# Characterization of gut microbial composition and diversity of New Zealand wild abalone (*Haliotis iris*) under potential environmental influences

**Jinchen Guo**

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Primary Supervisor: Professor Donnabella C. Lacap-Bugler

Co-supervisor: Dr. Leonie Venter

External Supervisor: Professor Andrea C. Alfaro

**Thesis Abstract**

Abalone (*Haliotis* spp. in Phylum Mollusca) are marine gastropods that have a wide distribution between intertidal and subtidal zones from tropical to subarctic waters in both hemispheres. Abalone are exclusively herbivores grazing on various seaweed in their natural habitats that contribute to maintaining healthy algal reef ecosystems. Additionally, abalone also possess high economic value as a popular shellfish seafood choice in Asia and Pacific Islands. In Aotearoa New Zealand (NZ), *Haliotis iris* is an endemic abalone species that is both farmed and caught in the wild. Differentiated abalone growth rates among the wild abalone populations are historically documented. While the exact explanations for this growth rate phenomenon are unclear, food digestion and nutrient absorption is one research avenue being looked at due to their energy and nutrient support roles. The gut microbiome (the microorganisms, mostly bacteria, and their genes in the gastrointestinal region of the digestive tract) of abalone plays an essential role in the host's food digestion. Previous studies on abalone gut microbiomes revealed that abalone gut microbiomes could be mainly influenced by diet and environmental conditions. However, such gut microbiome investigations have only been conducted on farmed stock of *H. iris* and not on wild populations. Commercial fisheries of wild *H. iris* contribute substantially to the total abalone production in NZ. Given the differentiated growth rate concern and the significance of the wild abalone populations to NZ's abalone fisheries, it would be beneficial to evaluate the gut microbiome of wild *H. iris* and explore how and why the gut microbiomes change.

An initial step in evaluating the gut microbiome and the digestion assistance function is to explore the composition and diversity of the gut microbial communities, or "gut microbiota". The present thesis utilized the amplicon metagenomic sequencing technology to investigate the gut microbiome of wild *H. iris* populations in Cook Strait and Chatham Islands. The overall thesis goals were to: 1) investigate the gut microbial composition and diversity of wild *H. iris* populations and 2) investigate some environmental factors that could potentially influence the host's gut microbiota. This thesis includes an introduction chapter, literature review chapter, three experimental chapters, and a synthesis and conclusion chapter. The literature review (Chapter 2) defines key concepts related to microbiome research, how microbiomes are generally assessed, provides some major influencing factors on abalone gut microbiomes, with an emphasis on dietary and aquatic physical and chemical factors, and pinpoints some suggested future research directions. The experimental chapters (Chapters 3 - 5) utilized Illumina MiSeq sequencing technology to evaluate the gut microbiomes of five wild *H. iris* populations with a specific focus on the associations of gut microbiome shifts to seaweed diet and over time in NZ. Chapter 3 compared the gut microbial composition and diversity among three abalone digestive regions and the microbiota between abalone's gut and the surrounding seaweed and sediment in Cook Strait. This

revealed that the microbial composition was similar between the foregut (esophageal pouch) and hindgut (intestine), and the microbiomes of the lower section of the digestive tract (foregut and hindgut) were different from that of the buccal cavity of the animals. Moreover, abalone gut microbiome was significantly different from that of the ambient seaweed and sediment samples. In Chapter 4, assessments on the gut microbiota and consumed algal content of four wild abalone populations in the Chatham Islands (CI) revealed differentiated gut microbiota across study sites and between age groups. Moreover, gut content microscopic results also revealed that the observed gut microbiome differences could be related to consumed seaweed type and algal availability. Lastly in Chapter 5, a two-year abalone gut microbiota evaluation on CI was executed and observed that the gut microbiomes of the wild *H. iris* populations at Ascots Beach and Owenga Harbour changed significantly overtime, with the gut microbial diversity was lower between March-May in 2021 compared to March 2021, November 2021, and April 2022. The observed gut microbiome changes presented in Chapters 4 and 5 could be related to specific seaweed diets and/or oceanographic condition changes over the sampling period, which need to be further investigated through additional field observations and targeted feeding experiments.

The present thesis is the first gut microbiota documentation on NZ wild *H. iris*. The results indicated that seaweed and sediment microbiota by themselves are unlikely to influence the abalone's gut microbiota. Instead, the gut microbiota of wild *H. iris* could be potentially affected by consumed seaweed availability and type as well as the changes of oceanographic conditions. Microbiome data collected from wild *H. iris* digestive tract and the ambient seaweed and sediment in this thesis can be the baseline for future gut microbiome research in NZ. While targeted experiments under controlled conditions need to be further conducted to specifically test the seaweed and environmental parameter hypotheses, seaweed- and oceanography- associated findings from this thesis provide informative predictions on the food digestion efficiency and nutritional and health states of local wild abalone stocks, ultimately influencing the harvesting time and quantity of this iconic species.

**Keywords:** Abalone; *Haliotis iris*; gut microbiome; Illumina MiSeq; abalone digestion; seaweed; environmental factors

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## Attestation of Authorship

I declare that the work contained in this thesis has not been previously published nor submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge, this thesis is not written by another person except where co-author contributions are stated and where due reference is made.

**Signature:**

**Date:** 05.09.2025

## Co-authors Contribution

**STUDENT AND SUPERVISOR APPROVALS**

***By signing you are confirming that the co-author contributions stated in the table(s) below are accurate.***

| | | | | |
|---|---|---|---|---|
| **Student Nam** | Jinchen Guo | **Signature** | **Date** | 08.09.2025 |
| **Primary Supervisor Name** | Donnabella C. Lacap-Bugler | **Signature** | **Date** | 11.09.2025 |
| **Co-Supervis Name** | Leonie Venter | **Signature** | **Date** | 08.09.2025 |

| | |
|---|---|
| **Chapter Number:** | **2** |
| **Manuscript Title:** | **Microbiome modulators in abalone: A review linking gut physiology, microorganisms, diet, and the environment** |
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| **AUTHOR SURNAME: (order as per manuscript)** | **CONTRIBUTION (May copy from the guidelines above)** |
| **Jinchen Guo** | Concept & structure;<br>Article retrieval;<br>Article assessments;<br>Writing |
| **Leonie Venter** | Concept & structure;<br>Article retrieval;<br>Article assessments;<br>Writing |
| **Donnabella C. Lacap-Bugler** | Reviewing & editing |
| **Andrea C. Alfaro** | Reviewing & editing |

| | |
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| | |
|---|---|
| **published:** | |

| AUTHOR SURNAME: (order as per manuscript) | CONTRIBUTION (May copy from the guidelines above) |
|---|---|
| **Jinchen Guo** | Sample collection & processing;<br>Data analysis;<br>Data interpretation;<br>Writing |
| **Andrea C. Alfaro** | Experimental design;<br>Sample collection & processing;<br>Reviewing & editing |
| **Leonie Venter** | Experimental design;<br>Sample collection & processing;<br>data analysis;<br>Writing |
| **Donnabella C. Lacap-Bugler** | Reviewing & editing |

| | |
|---|---|
| **Chapter Number:** | **4** |
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| AUTHOR SURNAME: (order as per manuscript) | CONTRIBUTION (May copy from the guidelines above) |
|---|---|
| **Jinchen Guo** | Sample collection & processing;<br>Data analysis;<br>Data interpretation;<br>Writing |
| **Leonie Venter** | Experimental design;<br>Sample collection & processing;<br>Data analysis;<br>Writing |
| **Shaneel S. Sharma** | Sample collection & processing;<br>Data analysis |
| **Stephen D. J. Archer** | Reviewing & editing |
| **Donnabella C. Lacap-Bugler** | Reviewing & editing |
| **Andrea C. Alfaro** | Experimental design;<br>Reviewing & editing |

| | |
|---|---|
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| AUTHOR SURNAME: (order as per manuscript) | CONTRIBUTION (May copy from the guidelines above) |
|---|---|
| **Jinchen Guo** | Sample collection & processing;<br>Data analysis;<br>Data interpretation;<br>Writing |
| **Leonie Venter** | Experimental design;<br>Sample collection & processing;<br>Writing |
| **Donnabella C. Lacap-Bugler** | Reviewing & editing |
| **Andrea C. Alfaro** | Experimental design;<br>Reviewing & editing |

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# Chapter 1: Thesis Introduction

## 1.1 General Introduction

Abalone (*Haliotis* spp.) are marine gastropods whose fossil record indicates their first appearance on Earth was no later than the Late Cretaceous and progressively became more abundant since the Late Miocene (Beu, 2011; Geiger & Owen, 2012). Abalone are univalves, recognized by their ear-shaped shells with a row of holes for spawning and waste excretion and short tentacles around the mantle. They are commonly found living in tropical, sub-tropical, and temperate rocky intertidal and subtidal regions. With notable taxonomic diversity observed in Asia, Australia, South Africa, Europe, and North America, more than 50 accepted abalone species have been globally identified (Ahyong et al., 2025), including three endemic abalone (or "pāua" in Māori language) species found in New Zealand (NZ): *Haliotis iris*, *H. australis*, and *H. virginea* (Peters et al., 2024; Walton et al., 2024). Despite their diverse morphologies, abalone are universally herbivores grazing on seaweed at a lower trophic level and considered keystone species, along with other herbivores such as sea urchins, in many seaweed-dominated ecosystems (Poore, 1973). As abalone maintain rocky substrates healthy by reducing seaweed overgrowth and recycling nutrients, they are also prey items for marine mammals such as seals and sea otters (Fanshawe et al., 2003; Rogers-Bennett, 2023), and the edible muscular foot of these shellfish is also attractive to human beings.

Abalone are culturally and economically important to human society for a long time. Historically, abalone shells and meat were common trading goods in many North American and Pacific aboriginal communities (Sloan, 2003; Goolmeer et al., 2022). Archaeological evidence indicates that native North Americans have harvested abalone for more than 10,000 years (Braje et al., 2021), and modern abalone culturing among the indigenous Pacific Islanders, such as the Māori communities in NZ, started in the 1980s (Wehi et al., 2013; McCarthy et al., 2014). It was traditionally used as a form of monetary currency for commercial exchanges or religious decorations, the hand-polished and crafted abalone shells mostly become beautiful jewelry and souvenirs nowadays (Menzies, 2010; Ainis et al., 2019). The economic contributions of abalone have been extended to a broader human society as the gastropods have made up a considerable part of global shellfish seafood production.

Abalone fisheries and aquaculture are practiced and can generate a great proportion of revenue, mostly around the Pacific Rim and South Africa and sporadically in Oman and Europe. Globally, China, South Korea, South Africa, Chile, and Australia being the largest abalone-producing countries, and the total abalone production from all sources was approximately 248,000 metric tons (mt) between 2020 and 2021, with legal fisheries contributed to around 4,500 mt (Cook, 2023c). In NZ, *H. iris* is the predominant abalone species for recreational fishery, commercial fishery and aquaculture, but abalone export from the

country is largely dependent on wild-caught fisheries, and aquaculture has small contributions to the total production (FAO, 2024). While the total estimated abalone aquaculture production was 60-70 mt between 2020 and 2021, the estimated total abalone production from legal fisheries at the same time was 753 mt (Cook, 2023c). Abalone production in NZ has been stable in recent years, with aquaculture production increasing slowly and the wild-caught becoming decreased, mostly due to the national quota management systems (Ryder et al., 2025). Aside from overfishing and poaching that are commonly observed in other abalone-fishery countries like South Africa and Australia, slow growth rate among the wild *H. iris* populations is one of the biggest challenges that the NZ abalone industry is currently facing (Naylor et al., 2006; Cook, 2023b). Moreover, the wild and farmed abalone stocks in NZ are also threatened by infectious diseases associated with pathogens and parasites (Haliotid herpesvirus, *Perkinsus olseni*, haplosporidian, etc. Diggles & Oliver, 2005; Corbeil et al., 2017; Muznebin et al, 2023), especially under the increasing climate change stressors and biological invasions mediated by anthropogenic activities.

