylum and class levels, though its abundance was changed in different seasons. The previous literature also supports our present findings. For example, taxonomic similarity and compositional differences were documented in eastern oyster

Crassostrea virginica among digestive glands, shells and the surrounding environment (Arfken *et al.,* 2017). Seasonal difference was also reported in bacterial abundance in eastern oyster *Crassostrea virginica* and blue mussel *Mytilus edulis* (Pierce and Ward, 2019). In particular, I found Tenericutes as the main taxon driving the change of relative abundance of bacteria in gut between two bivalve species. At the family level, *Mycoplasmataceae* belongs to class Mollicutes (Phylum: Tenericutes) showed both seasonal and species-specific influence in the bacterial abundance. The higher abundance of several members of *Mycoplasmataceae* in both bivalve guts and their almost absence in seawater indicate a high level of host fidelity of Mollicutes in bivalves. This result is consistent with previous studies such as the dominance of Mollicutes in digestive gland of Sydney rock oysters *Saccostrea glomerata* (Green and Barnes, 2010), freshwater mussel *Villosa nebulosa* (Aceves *et al*., 2018), and the gut of abalone *Haliotis discus hannai* (Tanaka *et al*., 2004) and mussel *Brachidontes* (Cleary *et al*., 2015).

Together with the intergeneric differences in oysters and mussels, some intra-specific differences in the global bacterial community in Mediterranean mussel *M. galloprovincialis* were also reported in distinct size cohorts (>40 mm and <60 mm). The shell length of Mediterranean mussels ranges 41.9–48.9 mm at age 1yr and 51.2–63.1mm at age 2yrs (Okaniwa *et al*., 2010). The microbiome changes with the developmental stage, such as juveniles versus adults in *Crassostrea gigas* and *Crassostrea corteziensis* (Trabal *et al*., 2012). Moreover, both bivalve species were more diverse in winter in terms of bacterial species diversity (species richness), Shannon index, Simpson index and species evenness in the community. On the other hand, low microbial diversity was reported in *Mytilus coruscus* gut (Li *et al.,* 2018) and the *C. gigas* haemolymph (Lokmer and Wegner, 2015) at high temperatures. My

study also indicates that bivalve species has a more significant influence on the global gut bacterial community structure than the season.

6.3.2. Different Environmental Conditions

More bacterial diversity in oyster gut of nutrient-rich habitat (Coffin Bay) than nutrient lean habitat (Franklin Harbor) in terms of species richness, species diversity, species evenness, and Shannon and Simpson index suggests that Coffin Bay oysters are more resistant to environmental changes than Franklin Harbor oysters. In contrast, low bacterial diversity in nutrient lean Franklin Harbor may be less resilient to environmental stress, triggering oyster disease or mass mortality in summer. In addition, high bacterial diversity was reported in winter oysters in both habitats. High bacterial diversity was also found in the digestive gland of Manila clam, *Ruditapes philippinarum* in winter (Milan *et al*., 2018), and low bacterial diversity was also found in mussel gut *Mytilus coruscus* with increasing temperature from 27°C to 31 °C (Li *et al*., 2018). Consequently, low nutrients at high temperatures (Cliff, 1982) might result in low bacterial diversity and trigger environmental stress, leading to disease caused by opportunistic pathogens and mass mortality in summer.

The most dominant phylum was Tenericutes in oyster guts, whereas Proteobacteria in the water of both habitats. This result is consistent with Arfken *et al*. (2017), where Tenericutes was also dominant phylum in the digestive gland of eastern oyster (*Crassostrea virginca*) and Proteobacteria in sediment. Moreover, Mollicutes (Tenericutes), Sericytochromatia (Cyanobacteria) and Spirochaetia (Spirochaetes) were almost absent in water of both habitats indicating host specificity in colonizing specific bacteria in oyster gut. Likewise, microbial communities were different in eastern oyster, *Crassostrea virginica,* from two areas in Maine, USA (La Valley *et al*., 2009). When season changes, the gut bacteria respond differently in different habitats,

suggesting both seasonal and habitat impacts in the bacterial assemblages in oyster gut.