The two challenges (i.e., slow growth rate and microbial diseases) post to NZ abalone production is highly associated with and could be mitigated by modulating the external and internal microbiota (the microbial communities), particularly the gut microbiota, of the gastropods due to its microbial functions such as food digestion and immunological enhancement. Abalone gut includes the intestine and anus region of the host's digestive tract, and microorganisms, predominantly bacteria, are present in the gut to primarily assist food digestion. While *Mycoplasma*, *Psychrilyobacter*, and *Propionigenium* are the core bacterial genera found in most seaweed-fed abalone globally, there are also many unique bacteria that are specialized in biochemically breaking down specific nutrients (Tanaka et al., 2016; Bullon et al., 2025). Yet, such gut microbiota surveys have not been adequately conducted on *H. iris*. Some of these detrimental microorganisms found either in the surrounding environments (e.g., sediment, seawater, seaweed, etc.) or on abalone's external surfaces (e.g., shell, gill, mantle, foot, etc.) could be carried through the abalone's digestive tract and become a detectable constituent of the gut microbiota. There are two potential factors that could be linked to the slow growth phenomenon. First, slow growth could be a specific genotype (e.g., genes unfavorable for growth when turned on) that is inheritable to the next generation (i.e., genetic effect; Tshilate et al., 2024). Second, dietary and environmental parameters such as food availability, food nutrient profiles, and seawater temperature could also influence abalone's gut microbiome and ultimately alter their growth rates, especially in their natural habitats where these parameters are highly unpredictable (You et al., 2020; Wang et al., 2021b). The slow growth potentially associated with suboptimal bacteria-assisted digestion and the microbial infections of wild abalone are both related to the hosts' microorganisms and could be difficult to monitor and control. Therefore, it is essential to prioritize baseline gut microbiota investigations among the wild abalone populations in NZ

because the wild abalone stocks are not only essential to the nation's recreational and commercial fisheries but also a vital brooding stock source for local abalone aquaculture facilities to sustain genetic diversity.

Metabarcoding amplicon sequencing is a semi-quantitative way to use genetic sequence abundance data to approximate real abundance of the targeted organisms. This method has been widely used in various ecological studies, such as benthic invertebrate community surveys (Pits et al., 2020), bird and mammalian diet investigations (Boukhdoud et al., 2021; Garfinkel et al., 2021), and marine invasive species detection and monitoring (Pagenkopp et al., 2019; Westfall et al., 2020). In the field of gut microbiota research, people often utilize the V3-V4 regions of the microbial 16S ribosomal RNA (rRNA) gene (conserved across prokaryotic organisms and has been conventionally used for microbial species identification) for gut microbial community characterizations. Compared to other next-generation sequencing technologies (e.g., 454 pyrosequencing, Ion Torrent, etc.), Illumina *MiSeq* is a relatively more cost-effective sequencing platform that generates high-throughput sequence data (Rapin et al., 2017; Salamon et al., 2022). While several studies have utilized Illumina *MiSeq*-based metabarcoding method to evaluate abalone gut microbiota under different dietary treatments and environmental conditions (Guo, 2017; Gobet et al., 2018), such culture-independent gut microbiota investigations have not been conducted on wild NZ black-footed abalone.

### 1.2 Thesis Aims and Structure

By using the 16S rRNA metabarcoding tool on the Illumina *MiSeq* sequencing technology, **the present thesis aims to investigate the gut microbiota of wild NZ abalone (*Haliotis iris*) with a focus on the potential factors of seaweed availability and type and environmental conditions overtime that might influence the host's gut microbiota.**

The present thesis is structured into six chapters. **Chapter 1** is the thesis introduction and structure chapter. **Chapter 2** is the literature review of the observed and potential effects of dietary seaweed and aquatic physical and chemical conditions, tailored on seawater temperature changes, on abalone gut microbiota. **Chapter 3** is the original research project to characterize the microbiota of the digestive tract of wild *H. iris* in Cook Strait and the seaweed and sediment collected in the surrounding environment. This chapter investigated whether the abalone's gut microbiota would resemble the microbial compositions of the ambient seaweed and sediment. The next two original research chapters focused on wild *H. iris* populations on Chatham Islands, which are approximately 800 kilometers away from the main islands. Briefly, **Chapter 4** is the baseline exploration of the gut microbiota of adult and juvenile *H.*

*iris* populations collected from four study sites with different growth rates on Chatham Islands. This chapter investigated whether the gut microbiota of the wild black-footed abalone would be differentiated by developmental stage and/or growth rate and whether the observed gut microbiota patterns would be associated with digested seaweed. **Chapter 5** assessed the gut microbiota of wild abalone populations at two study sites, which were surveyed in Chapter 4, overtime between November 2020 and April 2022. The aim of Chapter 5 is to explore whether abalone collected from the same sites would show differentiated gut microbial compositions and diversity between the two study sites and across the different sampling points. Another purpose of Chapter 5 is to discuss the potential influencing factors of environmental condition shifts, mostly seawater temperatures, on the gut microbial communities of the surveyed wild abalone populations on Chatham Islands. The last chapter, **Chapter 6**, is the synthesis chapter that discusses the connections among the thesis chapters, points out some research limitations of this thesis, suggests future abalone gut microbiome research directions and addresses the overall thesis conclusions and significance.

This thesis contributes novel information on wild *H. iris* gut microbiota observations to the molluscan microbiome research field and suggests future controlled experiments targeted at further evaluating the effects of seaweed diet and seawater temperature on this iconic species in NZ. Results of these investigations can guide targeted experiments to optimize abalone digestion and growth as well as to assess pathogenic prevalence and virulence among the wild populations, which will ultimately contribute to the sustainable management and conservation of this ecologically and economically important shellfish in NZ.

**Chapter 2: Microbiome modulators in abalone: A review linking gut physiology, microorganisms, diet, and the environment**

**Prelude: This chapter aims to provide a general understanding of abalone gut microbiome, relevant research methods, and environmental factors that may influence abalone gut microbial composition and diversity. Some bivalve and non-molluscan gut microbiome examples are also provided as comparative references.**

***This review manuscript is being finalized to be submitted to Molluscan Research or Reviews in Aquaculture journals.***


**Abstract**

Abalone are marine mollusks that contribute to ecological, recreational, cultural, and economic initiatives. Within their specialized gut, they are adapted to digest complex macroalgae and formulated feeds using a digestive system of four distinct regions. The digestive system facilitates efficient nutrient breakdown, absorption, and dynamic microbial interactions. The characteristic microbial communities that occupy the digestive system constitute the gut microbiome and facilitate multiple aspects of the host's physiological health status, ranging from nutritional regulation to immune modulation. Looking at research published in the last 10 years (2015 to mid-2025), a total of 34 studies were assessed to characterize the core microbiome of abalone. At the genus level, abalone dominant taxa include *Mycoplasma*, *Psychrilyobacter*, and *Vibrio*, responsible for functions such as protein degradation, immune modulation and pathogenicity, digestion, nutrient absorption, and fermentative metabolism. As a next section, the discussion leads with microbial changes induced by diet and dietary components constitute a large part of the available literature, with seaweed specific bacterial types, dietary building blocks (such as lipids and proteins) and dietary supplementation (such as probiotics and bile acids). The text transitions to the influence of environmental variability on the structure and function of the abalone gut microbiome, outlining abalone microbiome studies considering season, sampling location, temperature, and species. Also listed are microbial findings of abalone infections and diseases. Lastly the manuscript focuses on shortcomings and future directions. Detailed investigations on the relationships between abalone, their gut microbiome, and confounding factors such as diet and environmental parameters are necessary and pressing for predicting the compositional and functional changes of the microbial communities and managing the fishery and aquaculture stocks worldwide more effectively and efficiently, especially under the context of changing environments and future-proofing production facilities.

**Keywords:** Abalone, Gut Microbiome, Seaweed diet and nutrition, Marine sediment, Environment stressors

## 2.1 Introduction

Abalone (Haliotidae) are among the most valuable marine mollusks in global aquaculture, prized not only for their high market value, but also for their nutritional qualities, culinary appeal, and contributions to local economies and ecosystems (Negara et al., 2021, Cook, 2023b). Beyond consumption, abalone products include pharmacological extracts, decorative shells, and ecological applications such as reef restoration (Chung et al., 2024, Zhao et al., 2025). Despite their economic and ecological value, abalone populations and production systems face unprecedented pressures, posed by overfishing, recruitment bottlenecks, environmental changes, disease, handling, stocking density, etc. (Ragg and Watts, 2015, Morash and Alter, 2016, Lachambre et al., 2017, Hart et al., 2020).

Abalone holds a specialized gut system adapted to digest complex macroalgae and formulated feeds, with microbial symbionts providing key enzymatic functions such as alginate lyase and cellulase production (Frederick et al., 2022). Evidence increasingly suggests that these microbial communities are shaped by host development, dietary inputs, and environmental factors, including temperature and water quality, making them both sensitive indicators and active mediators of abalone health and performance (Mabuhay-Omar et al., 2019, Yu et al., 2025). The microbiome is increasingly studied for its potential as an indicator of physiological or environmental changes in the host (Apprill, 2017). Microorganisms coincides with the function of microbial communities in aquatic ecosystems and within aquatic organisms and their interactions within an abalone farm or fishery (Zhang et al., 2022). Also, microbial diversity can be linked to changes in the environment due to seasonal changes, such as bacterial load or temperature (Danckert et al., 2021). Thanks to technological advances, high-throughput sequencing enables the visualization of microbial communities associated with abalone farming and fisheries (Zhang et al., 2022).

The microbiota of abalone plays a crucial role in digestive physiology, nutrient assimilation, immune defense, and overall health and growth (Iehata et al., 2014; Gobet et al., 2018; Hur et al., 2023). Abalone microbiome supports food digestion, nutrient absorption, immunity, epithelial renewal and overall growth and health (Yu et al., 2022b, Zou et al., 2022). Despite advancements in this field, the dynamic triangular relationship between host, microbiota, and environmental parameters remains poorly understood in abalone. Disruption of microbial symbiosis, or dysbiosis, is implicated in disease susceptibility, poor nutrient utilization, and impaired growth. Conversely, a stable and functionally diverse microbiota may

confer resilience by enhancing immunity and digestive efficiency (Apprill, 2017, Wu et al., 2024). This can be useful in an aquaculture context to improve organismal health and the maintenance of water quality in rearing systems (Zhang et al., 2025b).

This review provides context on the microbiome, factors that influence it, aspects relating to the assessment thereof and its link to abalone biology, digestion and production. Novelly, we present a summary of regions of the abalone digestive systems classified as the buccal region, foregut, midgut and hindgut with associated functions. Next, by focusing on published research from the last ten years, this review seeks to elucidate the microbial compositions and their functional roles in various abalone species by defining the core abalone microbiome, and outlining microbiota changes due to diet, environmental stressors, infections and disease. The significance of microbiota in abalone health in aquaculture and wild settings is outlined along with shortcomings and potential avenues for future improvements. Understanding the microbial communities of abalone and the surrounding environments are essential for improving cultivation and population growth and ensuring lasting stocks of this valuable mollusk.

### 2.2 The microbiome and factors that influence it

Microbiota is a characteristic microbial community, of a resealable well-defined habitat which may include microorganisms, such as archaea, bacteria, fungus, protozoans, and invertebrate metazoans (Berg et al., 2020). The gut microbiome is shaped by intrinsic factors like genetics (horizontal gene transfer), developmental stage (e.g., post-settlement, juvenile, and adult), physiological condition, sex and age, and by extrinsic factors such as diet (i.e., feeding habits, dietary supplements, nutrients, probiotics) and environmental factors such as season, water quality, temperature, contaminants, pathogens, etc. (Chen et al., 2022, Diwan et al., 2023). The interactions between the host and its microbiota, according to Apprill (2017), can be classified as symbiosis and dysbiosis (Fig. 2.1). Symbiotic factors constitute a healthy, stable microbiome that supports normal physiological functions in the host, largely attributed to diet, a stable environment, and host genetics (Carey and Duddleston, 2014). Dysbiosis on the other end, are stressors or changes that disrupt the microbial community, function and structure of the host, these constitute changes due to pathogens, environmental stressors or malnutrition (Egan and Gardiner, 2016, Infante-Villamil et al., 2021). In a symbiotic state the gut microbiome improves host immunity and development and aids in digestion. In contrast, a dysbiosis state is associated with disease, inflammation and a compromised immune status (Danckert et al., 2021). Generally, host-microbiome relationships occur in a symbiotic state, where natural variations take place due to environmental or host factors regulated by host adaptive response mechanisms. However, when faced with a stressful event the host

will implement a stress response, resulting in an unstable state within the microbial community, due to unpredictable nature of these, shifts (Carey and Duddleston, 2014, Apprill, 2017, Chen et al., 2022). Additionally, dysbiosis can negatively affect immune regulation mechanisms and nutrient absorption efficiency, thereby influencing host health and growth performance (Li et al., 2025). The classification of microbiome in a state of symbiosis or dysbiosis is useful in determining if microbial changes are adaptive (i.e., aiding digestion) or harmful (i.e., driving disease). Besides that, this distinction is valuable for interpreting microbiome data in aquaculture and fisheries settings and adding functional outcomes to promote optimal host performance.

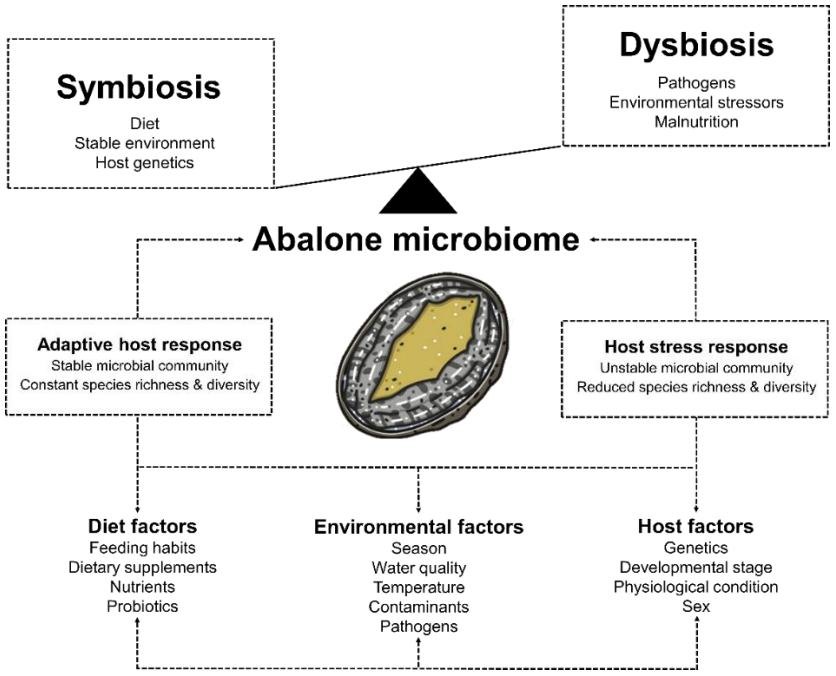


**Figure 2.1: Homeostasis of the microbiome is subjected to symbiosis or dysbiosis of dietary, environmental, and host factors creating a stable or unstable microbial community.**

The gut microbiome is known to showcase a functional core community in hosts, useful towards physiological functions and adaptations to changes (Zhang et al., 2025b). Research advances have revealed vital purposes of the molluscan microbiome in facilitating interactions between mollusks and their associated microbiomes (Salloum et al., 2025). Findings promote microorganisms to drive host physiological processes like food digestion, nutrient absorption, pathogen resistance, host health, growth, feeding efficiency, enhancement of intestine development, stimulation of mucosal immunity and establishing of ecosystems (Villasante et al., 2020, Wang et al., 2020, Choi et al., 2021, Yu et al., 2022b).

Host factors can be understood as genetic influences, with microbiota transferred via vertical transmission from parents to offspring, or among individuals and populations with high genetic similarity (Lasa and Romalde, 2021). On the other hand, environmental factors are often referred to as the horizontal transmission of microorganisms between shellfish hosts and their surrounding environments such as seawater, sediment, diets, etc., (Kijewska et al., 2023). While evidence has shown that molluscan microbiota could be influenced by both vertical and horizontal transmissions, environmental factors such as aquatic abiotic conditions (temperature, salinity, pH, dissolved oxygen level, etc.) and diets could be more predominant (Scanes et al., 2021b, Unzueta-Martínez et al., 2022, Salloum et al., 2025). The abalone intestinal tract has shown dominance of bacteria, likely linked to factors such as gut colonization, aquaculture practices (e.g., cleaning, handling), diet, age, and seasonal changes, that have created conditions that support the growth of specific microbes in the abalone gut (Danckert et al., 2021). As the composition of the microbiome is taxonomically and ecologically diverse and influenced by the environment, health status and species under study, it remains critical to firstly understand stability among complex microbiota, whereafter changes can be quantified and interpreted (Chen et al., 2022).

Microbial communities colonize both internal and external host tissues based on selective pressures that influence the assembly. Generally, internal communities such as the gut will be affected by diet, while external communities will be greatly affected by environmental changes (Ochoa-Sánchez et al., 2023). Host tissues, such as the skin, digestive glands, hemolymph and nasal cavities can comprise different microbiota which is hypothesized to be driven by niche specialization and host selective pressure (Lorgen-Ritchie et al., 2023). For example, microbial communities on aquatic animal shells were found to involve in shell biomineralization (Dade-Robertson et al., 2015, Brugman et al., 2018), and shellfish gill microbiota could provide respiration support via organic and inorganic carbon fixation (Mizutani et al., 2020, Chalifour and Li, 2021). While some microorganisms are beneficial to the hosts, certain microbes can be pathogenic and may induce diseases in both ectodermal and endodermal tissues (Moore, 2023). In abalone, complex microbial communities are present across various tissues, and some of these microbes may act as potential foodborne pathogens when raw abalone is consumed (Lee et al., 2016).

Understanding the composition, transmission, and functional roles of microbiota across various host tissues is essential for assessing host health, particularly in aquaculture species such as abalone. The dynamic interplay between host genetics, environmental conditions, and microbial communities underscores the complexity of maintaining microbial homeostasis. Ultimately, it is essential to qualitatively and quantitatively identify and measure the diversity and abundance of the microbial communities on molluscan hosts. Such practice not only advances fundamental insights into host–

microbe interactions but also provides practical applications in aquaculture and fisheries, where microbiome-informed strategies can be leveraged to promote disease resistance, optimize growth, and enhance overall host performance.

**2.3 Assessing the microbiome**

Efficiently quantifying the microbial richness, abundance and diversity (collectively referred as microbiota) of an animal host is a key start towards exploring the underlaid microbial functions and the involved genes of the microorganisms (i.e. microbiome) (Djemiel et al., 2022). Microbiota quantifications can be broadly categorized as culture-dependent and culture-independent methods. Culture-dependent assessment methods mostly rely on growing and isolating microorganisms on selected media under laboratory conditions, followed by direct microbial cell counting or biomass estimation (Fusco and Quero, 2014). Although the culture-dependent methods allow direct isolations of live microorganisms and facilitate downstream microbial functional studies via biochemical tests (Demko et al., 2021, Wang et al., 2021b), the entire process is time-consuming and often fails to capture the entire microbial communities, especially for microorganisms that were alive but no longer culturable at the time of laboratory processing (Koerner et al., 2023). By comparison, culture-independent methods mainly refer to next-generation sequencing techniques (e.g., Illumina, Ion Torrent, Shotgun sequencing, etc.) that analyse microbial communities without culturing microorganisms on petri dishes (Ditz et al., 2020). While all next-generation sequencing technology can generate high-throughput data, each sequencing system has its own characteristics. The Illumina system is a high cost-to-performance sequencing platform that follows a sequencing-by-synthesis workflow to generate high accurate reads in moderate lengths (often 75-300 bp). Ion Torrent system utilizes sensor chips to detect electrical signals altered by pH changes. While Ion Torrent can generate relatively longer read lengths (often 200-400 bp) and requires lower cost on instrumentation compared to Illumina, this sequencing technology often requires more pre-sequencing preparation and yields less accurate data. Shotgun metagenomic sequencing targets multiple genes which are often used for advanced functional evaluations and rely on complex bioinformatics.

There are two major advantages of utilizing these semi-quantitative next-generation sequencing-based methods, where data analysis is based on genetic sequences rather than direct microorganism counts. Firstly, they offer a faster, more comprehensive view of microbial diversity and abundance by detecting unculturable and rare species better than traditional culturing methods using the same sample size (Su et al., 2012). Secondly, sequencing-based methods are high-throughput, enabling the simultaneous processing of hundreds of samples and the generation of large-scale genetic data, such as millions of amplicons or whole-genome sequences (Satam et al., 2023). The technological advancements and

efficiency of the next-generation sequencing methods have substantially empowered microbiome assessments in the past decades; therefore, it is necessary to have a conceptual understanding of the overall workflow of these sequencing pipelines.

A general next-generation sequencing and microbiome assessment workflow includes laboratory processing and computational bioinformatics (Fig. 2.2). The laboratory procedure aims to produce targeted genetic amplicons for sequencing, and it includes genomic deoxyribonucleic acid (gDNA) isolation and quantification from animal tissues, polymerase chain reaction (PCR) amplifications of targeted microbial genes (e.g., 16S/18S ribosomal RNA), PCR amplicon purification and pooling and eventually quantification on the pooled amplicon libraries (Athanasopoulou et al., 2023). After the amplicon libraries are sequenced through one of the next-generation sequencing platforms, the sequencing data needs to be bioinformatically processed using sophisticated computer software to generate a sequence abundance table with microbial taxonomic information added for downstream ecological data analysis (Gao et al., 2021). In the case of Shotgun sequencing, metagenomic sequencing data are generated for functional analysis. The bioinformatic data processing step often includes PCR/sequencing primers trimming and erroneous and chimera sequences removal for generating high-quality sequencing abundance and functional data (Infante-Villamil et al., 2021). Therefore, both laboratory microbiome amplicon preparation for high-throughput sequencing and the subsequent data processing workflows are super critical for accurate, precise, and comprehensive microbial ecological data analysis and representation.

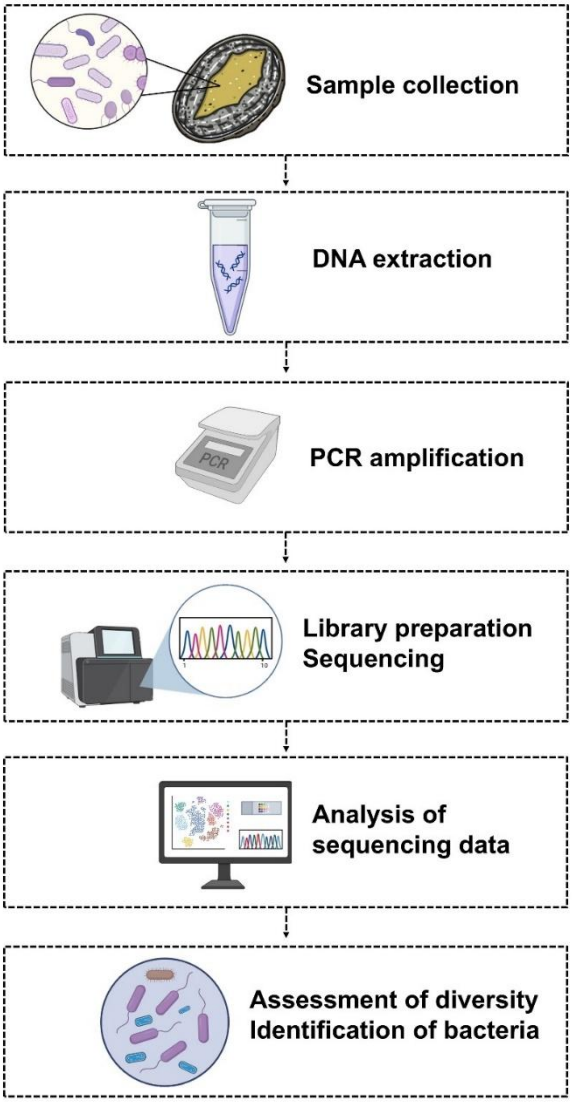


**Figure 2.2: Overview of gut microbiome analysis using 16S rRNA sequencing, including sample collection, DNA extraction, PCR amplification of the 16S rRNA gene, library preparation and sequencing, followed by bioinformatic analysis of sequencing data, assessment of microbial diversity, and taxonomic identification of bacterial communities.**

Microbial richness and diversity are two fundamental concepts for microbiome assessments, and the two terms evaluate the microbial communities differently. Richness exclusively refers to the number of different species present in a community, and some richness calculations, such as the Chao1 richness, specially weigh rare microbial species into consideration (Xia et al., 2018). Diversity, on the other hand, characterizes not only species richness but also abundance of each species, reflecting the evenness of the community structure (Vitorino and Bessa, 2018). Given the differences between the two terms, diversity often provides a more comprehensive description, as a microbial community can have high richness but

low diversity if a few species dominate the population (Wagner et al., 2018). The concept of diversity can be further partitioned into three scopes: alpha-diversity, beta-diversity and gamma-diversity. Alpha-diversity refers to the diversity within a specific microbial habitat or sample groups and is often presented as microbial richness, evenness, and diversity indices such as Shannon's and Simpson's diversity indices (Fasolo et al., 2024). Beta-diversity examines the variation in species composition between different habitats or communities, and the microbial abundance data of all samples in each pair of habitats or groups need to be converted into similarity/dissimilarity matrices to allow the between-group diversity comparisons (Walters and Martiny, 2020). Lastly, gamma-diversity captures the total diversity of all habitats or microbial communities of interest and serves as a collective measure of biodiversity at a broader spatial scale. In the context of microbiome assessment, a core microbiota is often calculated to describe a set of microbial species that are found in all habitats or sample groups (Marathe et al., 2021). Therefore, microbial richness and diversity metrics are collectively utilized to assess and compare microbiome dynamics and to assist in understanding the microbial ecological implications under influential factors of interest.