This study builds a baseline of a healthy oyster aquaculture system. On the other hand, Coffin Bay water was alpha-proteobacteria rich, regardless of season, and Franklin Harbor was alpha-proteobacteria rich in summer and gamma-proteobacteria rich in winter. Alpha-proteobacteria was also prevalent in the mesotrophic Lake Biwa, Japan, throughout the seasons (Nishimura & Nagata, 2007). Franklin Harbor has shallow coastal water enclosed with a large mangrove area, which might be why a higher abundance of gamma-proteobacteria was in Franklin Harbor water. Most gamma-proteobacteria are sulfer-oxidizing (Yamamoto and Takai, 2011; Patwardhan *et al*., 2018), and waters in mangrove areas are predominant in sulfer-oxidizing bacteria (Al-Sayed *et al*., 2005). In the present study, exclusively higher abundance (24.65%) of *Vibrio* (Gamma-proteobacteria) was reported in Franklin Harbor water in summer. Though the mangrove sediments are low-nutrient environments (Alfaro-Espinoza and Ullrich, 2015), iron-rich (Kristensen *et al*., 2000), the iron-enrichment of the North Sea and East Mediterranean seawater can stimulate gamma-proteobacteria such as *Vibrio* spp. (Pinhassi and Berman, 2003). Thus, in our current study, Franklin Harbor as a mangrove area might be enriched with iron, favouring the higher abundance of *Vibrio* (Gamma-proteobacteria).

6.3.3. Different Seasons in a Year

The seasonal pattern of bacterial composition in the gut of Pacific oysters and the surrounding environment in Coffin Bay has revealed some degrees of seasonal differences in composition and its relative abundance year-round. The beta diversity reveals different bacterial clusters of the global bacterial community in oyster gut and seawater. The oyster gut can establish different microbial communities from the

seawater. The difference of microbiota between animal tissues and the surrounding water was also found in eastern oyster *Crassostrea virginica* (La Valley *et al*., 2009) and Pacific oyster *C. gigas* (Asmani *et al*., 2016). For instance, Mollicutes belonging to the Phylum Tenericutes was prevalent in oyster gut throughout the year, although it was almost absent in seawater.

The dominance of Mollicutes as permanent residential microbes was also evident in the gut or digestive gland of eastern oyster *Crassostrea virginica* (Pimentel *et al*., 2021), Sydney rock oysters *Saccostrea glomerata* (Green and Barnes, 2010), and freshwater mussel *Villosa nebulosa* (Aceves *et al.,* 2018). In the present study, however, the alpha-proteobacteria and Bacteroidia were relatively abundant in seawater but were very low in oyster gut, suggesting that oysters could obtain and enrich transient microbes from the environment to oyster gut. The marine microbial communities are quite complex to maintain their ecological succession over time. The phyla Proteobacteria and Bacteroidetes are ubiquitous in the marine and coastal surface waters (Eilers *et al*., 2000; Yin *et al*., 2013). In the present study, the oyster gut was only enriched with gamma-proteobacteria (34%) in winter but was comparatively low in other seasons. Similarly, the abundance of gamma-proteobacteria is high in winter in the gill of northern red sea oysters *Spondylus spinosus* but low in summer (Roterman *et al.,* 2015).

The alpha-diversity also indicates some seasonal differences of the gut bacterial community in oyster. I found more taxonomic diversity in the second winter oyster samples than in other four seasons. Likewise, microbial diversity decreases in the *Mytilus coruscus* gut at higher temperatures (Li *et al*., 2018). Though the species richness of two consecutive winter samples was similar, the taxonomic diversity was higher in the first winter oysters. This result indicates the effect of other confounding

factors on the microbial community, such as upwelling (Wilson *et al*., 2018), salinity (Pinnell and Turner, 2020; Song *et al*., 2021), dissolved oxygen (Cao *et al*., 2018), and pH (Meron *et al*., 2012; Crummett, 2020; Nelson *et al*., 2020).