### 2.4 Abalone production, physiology, and digestive interactions

Abalone are classified as one of the most commercially valuable farmed marine mollusks due to its high nutritional quality and meat flavor, causing the price of live abalone of various species to reach up to US$100 per kilogram in the Asian market (Cook, 2023b). In addition to live product, abalone can be processed into several forms, including dried, canned or frozen (Negara et al., 2021) which are often consumed as raw, boiled or cooked products (Lee et al., 2016). Abalone support ecological, recreational and cultural initiatives (Orchard et al., 2024), while also playing an important part in the food web, being a food source for various predators, including starfish, octopuses, whelks, and lobsters (Hofmeister et al., 2018). Moreover, abalone extracts and active components, such as polysaccharides, amino acid peptides, oils, lipids and protein are utilized as nutraceuticals and pharmaceuticals (Suleria et al., 2017, Zhao et al., 2025). Remarkably abalone shell has found purpose as decorative items, jewelry and for reef restoration materials (Chung et al., 2024).

Abalone are cultivated and fished in numerous countries worldwide. The largest abalone producers are China, Korea, South Africa and Chile, with China alone accounting for over 95% of the total abalone production (You, 2023). Most abalone production now comes from aquaculture rather than wild fisheries. In 2021, abalone production totaled 243,506 metric tons through farming, while only 4,510 tons came from wild sources (Cook, 2023c). Wild fisheries are managed within quota management systems, which

sets a total allowable commercial catch within a specific region. Generally, the status of the wild abalone population varies (Neubauer, 2022, Ryder et al., 2025), and as such the total allowable commercial catch is adapted to best support the health of the population (Ryder et al., 2023). New Zealand, for example, is uniquely placed as it hosts one of the last remaining wild abalone fisheries worldwide (Gerrity and Schiel, 2025). The future of the wild abalone industry is at risk, as similar trends of population decline driven by overfishing from both recreational and commercial fishers have occurred in multiple countries, consistently resulting in the collapse of their abalone fisheries (Mundy et al., 2023). Accordingly, various countries, have adopted aquaculture, ocean ranching, or heavily subsidized 'put and take' fisheries to sustain production and support the recovery of remaining wild stocks (Gerrity and Schiel, 2025).

Abalone aquaculture comprises, a seed production phase, a larval rearing phase and a grow-out phase where juveniles are tented to a marketable size (Mamat et al., 2025). Additionally, broodstock animals are kept in abalone hatcheries to serve as a source of gametes (Daume, 2006). All of these abalone farming phases are affected by environmental factors and diseases, which in effect changes the bacterial communities of the ecosystem and of the animals itself (Zhang et al., 2022). Microorganisms and parasites threaten abalone culture in scenarios where aquaculture activities are intensified or where environmental conditions deteriorate (Mabuhay-Omar et al., 2019). Multi-dimensional factors such as seawater temperature, pH levels, salinity and nutrient concentrations influence abalone populations (Li et al., 2025) and negative impact abalone growth (Morash and Alter, 2016). Abalone have shown behavioral adjustments and physiological plasticity, as adaptive capacities, along with the potential for selection based on genetic polymorphisms within populations, as mechanisms to cope with fluctuating environmental conditions (Yu et al., 2023).

Abalone physiology comprises the biological and biochemical processes that support the survival, growth, reproduction, response to stressors and interaction with the environment (Morash and Alter, 2016). Abalone exhibit the classic molluscan body plan, consisting of a head-foot region and a visceral mass region, which is protected by a univalve with a role of holes to the shells left margin (Mamat et al., 2025). The heat-foot section contains a muscular foot structure, with hemolymph cavities that serve as a hydrostatic skeleton and supports attachment and locomotion. While the visceral mass houses the digestive, reproductive and circulatory organs, important for digestion and nutrient absorption, gamete production, delivering of oxygen and nutrients throughout the body, while also facilitating excretion and detoxification functions (Venter et al., 2018). Abalone physiology supports various systems and mechanisms to face stressors of physical, chemical, biological, or procedural nature. For example, Khan (2025), describes how environmental stressors, dietary stressors and disease related stressors affect

abalone physiology. While a review by Morash and Alter (2016) outlines abalone physiological responses to farming factors. Furthermore, within the multi-omics classification system, Nguyen et al. (2022) highlights how abalone respond on a genomic, transcriptomic, proteomic and metabolic level when faced with stressors. Together these studies reflect the ability of abalone to implement physiological changes to adapt to changing conditions to allow them to survive and thrive in a marine environment.

One way to counteract the effects of stressors is through dietary interventions and diet adjustments in aquaculture practices (Ciji and Akhtar, 2021). The incorporation of feed additives such as antimicrobial peptides, organic acids, plant-derived extracts, prebiotics, herbs and probiotics in abalone diets have been reviewed by Li et al. (2024). These additives aim to enhance abalone performance, improve product quality, and strengthen immune responses, thereby increasing resilience to environmental stressors (Bullon et al., 2023). Similarly, the inclusion of macroalgae as ingredients in abalone feed have been reviewed by Bansemer et al. (2016) demonstrating improved abalone feeding activity, health and marketability. Conversely, the negative effects of inadequate mixed diet approaches and poorly timed dietary transitions have been shown to reduce abalone production (Hannon et al., 2013). Given their nocturnal, herbivorous grazing behavior (Sales and Britz, 2001), abalone feeding patterns should be thoughtfully considered to ensure optimal feed delivery and intake in aquaculture settings. To effectively harness dietary strategies in mitigating stress impacts, it is essential to first understand the feeding behavior of abalone across different life cycle stages.

Abalone have distinct feeding patterns across the various production phases, reflecting ontogenetic dietary shifts from bacteria and microalgae to macroalgae (Won et al., 2010). In general, abalone larvae consume prokaryotes and diatoms, wild adults graze on macroalgae (Hur et al., 2023), and farmed adults are typically fed on formulated feeds in aquaculture grow-out facilities to promote growth (Bullon et al., 2023). Adult abalone possesses a complete digestive system with specialized tissues that can be broadly categorized into four sections: buccal region (mouth and buccal cavity), foregut (esophagus and crop), midgut (stomach and digestive gland), and hindgut (intestine, anus and rectum) (Harris et al., 1998a). Functions of the digestive system gradually shift from physical to biochemical (i.e., enzymatic activities through digestive glands and biochemical activities mediated by gut microbes) across the four digestive sections (Table 2.1). Food is brought into the mouth using the rhipidoglossan radula, which is flexible with outer marginal teeth that sweeps substratum along with a small number centre sturdy teeth (Kuehl and Donovan, 2021). Then food particles passes to the buccal region and mixes with large quantities of mucus (Dixon, 1992). Mucus ensures a barrier for host epithelial cells, and serves a source for microbial growth and attachment (Danckert et al., 2021). The ingested food moves through the salivary gland ducts

which extends to the esophagus followed by the muscular crop which houses digestive fluids (Dixon, 1992). The crop contains large numbers of secretory and mucous cells with limited ciliated cells, and serves as a storage organ (Harris et al., 1998a). Next the crop extends to the stomach, by forming a 180° loop at the posterior end to extend anteriorly adjacent to the crop (Johnston et al., 2005). The surface of the gastropod's stomach is rough, maximizing surface area for further crushing and facilitating subsequent biochemical interactions (Hur et al., 2023). The digestive gland overlays the crop and stomach via a series of ducts, which flows to the intestine and anus (Martin et al., 2011). The digestive gland consists of columnar epithelial cells, secretory or basophilic cells and digestive gland cells (Hur et al., 2023), which fulfills functions such as secretion of digestive enzymes, absorption of nutrients, intracellular digestion, assistance with detoxification and storing of glycogen, lipids and calcium (Lobo-da-Cunha, 2019). Enzymes of the digestive gland, like carboxypeptidases, aminopeptidases, chymotrypsin and trypsin, have been reported in abalone to break down polysaccharides and proteins (Frederick et al., 2022). The gastrointestinal tract (including the stomach and intestine) harbours microorganisms specialized in various microbial functions (e.g., digestion and antimicrobial activities) and is characterized by an acidic pH of 5.3 to 6, offering a stable internal environment that is either anaerobic or microaerophilic (Gobet et al., 2018). Undigested feed transfers through the hindgut comprising the intestine, rectum and is eliminated by the anus (Bullon et al., 2023).

The structural complexity and functional specialization of the abalone digestive tract often require different diet optimization methods to promote better food digestion and nutrient absorption efficiency. For example, a mixture of formulated diets (often enhanced with fatty acids) and fresh microalgae was provided to abalone broodstocks to maximize the dietary nutritional value (Daume, 2007), and customized probiotics and nutrients were encapsulated and intactly delivered to the intestine of *H. iris* to increase the feed conversion rate of the subjects (Dezfooli et al., 2023). These findings, along with other studies, have highlighted the intestine as the most important section of the entire digestive tract for nutrient breakdown and absorption. As an important component of the food digestion process in abalone, gut microbial communities have been particularly studied among various abalone species worldwide due to their roles in nutrient assimilation, metabolic regulation, and immune modulation.

**Table 2.1: Regions of the abalone digestive system comprising the buccal region, foregut, midgut, and hindgut with associated functions.**

| Classification | Region | Function | Description |
|---|---|---|---|
| Buccal region | Mouth | Collection of food | Radula brings food to the mouth and scraps the substrate into particles. |
| | Buccal cavity | Initial food processing | Mucus, houses microorganisms and supports the mixing of ingested food. |
| | | Transportation | Mucus assists with the movement of food from the buccal cavity into the esophagus. |
| Foregut | Esophagus | Transportation | The esophagus moves ingested food from the buccal cavity to the crop via salivary gland ducts. |
| | Crop | Temporary food storage | The crop temporarily stores ingested material. |
| | | Initial digestion | The digestive fluids assist with food breakdown, while secretory and mucous cells contribute enzymes and mucus to aid this process. |
| Midgut | Stomach | Mechanical digestion | Crushing and grinding food particles. |
| | | Preparation for enzymatic digestion | Physical breakdown of food enhances exposure to digestive enzymes from the digestive gland. |
| | Digestive gland | Digestive enzyme secretion | Secretes enzymes to enable the efficient breakdown of diverse dietary components. |
| | | Digestion and nutrient absorption. | Support intracellular digestion to ensure that nutrients are processed and assimilated at the cellular level for absorption and storage. |
| Hindgut | Intestine | Transit of undigested material | Undigested feed and residual digestive matter move through the intestine. |
| | | Microbial fermentation and degradation | The intestine is heavily colonized by bacteria driving the breakdown of complex polysaccharides. |
| | Rectum and anus | Excretion of waste | Final outlet for faecal material and waste disposal. |

### 2.5 Abalone gut microbiome

Abalone gastrointestinal (and digestive gland) microbiome research emerged in the 1990s (Wang et al., 2021b) and has now grown into a substantial body of research, expanding to studies of abalone gut bacteria within aquaculture production systems (Danckert et al., 2021). Looking at research published in the last 10 years (2015 to mid-2025), a total of 34 studies were documented (Table 2.2). For a study to be included in this list: empirical research had to be conducted on a *Halitois* species; utilizing technology to assess the microbiome with associated library and data processing steps; reporting core bacteria at phyla and/or genus level. The summarized research targets 14 abalone species including: *H. corrugata, H. discus, H. discus hannai, H. diversicolour, H. fulgens, H. gigantea, H. iris, H. laevigata, H. laevigata* x *H. rubra, H. midae, H. rufescens, H. sorenseni, H. tuberculata* and hybrid species *H. discus hannai* × *H. fulgens*. Concurring with global trends the 52% of these studies were done on *H. discus hannai*, with all the remaining species documented once or twice, accounting for 2.5% or 5% of the studies respectfully (Fig. 2.3a). These microbiome studies include research topics relating predominantly to nutritional themes, but also include aims linked to environmental, health, growth and rearing and profiling questions. The main findings from the body of literature (34 summarized studies) will target microbial findings in abalone due to dietary interventions, environmental changes and infections and diseases. Moreover, this section will highlight shortcomings, research gaps and advantageous points relating to microbiome studies and abalone.

**Table 2.2: Published original abalone gut microbiome studies since 2016.**

| Publication Title | Abalone species | Aquaculture or wild abalone | Tissue / sample investigated | Reference |
|---|---|---|---|---|
| Expanding the menu for New Zealand farmed abalone: dietary inclusion of insect meal and grape marc (effects on gastrointestinal microbiome, digestive morphology, and muscle metabolome) | *H. iris* | Aquaculture | Gastrointestinal tract | Bullon et al., 2025 |
| Effects of dietary supplementation of Bacillus, β-glucooligosaccharide and their synbiotic on the growth, digestion, | *Haliotis discus hannai* | Aquaculture | Gastrointestinal tract (hepatopancreas, stomach, and intestine) | Cadangin et al., 2024 |

| | | | | |
|---|---|---|---|---|
| immunity, and gut microbiota profile of abalone, *Haliotis discus hannai* | | | | |
| Effects of dietary bile acids levels on growth performance, anti-oxidative capacity, immunity and intestinal microbiota of abalone *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | intestine | Chen et al., 2023 |
| Intestinal microbial diversity is higher in Pacific abalone (*Haliotis discus hannai*) with slower growth rates | *Haliotis discus hannai* | Aquaculture | intestines | Choi et al., 2021 |
| Structure, dynamics and predicted functional role of the gut microbiota of the blue (*Haliotis fulgens*) and yellow (*H. corrugata*) abalone from Baja California Sur, Mexico | *Haliotis fulgens & Haliotis corrugata* | Wild | Post esophageal tissue | Cicala et al., 2017 |
| The role of diversity in mediating microbiota structural and functional differences in two sympatric species of abalone under stressed withering syndrome conditions. | *Haliotis fulgens and Haliotis corrugata* | Wild | Gastrointestinal tract | Cicala et al., 2023 |
| The intestinal microbiome of Australian abalone, *Haliotis laevigata* and *Haliotis laevigata* × *Haliotis rubra*, over a 1-year period in aquaculture. | *(Haliotis laevigata x H. rubra) and (Haliotis laevigata)* | Aquaculture | Intestine | Danckert et al., 2021 |
| Seasonal and algal diet-driven patterns of the digestive microbiota of the European abalone | *Haliotis tuberculata* | Aquaculture | Gonado-digestive gland | Gobet et al., 2018 |

| | | | | |
|---|---|---|---|---|
| *Haliotis tuberculata*, a generalist marine herbivore | | | | |
| *Bdellovibrio* and like organisms promoted growth and survival of juvenile abalone *Haliotis discus hannai* Ino and modulated bacterial community structures in its gut | *Haliotis discus hannai* | Aquaculture | Intestine | Guo et al., 2017 |
| Effects of different feeding patterns on growth, enzyme activity, and intestinal microbiome of the juvenile Pacific abalone *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | Intestine | Guo et al., 2024 |
| Dietary replacement of *Undaria pinnatifida* by *Sargassum horneri* in feed formulation for abalone *Haliotis discus hannai*: Effect on growth, gut microbiota, and taste sensory profile | *Haliotis discus hannai* | Aquaculture | Intestine | Hur et al., 2023 |
| Analysis of microbiota on abalone (*Haliotis discus hannai*) in South Korea for improved product management | *Haliotis discus hannai* | Wild | Intestine | Lee et al., 2016 |
| Effects of Seawater from Different Sea Areas on Abalone Gastrointestinal Microorganisms and Metabolites. | *Haliotis discus hannai* | Aquaculture | Gastrointestinal tract | Li et al., 2025 |
| Effects of dietary protein levels on growth performance, serum indexes, PI3K/AKT/mTOR/S6K signalling and intestinal microbiota of abalone *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | Intestine | Ma et al., 2021 |

| | | | | |
|---|---|---|---|---|
| Multi-omics reveal the effect of different dietary plant protein sources on the microbiota-gut-digestive gland axis of abalone *Haliotis dicus hannai* | *Haliotis discus hannai* | Aquaculture | Digestive gland and Intestine | Ma et al., 2025 |
| Microbial community analysis in the gills of abalones suggested possible dominance of epsilonproteobacteriu m in *Haliotis gigantea* | *Haliotis gigantea, H. discus and H. diversicolour* | Wild | Gill | Mizutani et al., 2020 |
| Effects of kelp *Ecklonia maxima* inclusion in formulated feed on the growth, feed utilisation and gut microbiota of South African abalone *Haliotis midae* | *Haliotis midae* | Aquaculture | digestive tracts, including the oesophagus, crop, stomach, style sac and intestines, | Nel et al., 2017 |
| The effect of juvenile abalone *Haliotis midae* (Linnaeus, 1758) weaning diet on gut-bacterial formation | *Haliotis midae* | Aquaculture | All soft tissues (including the foot muscle and viscera) | Nel et al., 2018 |
| Effect of oxytetracycline treatment on the gastrointestinal microbiome of critically endangered white abalone (*Haliotis sorenseni*) treated for withering syndrome | *Haliotis sorenseni* | Wild (research stock) | Gastrointestinal tract | Parker-Graham et al., 2020 |
| Effects of dietary EPA/DHA ratio on the growth performance, intestinal microbiota, immunity and resistance to heat stress in abalone *Haliotis discus hannai* Ino | *Haliotis discus hannai* | Aquaculture | Intestine | Tabuariki et al., 2024 |
| Microbiota of the Digestive Gland of Red Abalone (*Haliotis rufescens*) Is Affected by Withering Syndrome | *Haliotis rufescens* | Aquaculture | Digestive gland | Villasante et al., 2020 |

| | | | | |
|---|---|---|---|---|
| Effects of temperature, diet and genotype-induced variations on the gut microbiota of abalone | *1) Haliotis discus hanna and 2) hybrid Haliotis discus hannai*♀ × *H. fulgens*♂ | Aquaculture | Intestine, rearing water filtrates and diet samples | Wang et al., 2020 |
| Seasonal microbial profiling of left kidney and stomach of farmed adult greenlip abalone (*Haliotis laevigata*) | *H. laevigata* | Aquaculture | Left kidney tissue | Wang et al., 2021b |
| The differences in intestinal microbiota between common and orange-muscle of *Haliotis gigantea* and dietary influences on abalone's intestinal microbiota | *Haliotis gigantea* | Aquaculture | Intestine | Wei et al., 2025 |
| Effects of replacing fish meal with corn gluten meal on growth performance, intestinal microbiota, mTOR pathway and immune response of abalone *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | intestine | Yu et al., 2022b |
| Impacts of replacing dietary fish meal with poultry by-product meal on growth, digestive enzymes and gut microbiota, biomarkers of metabolic and immune response, and resistance to *Vibrio* challenge in abalone (*Haliotis discus hannai*) | *Haliotis discus hannai* | Aquaculture | Intestine | Wu et al., 2023 |
| Dietary Clostridium autoethanogenum protein has dose-dependent influence on the gut microbiota, immunity, inflammation and disease resistance of abalone *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | Intestine | Wu et al., 2024 |

| | | | | |
|---|---|---|---|---|
| Effects of replacing dietary fish meal with enzyme-treated soybean meal on growth performance, intestinal microbiota, immunity and mTOR pathway in abalone *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | Digestive gland | Yu et al., 2022a |
| Exploring the Intestinal Microbiota and Metabolome Profiles Associated With Feed Efficiency in Pacific Abalone (*Haliotis discus hannai*) | *Haliotis discus hannai* | Aquaculture | Intestine | Yu et al., 2022b |
| Comparative analysis of intestinal microbiota and its function on digestion and immunity of juvenile abalone *Haliotis discus hannai* fed two different sources of dietary soybean protein | *Haliotis discus hannai* | Aquaculture | Intestine | Yu et al., 2025 |
| Monitoring bacterial community dynamics in abalone (*Haliotis discus hannai*) and the correlations associated with aquatic diseases. | *Haliotis discus hannai* | Aquaculture | Intestine, artificial culture pond water and seawater filtrates | Zhang et al., 2022 |
| Effects of dietary supplementation of probiotics on growth, immune responses, and gut microbiome of the abalone *Haliotis diversicolour* | *H. diversicolour* | Aquaculture | Intestine | Zhao et al., 2018 |
| Dietary curcumin influence on growth, antioxidant status, immunity, gut flora and resistance to *Vibrio harveyi* AP37 in *Haliotis discus hannai* | *Haliotis discus hannai* | Aquaculture | Intestine | Zou et al., 2022 |

### *2.5.1 Characterization of abalone core gut microbiome*

Among documented abalone microbiome communities, the microbial composition, diversity, and the intrinsic microbial biochemical pathways of the digestive tract (stretching from buccal cavity to anus, collectively referred as “gut”) have been predominately studied in abalone due to two main reasons. Firstly, the gastropod digestive tract harbours taxonomically and functionally more diverse and complex microbes than any other body region as microorganisms can be introduced into the gut directly through food ingestion and seawater (Zhang et al., 2022, Li et al., 2025, Salloum et al., 2025). Secondly, the gut microbiome plays a crucial role in food digestion and nutrient absorption (Zou et al., 2022), and contributes to immunity, epithelial renewal and overall growth and health (Yu et al., 2022b).

Dominant bacterial taxa documented in the abalone digestive system have been reported across a broad range of anatomical terms, including samples reported as: intestine, gut, gastrointestinal tract, digestive gland, stomach, viscera, digestive gland merged with gonad, digestive track, esophagus, content of the gastrointestinal track, gill, left kidney and soft tissue (Fig. 2.3b; Table 2.2). From these reports 42% of the studies explicitly stated that intestinal samples were collected for microbiome analyses. This lack of anatomical specificity is problematic, as the abalone digestive system comprises morphological and functional distinct regions, each with unique cell types, microbial niches, and physiological roles, as previously outlined in this manuscript. Therefore, greater precision in anatomical terminology and methodological reporting is strongly encouraged in future abalone microbiome research.

The abalone studies analysed include research on a wide range of abalone species, sourced from both wild and aquaculture settings, spanning various sizes and life stages, and subjected to diverse experimental interventions or sampled from large natural populations, analysed from an array of samples with a multitude of techniques (Table 2.2). Despite these differences, a consistent set of dominant bacterial phyla has been identified across studies, namely: Proteobacteria, Tenericutes, Firmicutes, Fusobacteria, and Bacteroidota (Fig. 2.3c). At the genus level, abalone dominant taxa include *Mycoplasma, Psychrilyobacter*, and *Vibrio* (Fig. 2.3d). These genera are thought to play key roles in host physiology. Collectively, the consistent presence of these bacterial taxa across diverse host and environmental contexts suggests they represent a conserved core microbiota with essential roles in digestion, nutrient assimilation, and host-microbe interactions in abalone (Fig. 2.4).