6.3.4. Feed Composition

Both alpha and beta diversities revealed a direct impact of food type on the gut microbiota in both inter-generic bivalves *Crassostrea gigas* and *Mytilus galloprovincialis*, but the degree of impact differed between bivalve species. The beta diversity determines the distinct gut bacterial assemblages in pre-trial, micro-algae (*Isochrysis galbana*) and macro-algae fed (*Ulva* sp.) oysters and mussels, signifying the impact of diet type and host species in shaping gut bacterial composition. Miyake *et al*. (2015) reported distinct gut bacterial patterns of micro and macro-algavores, such as surgeonfish (Family: Acanthuridae) and three other coral reef fish species of the central Red Sea, and both host phylogeny and diet type can drive the gut bacterial changes. In the present study, principal coordination also suggests that the diet (algae) type has a more significant influence than the host species in structuring the gut bacterial community. The gut bacteria of mammalian species also show a similar result of clustering bacterial community along with diet rather than host phylogeny (Muegge *et al*., 2011). In particular, among significant changes, Mollicutes (Phylum: Tenericutes) was the most dominant bacteria (39.13%) in pre-trial oysters. However, it became less abundant in both micro-algae fed oysters (3.17%) and macro-algae fed oysters (8.22%), indicating the impact of diet type. Besides, the relative abundance of Mollicutes was changed after the feeding trial in pre-trial (7.76%), micro-algae fed (0.03%), and macro-algae fed (2.09%) mussels, indicating the change of gut microbiota depends on both type of dietary algae and host species. Mollicutes can be abundant in the gut of other oyster and mussel species such

as Sydney rock oysters *Saccostrea glomerata* (Green and Barnes, 2010), eastern oyster *Crassostrea virginica* gut (King *et al*., 2012; Pimentel *et al*., 2021), mussel *Brachidontes* sp. in an Indonesian lake (Cleary *et al*., 2015), and freshwater mussel *Villosa nebulosa* (Aceves *et al*., 2017). Surprisingly, Spirochaeta was absent in mussel guts at the start and end of the trial despite its prevalence in oysters, revealing host specificity in colonizing gut bacteria, which might have some specific function in oyster gut health. Spirochaetes occurs as symbionts in some oyster spp., such as Northern Red Sea oyster *Spondylus spinosus* (Roterman *et al*., 2015) and pearl oyster *Pinctada fucata* (Matsuyama *et al*., 2019), and helps with digestion in *Saccostrea glomerata* (Green and Barnes, 2010). Furthermore, the pattern of alpha diversity among pre-trial and two types of algae fed oysters and mussels clearly shows the effect of diet type in gut bacterial diversity. For instance, both species diversity and taxonomic diversity were high in micro-algae fed oysters. On the other hand, those measures were high in macro-algae fed mussels. This result indicates that dietary manipulation could affect the bivalve gut microbial community, but the bacteria response to diet varied with food types in two bivalve species.

6.4. Overall Conclusion

The present thesis has explored the dynamics of gut microbiota in bivalves from the perspectives of host phylogeny, habitat, season and feed. The research outcomes from characterizing gut bacteria community structure and their dynamics due to phylogenetic, seasonal, environmental and dietary change will improve our understanding of gut microbial interaction, effectively applying probiotics and prebiotics in bivalve farming. As a result, the health management of marine invertebrates can be improved through a better understanding of gut microbiota. The major conclusions are drawn as follows:

1. Host and season can shape the gut bacterial assemblages in inter-generic bivalves *C. gigas* and *M. galloprovincialis*. I found host-specificity in microbial colonization in bivalves. The low microbial diversity in oysters, especially in summer, may partially explain the vulnerability of oysters to bacterial infection and mass mortality in summer.
2. The variation of seasonal temperature and nutrient supply in the environment can influence bacterial population in water, thus affecting the gut bacteria in filter-feeding oysters. The high gut bacterial species diversity was found in a oceanic nutrient based habitat (Coffin Bay) compared to local nutrient based habitat (Franklin Harbor).
3. The present study shows the effect of seasonal temperature on microbe dynamics and characterizes the change of resident and transient microbes in oyster gut. Understanding the microbial community in oyster gut and environment will predict the occurrence of opportunistic pathogens in the ecosystem. The diversity and proliferation of opportunistic pathogens may serve as an indicator of oyster health.
4. The information about dietary manipulation related to microbial dynamics helps modulate microbial symbiosis in different molluscan species. Modulation of gut microbes could be used to favour the growth of beneficial bacteria in marine bivalves in aquaculture. In a low productive ecosystem, gut microbes may predict the event of mass mortality in bivalves.

6.5. Potential Implications in Aquaculture Industry/ Science Community

The profiling of bacterial community of the field-collected oyster gut samples helps explain community composition dynamics due to host species, seasons, and sites. In addition, bacterial community profiling mediated by diet types will be

helpful to understand gut bacterial symbiosis in controlled conditions. Finally, understanding microbial association will pave the way towards ecosystem-based aquaculture management particularly where multiple species are involved. The potential implications in the aquaculture industry and/or science community are as follows:

- To improve oyster fitness by characterizing resident and transient opportunistic pathogenic bacteria. The diversity and proliferation of opportunistic pathogens may serve as an indicator of oyster health.
- To predict the source of pathogenic bacteria in the environment, water quality, pollution sources. Maintaining a healthy aquatic system can increase productivity and prevent disease and oyster mortality.
- To predict the condition when pathogenic transient microorganisms can invade a host and weaken the host immunity, leading to susceptibility of the host to diseases.
- To predict the likelihood of bacterial epidemics in oysters by monitoring microbiota dynamics in oyster gut and the environment.
- To understand the gut-microbiota interactions for the application of probiotics. Modulating beneficial gut microbes can enhance growth and immunity.

6.6. Future Research Indications and Recommendations

The progress of marine invertebrate aquaculture is driving scientific interest to unveil the understanding of gut bacteria in nutrient metabolism and immune function. With the advent of advanced molecular approaches, the areas of research interest from taxonomic profiling to functional roles will need to be defined in the following decades. Future research directions are also proposed as follows,

- Future functional metagenomics with taxonomic characterization might reveal the link of maintaining gut bacterial communities in oysters of different habitats with their specific functional role in a specific organ, which will, in turn, give possible insight into host-microbiota symbiosis in a specific organ such as gut, digestive gland, gill, and skin.
- Future studies should consider other confounding intrinsic factors of the host, such as host developmental stages, age, and sampling tissues.
- Besides, extrinsic factors other than seasonal temperatures such as pH, salinity, and dissolved oxygen level monthly might better represent the host-associated microbial community and its surroundings.
- The impact of ecological processes to the gut bacterial assembly in bivalves should also be considered in future research.
- Further research needs to be conducted between two reproductive generations of the host to explore vertical transmission dynamics of microbiota from parents to offspring.
- Our present study recommends studying functional metagenomics with the taxonomic characterization of microbial community and its transmission dynamics (e.g., vertical, or horizontal) in different tissues together to get a clear representation of the microbiome of healthy organisms.

6.7. References

Aceves, A. K., Johnson, P., Bullard, S. A., Lafrentz, S., & Arias, C. R. (2018). Description and characterization of the digestive gland microbiome in the freshwater mussel *Villosa nebulosa* (Bivalvia: Unionidae). *Journal of Molluscan Studies*, *84*(3), 240-246.

Alfaro-Espinoza, G., & Ullrich, M. S. (2015). Bacterial N2-fixation in mangrove ecosystems: insights from a diazotroph–mangrove interaction. *Frontiers in Microbiology*, *6*, 445.

Alfaro-Espinoza, G., & Ullrich, M. S. (2015). Bacterial N2-fixation in mangrove ecosystems: insights from a diazotroph–mangrove interaction. *Frontiers in Microbiology*, *6*, 445.

Al-Sayed, H. A., Ghanem, E. H., & Saleh, K. M. (2005). Bacterial community and some physico-chemical characteristics in a subtropical mangrove environment in Bahrain. *Marine Pollution Bulletin*, *50*(2), 147-155.

Amaro, T., Witte, H., Herndl, G. J., Cunha, M. R., & Billett, D. S. (2009). Deep-sea bacterial communities in sediments and guts of deposit-feeding holothurians in Portuguese canyons (NE Atlantic). *Deep Sea Research Part I: Oceanographic Research Papers*, 56(10), 1834-1843.

Arfken, A., Song, B., Bowman, J. S., & Piehler, M. (2017). Denitrification potential of the eastern oyster microbiome using a 16S rRNA gene based metabolic inference approach. *PloS One,* 12(9).

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., & Nicolas, J. L. (2016). Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture*, *464*, 434-444.

Cahill, M. M. (1990). Bacterial flora of fishes: a review. *Microbial Ecology*, 19(1), 21-41.

Cao, Y., Kwok, B. H., van Loosdrecht, M., Daigger, G., Png, H. Y., Long, W. Y., & Eng, O. K. (2018). The influence of dissolved oxygen on partial nitritation/

anammox performance and microbial community of the 200,000 m3/d activated sludge process at the Changi water reclamation plant (2011 to 2016). *Water Science and Technology*, *78*(3), 634-643.

Chen, X., Di, P., Wang, H., Li, B., Pan, Y., Yan, S., & Wang, Y. (2015). Bacterial community associated with the intestinal tract of Chinese Mitten Crab (*Eriocheir sinensis*) Farmed in Lake Tai, China. *PloS One*, 10(4).

Cleary, D. F., Becking, L. E., Polónia, A. R., Freitas, R. M., & Gomes, N. C. (2015). Composition and predicted functional ecology of mussel-associated bacteria in Indonesian marine lakes. *Antonie van Leeuwenhoek*, 107(3), 821-834.

Cliff, G. (1982). Seasonal variation in the contribution of phytoplankton, bacteria, detritus and inorganic nutrients to a rocky shore ecosystem. *Transactions of the Royal Society of South Africa*, *44*(4), 523-538.

Cooper, E. L., Rinkevich, B., Uhlenbruck, G., & Valembois, P. (1992). Invertebrate immunity: another viewpoint. *Scandinavian Journal of Immunology*, 35(3), 247-266.

Crummett, L. T. (2020). Acidification decreases microbial community diversity in the Salish Sea, a region with naturally high pCO2. *Plos One*, *15*(10), e0241183.

Eilers, H., Pernthaler, J., Glöckner, F. O., & Amann, R. (2000). Culturability and in situ abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology*, *66*(7), 3044-3051.

Frank, U., Oren, U., Loya, Y., & Rinkevich, B. (1997). Alloimmune maturation in the coral *Stylophora pistillata* is achieved through three distinctive stages, 4 months post–metamorphosis. Proceedings of the Royal Society of London B: *Biological Sciences*, 264(1378), 99-104.

Gao, F., Li, F., Tan, J., Yan, J., & Sun, H. (2014). Bacterial community composition in the gut content and ambient sediment of sea cucumber *Apostichopus japonicus* revealed by 16S rRNA gene pyrosequencing. *PloS One*, *9*(6), e100092.

Green, T. J., & Barnes, A. C. (2010). Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology*, 109(2), 613-622.