***Mycoplasma***, is suggested as core microbiota in abalone (Wang et al., 2021b). These bacteria are described as facultative anaerobes which produce lactate and acetoacetate (Guo et al., 2024), act as mutualists (Zou et al., 2022), and ferment glucose and hydrolyze arginine (Wang et al., 2020). Generally,

*Mycoplasmas* are considered members of the class Mollicutes, a group of Gram-positive bacteria that evolved from the Bacillus/Clostridium lineage within the Firmicutes phylum (Yu et al., 2025). *Mycoplasmas* are well adapted to the unique environmental conditions of the abalone intestinal tract, including low oxygen levels and the presence of mucus (Wei et al., 2025). They are useful in the catabolism of complex substances such as oligosaccharides, polysaccharides, glycans and proteins (Ma et al., 2021, Hur et al., 2023). They provide their hosts with simple carbohydrates and amino sugars (Cicala et al., 2018). *Mycoplasma* spp. are believed to aid in abalone digestive processes (Danckert et al., 2021). *Mycoplasma* supports hosts metabolic functions and contributes to digestion of nutrient-poor foods (Villasante et al., 2020). *Mycoplasma* spp. has also been found as pathogens linked to infections (Li et al., 2025), which may disrupt host digestive and absorptive capabilities (Wu et al., 2023). *Mycoplasma* may help protect their host against microbial pathogens by degrading the sialic acid coating on bacterial cell walls (Cicala et al., 2018). Reports suggest *Mycoplasma* protect the host by competitively inhibiting pathogen attachment through occupation of cellular binding sites (Cicala et al., 2023). In the last 10 years, the genus *Mycoplasma* has been reported in: *Haliotis gigantea* (Mizutani et al., 2020, Wei et al., 2025), *H. fulgens and H. corrugata* (Cicala et al., 2018, Cicala et al., 2023), *H. laevigata* (Danckert et al., 2021, Wang et al., 2021b), *H. laevigata* × *H. rubra* (Danckert et al., 2021), *H. tuberculata* (Gobet et al., 2018), *H. midae* (Nel et al., 2017), *H. rufescens* (Villasante et al., 2020), hybrid *H. discus hannai* × *H. fulgens* (Wang et al., 2020) and *H. discus hannai* (Nam et al., 2018, Wang et al., 2020, Choi et al., 2021, Ma et al., 2021, Wu et al., 2022b, Yu et al., 2022a, Yu et al., 2022b, Zhang et al., 2022, Zou et al., 2022, Chen et al., 2023, Hur et al., 2023, Wu et al., 2023, Cadangin et al., 2024, Guo et al., 2024, Li et al., 2025, Tabuariki et al., 2024, Wu et al., 2024, Yu et al., 2025).

***Psychrilyobacter*** is classified as an obligate anaerobe, part of the phylum Fusobacteria, with functions useful in digestion, nutrient absorption, the processing of fermentative metabolism (Guo et al., 2024), protein degradation (Hur et al., 2023). They are associated with marine sediments (Danckert et al., 2021). *Psychrilyobacter* metabolize carbon sources (sugars, organic substances, and amino acids) to produce hydrogen and acetate useful in metabolic processes (Wang et al., 2020, Wei et al., 2025). Moreover this bacteria has been documented in the production of bioactive compounds such as short-chain fatty acids and antimicrobial agents (Li et al., 2025) the production of acetyl-CoA useful for degrading of undigested oligo-polysaccharides (Choi et al., 2021). Their role further extents to the fermentation of pyruvate to produce short-chain fatty acids (Zou et al., 2022), which are important in energy metabolism and immune homeostasis (Danckert et al., 2021). Abalone studies reporting *Psychrilyobacte* at genus level utilized, *H. gigantea* (Wei et al., 2025), *H. laevigata* (Wang et al., 2021b), *H. tuberculata* (Gobet et al., 2018), *H. laevigata* and *H. laevigata* × *H. rubra* (Danckert et al., 2021), hybrid *H. discus hannai* × *H. fulgens*

(Wang et al., 2020) and *H. discus hannai* (Wang et al., 2020, Choi et al., 2021, Zou et al., 2022, Chen et al., 2023, Hur et al., 2023, Li et al., 2025).

Also, ***Vibrio*** is widely reported as genus linked to the abalone core microbiome and regarded as a dominant digestive tract species (Cadangin et al., 2024). These are members of the Proteobacteria, classified as facultative anaerobes (Danckert et al., 2021), with the capacity to produce fermentation products (Choi et al., 2021). *Vibrio* is believed to partake in the degrading of complex polysaccharides via the production of exogenous digestive enzymes (Hur et al., 2023) and often been used as probiotic feed supplementation due to their ability to assimilate macroalgae (Danckert et al., 2021). The ability of *Vibrio* to degrade seaweed polysaccharides (like alginic acid and laminarin) makes it important for host digestion (Wei et al., 2025). Moreover, they are often regarded as pathogenic (Guo et al., 2024), hand have been associated with marine disease outbreaks (Li et al., 2025). *Vibrio* pathogens produce toxins with oxidative stress and inflammatory responses to follow, resultantly affecting overall immune function (Yu et al., 2025). The specific role and interaction of *Vibrio* spp. with the host (symbiotic or parasitic) depends on the strain involved and the environmental conditions at sampling time (Danckert et al., 2021). Their presence at genus level has been reported in the abalone: *H. gigantea* (Mizutani et al., 2020, Wei et al., 2025), *H. fulgens* and *H. corrugata* (Cicala et al., 2018, Cicala et al., 2023), *H. laevigata* and *H. laevigata* × *H. rubra* (Danckert et al., 2021), *H. tuberculata* (Gobet et al., 2018) and *H. discus hannai* (Nam et al., 2018, Choi et al., 2021, Zhang et al., 2022, Hur et al., 2023, Cadangin et al., 2024, Guo et al., 2024, Li et al., 2025, Yu et al., 2025).

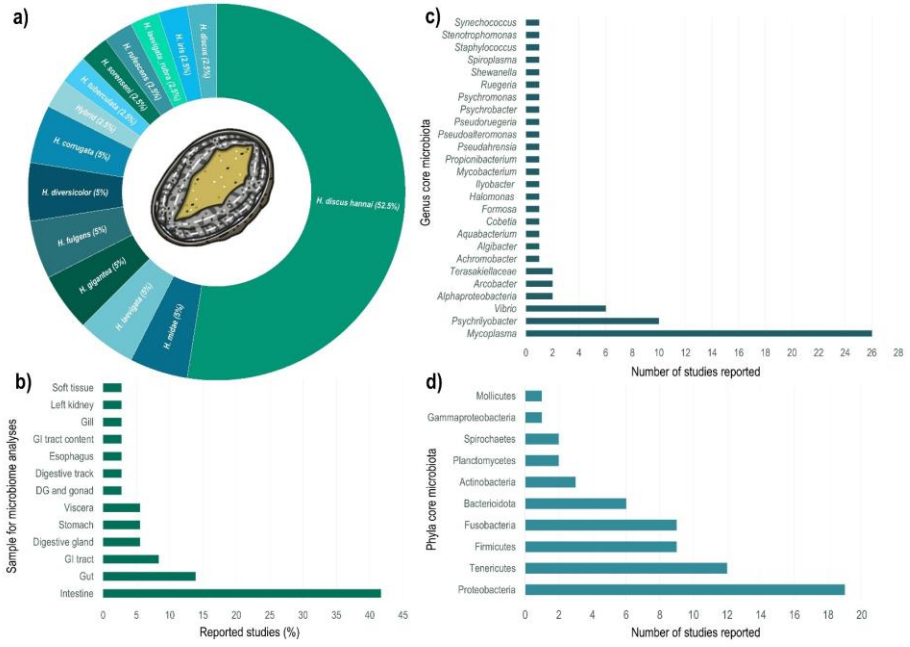

**Figure 2.3: Overview of 10-years' (2015 - mid-2025) worth of abalone microbiome studies including a) the percentage of studies per *Haliotis* species, b) the tissue types reported for microbiome analyses, the c) dominant bacterial genus and d) phyla reported in abalone gut microbiome composition.**

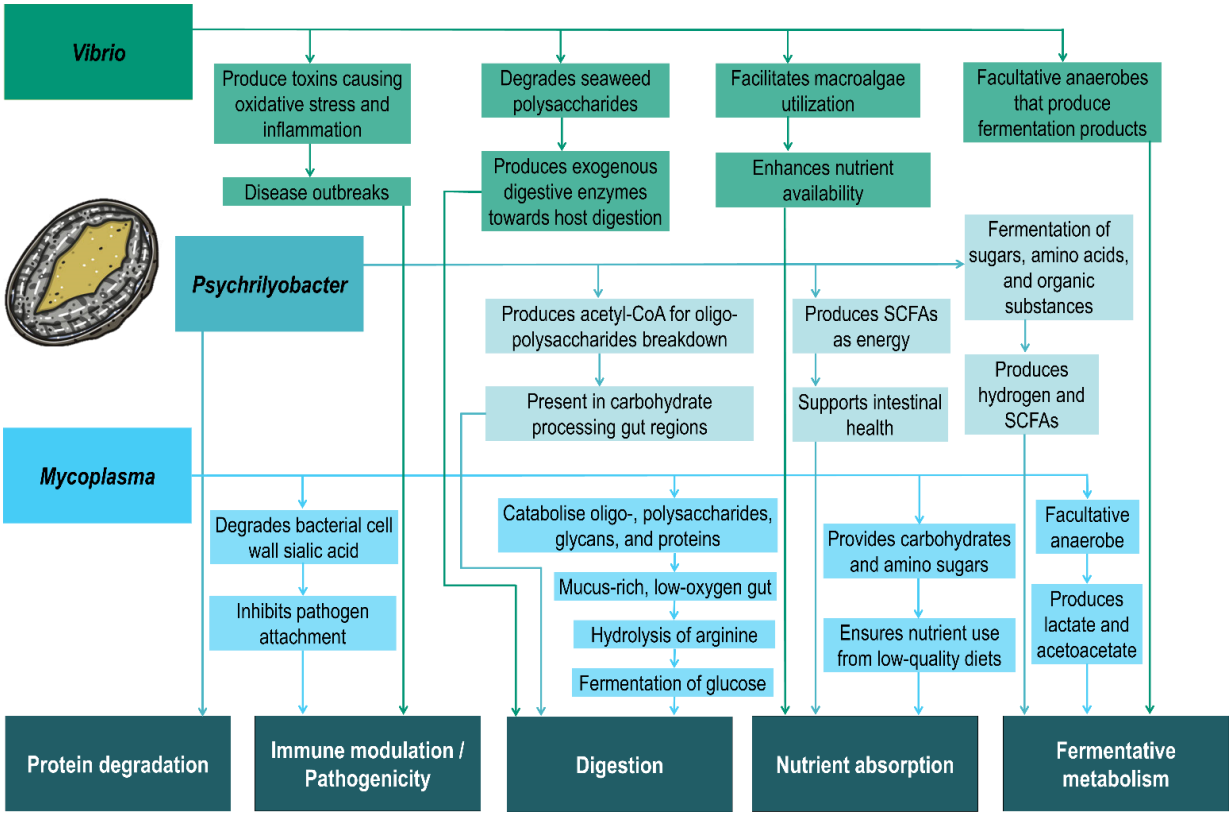


**Figure 2.4: Dominant genus-level taxa in abalone as *Mycoplasma, Psychrilyobacter* and *Vibrio* fulfilling functions linked to protein degradation, immune modulation and pathogenicity, digestion, nutrient absorption and fermentative metabolism.**

### *2.5.2 Abalone microbiome changes associated with dietary interventions*

Indeed, gut microbial communities modulate and are themselves modulated by the physical and biochemical environment of the digestive tract and the food that passes through (Vernocchi et al., 2020). Abalone naturally feeds on seaweed, which is broadly classified as microalgae (e.g., diatoms and dinoflagellates) and macroalgae (including red, green, and brown seaweed). The nutritional makeup of both seaweed types predominantly comprises lipids, polysaccharides, and proteins (Xie et al., 2024). Generally, seaweed contains approximately 65% fiber (in the form of polysaccharides), 25% proteins, and 5% lipids (Dawczynski et al., 2007, Viera et al., 2011). Moreover, bacteria belonging to the classes *Bacilli, Actinomycetia, Gammaproteobacteria* and *Clostridia* are needed for effectively breaking down these macronutrients (You et al., 2020, Yesiltas et al., 2021, Abomohra et al., 2022). In addition to seaweed,

formulated feeds have gained widespread use in the abalone aquaculture industry over the past decades (Lee et al., 2017), and they have been found to enhance abalone growth by balancing the microbiota in the digestive tracts of farmed abalone (Bullon et al., 2025). Utilization of these artificial diets is advantageous since the nutritional compositions of the formulated feeds can be meticulously designed and precisely measured (Van der Poel et al., 2020). Additionally, special additives, such as probiotics (beneficial bacterial strains), for example, can be incorporated into the feeds and provided to abalone to achieve better performance (Dezfooli et al., 2023) which also alters the microbial composition and diversity in different gut regions of abalone (Zhao et al., 2018). The nutritional composition of the diets significantly affects the microbiome of the digestive tract (Beam et al., 2021). Studies published from the last 10 years (Table 2.2), have reported changes in the intestinal microbiota of abalone in response to dietary interventions linked to the use algae, lipids, protein, probiotics and additional supplementation ingredients. The following section summarizes key studies from the literature that have investigated these dietary influences on the gut microbiota of abalone.

***Algal diets***

Abalone aquaculture largely relies on cultured algae during the early life stages, providing both a settlement cue for larvae and a primary food source for post larvae and juveniles, until they transition to formulated feeds in the grow-out phase (Daume, 2006). The consumption of macroalgae in aquaculture settings as broodstock or as seasonal feed, and wild abalone are mainly dependent on carbohydrate-rich macroalgae (Bullon et al., 2023). Abalone can digest most of the carbohydrates in macroalgae due to gut-associated bacteria which can hydrolyze structural and storage carbohydrates using a variety of carbohydrases, including laminarinase, α-amylase, maltase, agarase, alginate lyase, carrageenase, carboxymethylcellulase, sucrase, and β-galactosidase, etc. (Bansemer et al., 2016). Findings from microbiome studies are less focused on the enzymes involved in carbohydrate digestion but do report the associated microbiota due to consumption of an algal diet. The types of macroalgae (as brown, red or green seaweed) are often associated with polysaccharide digestion. To illustrate, alginate, laminarin, and fucoidan are the three primary polysaccharides present in brown macroalgae (Phaeophyta), consumed by abalone worldwide (Hay, 1990, Wernberg et al., 2003, Almanza and Buschmann, 2013, Abe et al., 2020). Then again, red seaweed (Rhodophyta) contains the polysaccharides agar and carrageenan which are crucial ingredients in formulated aquaculture diets (Cotas et al., 2020). Abalone also commonly feed on green seaweed (Chlorophyta), such as sea lettuce (*Ulva* spp.) with sulfated polysaccharides, especially in hatcheries as habitat substrates to facilitate early growth of abalone (Wikfors and Ohno, 2001, Bansemer et al., 2016).

Bacterial strains capable of digesting brown, red and green seaweeds (e.g., *Sargassum horneri, Ecklonia maxima, Laminaria japonica, Gracilaria lemaneiformis*) were notably abundant in the gut of *H. discus hannai*, *H. midae*, *H. gigantea,* hybrid species and *H. tuberculata,* and are outlined below in the content of reported microbiota. For instance, following a 12-week feeding trial in post-larval *H. discus hannai*, *Undaria pinnatifida* were replaced with varying levels of *Sargassum horneri* (brown macroalgae). Herein *Psychrilyobacter* was found in high levels in the absence of *S. horneri*, but lower levels were present when only *S. horneri* was consumed. *S. horneri* was found to enhance growth and support gut microbial communities in this abalone species (Hur et al., 2023). In *H. midae* consuming brown kelp, *Ecklonia maxima* incorporated into formulated feeds, *Mycoplasma* was found as abundant in the gut alongside a balanced gut bacterial composition, attributed to the phytochemicals found in kelp and the gut bacterial regulation associated with dietary kelp (Nel et al., 2017). In a next study by Nel et al. (2018) post-settled, *H. midae* weaned onto fresh brown kelp (*Ecklonia maxima*), reported anaerobic Clostridium species as dominated the guts due to the large volume fermentable polysaccharides present in this macroalgae. In two-year-old *H. gigantea* the dominant bacterial genus in abalone fed with fresh brown kelp (*Laminaria japonica)* for six- months, were *Stenotrophomonas* and *Vibrio.* Also in this study the use of red macroalgae (*Gracilaria lemaneiformis)* was tested and showed *Psychrilyobacter* with a higher relative abundance in the intestinal tract of abalone fed with *G. lemaneiformis* (Wei et al., 2025). The hybrid *H. discus hannai* × *H. fulgens*, showed stable dominant bacteria reported as unidentified Oxyphotobacteria when consuming either *Gracilaria lemaneiformis* - red macroalgae*, Laminaria japonica* - brown macroalgae or an artificial diet (Wang et al., 2020). A 6-month feeding trial of monospecific algal species using juvenile *H. tuberculata* reflected a diet-specific core microbiota, with genera representative of aerobic algal polysaccharide degraders. In this study the genera, *Polaribacter* and *Pseudahrensia* were found in abalone consuming the *Palmaria palmata* (red algae) diet, while *Escherichia-Shigella* was linked to the *Ulva lactuca* (green algae) diet. Then again, *Roseibacillus* was found in the *Laminaria digitata* (brown algae) diet group, and *Ulvibacter* was associated with the *Saccharina latissima* (brown algae) diet. Interestingly this study concludes that feed type only accounts for a small proportion of microbiome variability and that other factors such as microbiota gene transfer may play a bigger part (Gobet et al., 2018). Guo et al. (2024) reported better growth performance and digestive enzymes in juvenile *H. discus hannai* when fed a formulated diet (compared to starvation and fresh kelp), while also boasting higher Proteobacteria.

Overall, macroalgal diets shape distinct gut microbial profiles in abalone, with specific bacterial taxa responding to the structural complexity of algal carbohydrates. Future research should adopt enzyme profiling to uncover carbohydrate-active enzymes (CAZymes) produced by marine algae (Hehemann et

al., 2010) to infer degradation capacity of dietary polysaccharides. This was demonstrated by Gobet et al. (2018) which explored the algal polysaccharide digestion potential of the core abalone digestive microbiota by means of a model bacterium for the bioconversion of algal polysaccharides. It is also important to keep in mind that diet alone does not fully explain microbiome variability, and that additional influences such as host genetics, developmental stage, and horizontal gene transfer (genetic information inherence from parents to offsprings) should also be considered,

***Lipids***

Intestinal microbiota contributes to new lipid molecules in the hosts gut composition. These lipids obtained by the host's dietary intake are typically processed by the intestinal microbiota's lipase enzymes. Also intestinal microbial species can produce lipids from non-lipid sources (e.g., some oleaginous yeasts and fungi utilize non-lipid precursors like sugars, acetate, and glycerol to synthesize lipids) (Yoon et al., 2024). The nutritional proportion of lipids in microalgae, such as diatoms and dinoflagellates, is relatively higher than that in macroalgae (Leyland et al., 2020). While small lipid molecules can be readily utilized by abalone larvae, relatively larger molecules (e.g., triacylglycerol and unsaturated fatty acids) could be comparatively more difficult to digest (Lemons and Liu, 2022). Previous studies have illustrated that the nutritional lipids selected and proliferated bacterial strains, such as *Acinetobacter*, Actobacilli, *Bifidobacterium Rhodococcus*, *Roseburia*, *Mycobacterium*, *Clostridium*, *Syntrophomonas*, Chloroflexi, and Bacteroidetes specialized in digesting fatty acids in the gut of juvenile abalone (Durazo-Beltrán et al., 2003, Guest et al., 2008, Pan et al., 2018). Similarly, the Pacific abalone *H. discus hannai* fed with an optimized eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-ratio diet showed high proportions of Clostridium, Mycobacterium, Bacilli and Bacteroides, which demonstrated high efficiency on metabolizing lipid molecules (Tabuariki et al., 2024).

***Protein***

Proteins are another group of macronutrients that are essential to abalone growth, and digesting protein molecules often require various specialized gut microorganisms through biochemical processes (Ringø et al., 2016). Research on how dietary proteins influence the gut microbiota of abalone has mostly focused on farmed abalone species due to the controlled formulation and consistent macronutrient availability in their diets. Moreover studies have shown that abalone can utilize proteins as dietary macronutrients to metabolized amino acids which supports protein synthesis (Tung and Alfaro, 2012, Bullon et al., 2023). The amino acids and peptides generated from protein digestions influence the gut microbiome composition. Also undigested feed (due to oxidation for example) contain proteins that serve as substrates for microbial fermentation and influence the gut microbiota diversity (Wu et al., 2022a). This is evident in

an experiment on farmed *H. discus hannai* fed formulated feeds with varying protein contents. Results showed the highest gut microbial diversity at 275.9 g/kg protein, with elevated *Flavobacteriaceae*, a group linked to iso-amylase production and higher intestinal α-amylase. *Acinetobacter*, associated with digestive enzyme support, was abundant at 230.3 g/kg protein inclusion. Herein it was reported that both excessively high and low protein levels negatively affect microbial diversity (Ma et al., 2021).

Plant protein ingredients and animal byproducts are more commonly used as an alternative to fish meal in aquafeeds (Hua et al., 2019). Alternative protein sources are popular ingredients of abalone feeds. Ingredients such as insect-meal, grape marc, soybean meal, soy protein concentrate, gluten meal, enzyme-treated soybean meal, soybean protein concentrate, corn gluten meal, poultry by-product meal, and ethanol fermentation protein products have been utilized as protein sources for abalone feeds, with changes observed in the gut microbial diversity after the hosts were fed with these alternative protein-based feeds. For instance, juvenile *H. iris* consuming insect-meal and grape marc (low-protein level) showed Firmicutes and Fusobacterium linked to polysaccharide breakdown in the intestines following a 165-day feeding trial (Bullon et al., 2025). In a 120-day trial, *H. discus hannai* fed corn gluten meal showed increased Marinifilaceae and Bacteroides, with reduced Proteobacteria, leading to lower pectinase activity and impaired digestion. The dehulled soybean-meal diet, increased *Bacteroidetes*, linked to disrupted energy metabolism. The soy protein concentrate diet enriched *Paraburkholderia*, *Ralstonia*, and *Ahrensia*, promoting the colonization of arginine-synthesizing microbes and enhancing nutrient metabolism (Ma et al., 2025). When fully replacing fishmeal with enzyme-treated soybean meal (ESBM) in a formulated diet for *H. discus hannai,* Yu et al. (2022b) reported an increased abundance of *Mycoplasma* and *Terasakiellaceae* and decreased abundance of *Halomonas*, *Zobellella* and *Bacillus* alongside decreased growth. The partial replacement of soybean protein concentrate (SPC) to 25% in a formulated diet for *H. discus hannai*, lowered harmful bacteria (such as *Mycoplasma* and *Vibrio*) in the abalone intestine; while a full replacement (100%) changed the intestinal microbiome structure and increased the risk of abalone viral infectious disease (Yu et al., 2025). Also in the study by Yu et al. (2025) soybean meal (SBM) replacement of 50%, resulted in significantly decreased *Dinoroseobacter* in the abalone intestine, along with the highest number of unique operational taxonomic units, supporting improved digestion and enhanced growth rates. The substitution of fish meal with corn gluten meal at levels of 10.8% and higher showed negative effects on the intestinal microbiome on *H. discus hannai*. Dominant genera in tested abalone gut samples included: *Mycoplasma,* Terasakiellaceae*,* Alphaproteobacteria and Spiroplasma, which the authors concluding that higher levels of corn gluten meal increased the harmful bacteria in abalone intestine, due to the hosts reduced ability to digest and absorb nutrients (Wu et al., 2022a). The use of a poultry by-product meal in place of fish meal resulted in

an increased abundance of *Pseudahrensia* in the gut samples of *H. discus hannai,* altering host physiology and coinciding with reduced growth under this treatment (Wu et al., 2023). The addition of *Clostridium autoethanogenum* protein (CAP), a by-product of ethanol fermentation, promoted the presence of probiotic bacterial genera *Ruegeria* and *Bacteroides* in *H. discus hannai*. This role of CAP in enhancing immunity, as indicated by the expression of inflammatory genes are concluded in this study by Wu et al. (2024).