Hernández-Zárate, G., & Olmos-Soto, J. (2006). Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. *Journal of Applied Microbiology*, 100(4), 664-672.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. *PLoS One*, *7*(12), e51475.

Kristensen, E., Andersen, F. Ø., Holmboe, N., Holmer, M., & Thongtham, N. (2000). Carbon and nitrogen mineralization in sediments of the Bangrong mangrove area, Phuket, Thailand. *Aquatic Microbial Ecology*, *22*(2), 199-213.

La Valley, K. J., Jones, S., Gomez-Chiarri, M., Dealteris, J., & Rice, M. (2009). Bacterial community profiling of the eastern oyster (*Crassostrea virginica*):

comparison of culture-dependent and culture-independent outcomes. *Journal of Shellfish Research*, *28*(4), 827-835.

Li, Y. F., Yang, N., Liang, X., Yoshida, A., Osatomi, K., Power, D., Batista, F. M. & Yang, J. L. (2018). Elevated seawater temperatures decrease microbial diversity in the gut of *Mytilus coruscus*. *Frontiers in Physiology*, *9*, 839.

Lokmer, A., & Wegner, K. M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *The ISME Journal*, *9*(3), 670-682.

Matsuyama, T., Takano, T., Nakayasu, C., Odawara, K., Tsuchihashi, Y., Tanaka, S., Yasuike, M., Fujiwara, A., Nakamura, Y. & Masaoka, T. (2019). Spatiotemporal dynamics of *Spirochaeta*, the putative etiologic agent of Akoya oyster disease in pearl oysters, as determined by quantitative PCR. *Aquaculture*, *513*, 734433.

Meron, D., Rodolfo-Metalpa, R., Cunning, R., Baker, A. C., Fine, M., & Banin, E. (2012). Changes in coral microbial communities in response to a natural pH gradient. *The ISME Journal*, *6*(9), 1775-1785.

Milan, M., Carraro, L., Fariselli, P., Martino, M. E., Cavalieri, D., Vitali, F., Boffo, L., Patarnello, T., Bargelloni, L. & Cardazzo, B. (2018). Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. *Aquatic Toxicology*, *194*, 195-207.

Miyake, S., Ngugi, D. K., & Stingl, U. (2015). Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology*, *24*(3), 656-672.

Mohapatra, S., Chakraborty, T., Kumar, V., Deboeck, G., & Mohanta, K. N. (2013). Aquaculture and stress management: a review of probiotic

intervention. *Journal of Animal Physiology and Animal Nutrition*, 97(3), 405-430.

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., Henrissat, B., Knight, R. & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, *332*(6032), 970-974.

Nelson, K. S., Baltar, F., Lamare, M. D., & Morales, S. E. (2020). Ocean acidification affects microbial community and invertebrate settlement on biofilms. *Scientific Reports*, *10*(1), 1-9.

Ninawe, A. S., & Selvin, J. (2009). Probiotics in shrimp aquaculture: avenues and challenges. *Critical Reviews in Microbiology*, 35(1), 43-66.

Nishimura, Y., & Nagata, T. (2007). Alphaproteobacterial dominance in a large mesotrophic lake (Lake Biwa, Japan). *Aquatic Microbial Ecology*, *48*(3), 231-240.

Okaniwa, N., Miyaji, T., Sasaki, T., & Tanabe, K. (2010). Shell growth and reproductive cycle of the Mediterranean mussel *Mytilus galloprovincialis* in Tokyo Bay, Japan: relationship with environmental conditions. *Plankton and Benthos Research*, *5*(Supplement), 214-220.

Patwardhan, S., Foustoukos, D. I., Giovannelli, D., Yücel, M., & Vetriani, C. (2018). Ecological succession of sulfur-oxidizing Epsilon-and Gammaproteobacteria during colonization of a shallow-water gas vent. *Frontiers in Microbiology*, *9*, 2970.

Pérez, T., Balcázar, J. L., Ruiz-Zarzuela, I., Halaihel, N., Vendrell, D., De Blas, I., & Múzquiz, J. L. (2010). Host–microbiota interactions within the fish intestinal ecosystem. *Mucosal Immunology*, *3*(4), 355-360.

Pierce, M. L., & Ward, J. E. (2019). Gut microbiomes of the eastern oyster (*Crassostrea virginica*) and the Blue mussel (*Mytilus edulis*): temporal variation and the influence of marine aggregate-associated microbial communities. *Msphere*, 4(6), pp.e00730-19

Pimentel, Z. T., Dufault-Thompson, K., Russo, K. T., Scro, A. K., Smolowitz, R. M., Gomez-Chiarri, M., & Zhang, Y. (2021). Microbiome analysis reveals diversity and function of mollicutes associated with the eastern oyster, *Crassostrea virginica*. *Msphere*, *6*(3), e00227-21.

Pinhassi, J., & Berman, T. (2003). Differential growth response of colony-forming α-and γ-proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean sea, and the Gulf of Eilat. *Applied and Environmental Microbiology*, *69*(1), 199-211.

Pinnell, L. J., & Turner, J. W. (2020). Temporal changes in water temperature and salinity drive the formation of a reversible plastic-specific microbial community. *FEMS Microbiology Ecology*, *96*(12), fiaa230.

Rungrassamee, W., Klanchui, A., Maibunkaew, S., Chaiyapechara, S., Jiravanichpaisal, P., & Karoonuthaisiri, N. (2014). Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). *PloS One*, *9*(3), e91853.

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the*

National Academy of Sciences of the United States of America, 101(13), 4596-4601.

Rinkevich, B. (1996). Immune responsiveness in marine invertebrates revisited: the concourse of puzzles. *New Directions in Invertebrate Immunology*, 55-90.

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., & Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, 5(10), 1595-1608.

Roterman, Y. R., Benayahu, Y., Reshef, L., & Gophna, U. (2015). The gill microbiota of invasive and indigenous *Spondylus* oysters from the Mediterranean Sea and northern Red Sea. *Environmental Microbiology Reports*, *7*(6), 860-867.

Rungrassamee, W., Klanchui, A., Maibunkaew, S., Chaiyapechara, S., Jiravanichpaisal, P., & Karoonuthaisiri, N. (2014). Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). *PloS One,* 9(3), e91853.

Song, Y., Gilleaudeau, G. J., Algeo, T. J., Over, D. J., Lyons, T. W., Anbar, A. D., & Xie, S. (2021). Biomarker evidence of algal-microbial community changes linked to redox and salinity variation, Upper Devonian Chattanooga Shale (Tennessee, USA). *Bulletin*, *133*(1-2), 409-424.

Tanaka, R., Ootsubo, M., Sawabe, T., Ezura, Y., & Tajima, K. (2004). Biodiversity and in situ abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture*, *241*(1-4), 453-463.

Trabal, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Asencio-Valle, F., Morales-Bojórquez, E., & Romero, J. (2012). Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. *Microbial Ecology*, *64*(2), 555-569.

Wilson, J. M., Litvin, S. Y., & Beman, J. M. (2018). Microbial community networks associated with variations in community respiration rates during upwelling in nearshore Monterey Bay, California. *Environmental Microbiology Reports*, *10*(3), 272-282.

Yamamoto, M., & Takai, K. (2011). Sulfur metabolisms in epsilon-and gamma-Proteobacteria in deep-sea hydrothermal fields. *Frontiers in Microbiology*, *2*, 192.

Yin, Q., Fu, B., Li, B., Shi, X., Inagaki, F., & Zhang, X. H. (2013). Spatial variations in microbial community composition in surface seawater from the ultra-oligotrophic center to rim of the South Pacific Gyre. *PLoS One*, *8*(2), e55148.