***Probiotics and other compounds***

Probiotics are beneficial live microorganisms (mostly bacteria) that primarily aim to enhance the growth of the farmed abalone in aquaculture by optimizing digestive efficiency (Preece, 2006, Silva-Aciares et al., 2011, Hadi et al., 2014). Prominent bacterial genera, such as *Bacillus*, *Vibrio*, *Enterococcus* and *Exiguobacterium*, have been cultured and isolated, and the contributions of these bacterial strains to abalone survival and growth have shown promising results (Van Doan et al., 2020, Masoomi Dezfooli et al., 2021). Specifically, dietary supplementation using two exogenous probiotics [*Bacillus sp*. KRF-7 (KRF-7), Bacillus sp. PM8313 (PM8313)] and one prebiotic β-glucooligosaccharide (BGO) candidates in juvenile *H. discus hannai* showed diet-specific core microbiota following three months of rearing. Their findings conclude that BGO, KRF-7, and PM8313 can be used in abalone aquaculture as growth-promoting and immune-stimulating supplements, with special mention to *Mycoplasma* and *Algibacter* bacteria (Cadangin et al., 2024). Also, a 180-day trial in *H. diversicolour* showed that diets supplemented with *Bacillus stratosphericus* A3440, *Phaeobacter daeponensis* AP1220, or both diets enhanced immune status and survival. *Bacillus* A3440 notably increased Enterobacteriaceae and Alcaligenaceae, while reducing Mycoplasmataceae (Zhao et al., 2018). An important goal of introducing probiotics to abalone diets is to diversify the microbial communities within their digestive tracts to increase the capability of biochemical functions, such as macronutrient digestion and anti-microbial mechanisms, as shown in the above-mentioned studies.

Other compounds with reported benefits in abalone feeding trials include bile acids and curcumin. The inclusion of dietary bile acids to *H. discus hannai* feed resulted in a decrease in Actinobacteria and *Mycobacterium* and increase of Firmicutes and *Mycoplasma* with increasing dietary bile acid concentrations. Bile acids at appropriate levels were said to promote intestinal health of Pacific abalone following the 105-day feeding trial (Chen et al., 2023). Next, in a 100-day trial, *H. discus hannai* fed 0.1 g/kg curcumin showed increased Firmicutes, Fusobacteriota, and a higher Firmicutes/Bacteroidetes ratio. Dominant genera included *Mycoplasma*, *Psychrilyobacter*, and *Vibrio*. Ultimately curcumin is said to promote bacterial proliferation that supports abalone growth and immunity (Zou et al., 2022).

In essence, diet strongly influences the gut microbiota of abalone, with direct implications for digestion, immunity, and growth. Certain bacterial groups are linked to specific seaweed types, and along with factors such as host species, environment, and microbial gene exchange, help to shape gut communities. Formulated feeds offer controlled nutrient delivery and the addition of probiotics and functional compounds, which can further modulate gut microbiota. Variations in protein and lipid sources, as well as supplemental ingredients like probiotics, bile acids and curcumin, significantly influence microbial composition and host outcomes. Understanding these interactions is key to optimizing feed formulations that support gut health and enhance abalone performance.

### *2.5.3 Abalone microbiome changes associated with environmental changes*

Aside from dietary macronutrients, abalone gut microbial composition, and diversity can also be significantly influenced by a range of environmental factors in the hosts' aquatic habitats (Harris, 1993). Physiological parameters of both wild and farmed abalone are subject to the influences of coastal oceanographic conditions (Morash and Alter, 2016). Fluctuations in the abalone's surrounding environment (e.g., temperature, salinity, wave exposure, pH, and sedimentation) can have a significant impact on their physiology, such as impeded larval settlement (Naylor and McShane, 2001, Onitsuka et al., 2008), reduced growth rates (Cummings et al., 2019), inhibited shell formation (Auzoux-Bordenave et al., 2020), mortalities (Samuel et al., 2019), changed metabolic rate (Fan et al., 2025), altered gene expression (Boamah et al., 2024), modified enzyme responses (Gao et al., 2024) etc. For animals to withstand changes in the environment they need to adapt, both genetically and physiologically. The gut microbiome contributes significantly to host resilience, aiding adaptation to environmental fluctuations (Zhang et al., 2025a). The gut microbiome of abalone showed significant changes in response to season, sampling location, temperature, and species. These factors have contributed to the forming of abalone intestinal microecology as outlined next.

When investigating the wild populations of *H. discus hannai* over different months, *Psychrobacter, Vibrio*, and *Shewanella* were reported as the dominant microbiome genera. Specifically, *Psychrobacter* was linked to the sampling times where lower temperatures were recorded, coinciding with the cold environment ecological niche of this bacterium. The opposite was also reported where the sampling points at higher water temperatures showed increased microbial diversity and bacterial loads. Findings from this study confirm that season, location, and temperature significantly influence abalone microbiota (Lee et al., 2016). In a next study, interested in the nutrient-rich conditions of different marine regions, the gut microbiome of *H. discus hannai* showed distinct microbial signatures across sampling groups. One

sampling site reported dominant *Pelagibacterium* as a contributor to oxidation and nitrification processes, while *Psychrilyobacter* correlated positively with improved host nutrient assimilation and increased seawater salinity. At a second site *Mycoplasma* and *Vibrionimonas* were the reported, often associated with infections and disease outbreaks, potentially increasing host vulnerability under environmental stress. At a third site, higher *Chitinophaga* was found associated with nutrient recycling through the degradation of chitin, a key source of carbon and nitrogen in marine ecosystems. This study indicates that changes in the abalone gut microbiomes are often correlated with regional (i.e., inter-site) differences in environmental conditions (Li et al., 2025). Fusobacteria were reported as a major contributor to seasonal differences in the microbiota of farmed *H. laevigata*. At genus level *Pseudoruegeria*, *Arcobacter*, *Shewanella* and *Pseudoalteromonas* showed distinct seasonal differences potentially because of geographical characteristics of the study sites. Also factors such as abalone life stage, species, organ and collection methods are described as reasons for the microbial differences over time. The findings indicate that the microbiome between summer and winter (indicative of microbiota population) was coupled with seasonal and nutritional changes (Wang et al., 2021b). Seasonal variability was also seen in the digestive gland of *H. tuberculata,* which was dominated by *Psychrilyobacter, Mycoplasma*, and *Vibrio* over a one-year sampling trial, yet the community structure showed changes attributed to season. Seasons with increased water temperature showed correlations to *Vibrio*, *Psychromonas*, an unclassified *Rhodobacteraceae*, *Polaribacter*, and *Pseudahrensia* likely in response to seasonality of the water column composition. Altogether, seasonal environmental variations, rather than specific algal diets, significantly shaped the composition and diversity of *H. tuberculata* associated microbiota (Gobet et al., 2018). The intestinal microbiomes of farmed Tiger, (*H. laevigata x H. rubra*) and greenlip (*H. laevigata*) abalone were significantly influenced by the sampling date, linked to seasonal changes and abalone age. *Psychrilyobacter, Vibrio*, and *Mycoplasma* were the dominant genera in both abalone species over a yearly average, yet the relative abundance of these changed with season (Danckert et al., 2021). Considering different temperature exposures, the gut microbiota of *H. discus hannai* was reported to be more sensitive to temperature than the hybrid *H. discus hannai*♀ × *H. fulgens*♂. Overall *Mycoplasma* and *Psychrilyobacter*, were the most abundant genus at different temperatures. At 20 °C the abalone gut microbiome supported higher functions towards carbohydrate metabolism, while degradation of abalone biochemical processes is suggested at 28 °C. This study concluded that there were alternations in the overall microbial community composition under different temperature treatments, but the dominant bacterial genera remained the same (Wang et al., 2020).

Taken together, these studies highlight the influence of environmental variability on the structure and function of the abalone gut microbiome. Indeed, specific microbial genera such as *Mycoplasma,*

*Psychrilyobacter*, and *Vibrio* appear consistently across species and regions, while their relative abundances and functional roles fluctuate in response to environmental cues. These shifts not only reflect microbial adaptation to local conditions but also have important implications for host health and responses. Notably temperature alters abalone microbial community composition and metabolic potential without necessarily replacing dominant taxa. Interestingly, Gobet et al. (2018), reports that digestive microbiota from abalone appears to be largely established within the first year of life, with environmental changes minimally contributing to the overall variability, an assertion that future studies should test across different species and contexts to refine our understanding of microbiome stability in abalone.

### *2.5.4 Abalone infections and diseases and microbial findings*

The growth of abalone aquaculture has resulted in increasing reports of abalone diseases that are worrisome as they hinders abalone physiology and health and ultimately degrade aquaculture advances, resulting in significant financial losses (Gao et al., 2022). To date, at least seven infections and diseases associated with or caused by prokaryotes and microscopic eukaryotes have been described and studied in abalone since 2010 (Table 2.3). Major diseases and infections include abalone herpesvirus, withering syndrome, perkinsosis, nematode parasitism, polychaeta infections and snail parasitism. Microbial agents such as *Vibrio* spp., *Candidatus Xenohaliotis californiensis*, *Pseudoalteromonas shioyasakiensis* and *Perkinsus* spp. are highly virulent in abalone, as seen in *H. rufescens, H. sorenseni, H. discus hannai, H. iris* and *H. rubra* (Nicolas et al., 2002, Cruz-Flores et al., 2015, Li et al., 2021, Zou et al., 2022, Muznebin et al., 2023). Such microbial infections and diseases could trigger two detrimental consequences. First, microbial infections and diseases could alter the abundance and diversity of other microorganisms within the same microbial communities, leading shifts in the entire microbial composition. For example, the withering syndrome observed in *H. discus hannai*, particularly in its gastrointestinal tracts, was found to coincide with alterations in the relative abundance of *Fusobacteria*, *Candidatus Xenohaliotis californiensis* and *Mycoplasma* in the same disease outbreaks, suggesting a possible connection between microbial composition and disease incidence (Zhang et al., 2022). Second, seafood shellfish diseases could bring potential health risks to humans upon exposure to the associated pathogens and parasites. Notably, five pathogenic species: *Lactococcus garvieae*, *Yersinia kristensenii*, *Staphylococcus saprophyticus*, *Staphylococcus warneri* and *Staphylococcus epidermidis* that were linked to food-borne diseases were previously identified (Lee et al., 2016). Furthermore, as the pathogen- and parasite-related disruptions in the microbiota occur, it could also lead to further colonization of opportunistic pathogens and/or parasites to potentially exacerbate the spread of microbial infections and diseases among the hosts, which creates a negative feedback loop (Dantan et al., 2024). Therefore, it is crucial to control microbial

infections and diseases to sustain the health of abalone and other shellfish stocks to ensure both host and human safety.

**Table 2.3: Major abalone disease and microbial infections described since 2010.**

| Disease/Disorder | Agents | Symptoms | Host Species | Geographic distribution | References |
|---|---|---|---|---|---|
| Viral ganglioneuritis | Virus | Irregular foot concave elevation; foot blisters; swollen mouth; radula intrusion; reduced foot muscle adhesion and movement; excess mucus secretion; death. | *H. diversicolour supertexta* | Taiwan | Chen et al., 2012 |
| | | | *H. laevigata, H. rubra, and hybrid H. laevigata* × *H. rubra* | Tasmania, Australia | Caraguel et al., 2019 |
| | | | *H. laevigata, H. rubra and H. conicopora* | Victoria, Australia | Corbeil et al., 2016 |
| | | | | | |
| Shriveling syndrome | Virus | Cracked shell; histological necrosis; abnormal hemocyanin macromolecules; death. | *H. diversicolour* | China | Jiang et al., 2014 |
| | | | | | |
| Withering syndrome | Bacterium (*Candidatus Xenohaliotis californiensis*) | Soft foot muscle; lethargy; retracted visceral tissues; death. | *Haliotis cracherodii* | California, USA; Baja California, Mexico | Orland et al., 2022 |
| | | | *H. rufescens* | | Moore et al., 2011; Friedman and Crosson, 2012; Vater et al., 2018 |
| | | | *Haliotis sorenseni* | | Friedman et al., 2014; Vater et al., 2018 |

| | | | | | |
|---|---|---|---|---|---|
| | | | *H. discus discus, H. discus hannai, H. gigantea, H. diversicolour aquatilis, H. diversicolour diversicolour* | Japan | (Nishioka et al., 2016) |
| | | | | | |
| Perkinsosis | Protozoa (*Perkinsus* spp.) | Blisters on foot and mantle; thin, watery tissues; pale digestive gland. | *H. iris* | New Zealand | Muznebin et al., 2023 |
| | | | *H. rubra* | South Australia | Abbas et al., 2025 |
| | | | | | |
| Nematode parasitism | Nematode (*Echinocephalus pseudouncinatus*) | Foot blisters; weakened foot muscle. | *H. rufescens* | California, USA | Lynch et al., 2022 |
| | | | | | |
| Polychaeta infection | Polychaetes (*Argyrosomus inodorus* and *Arenicola loveni loveni*) | Visible on abalone shell with thick mucus; burrowed, decoloured, and/or thinned shell; thickened shell edge; reduced growth rates. | *H. midae* | South Africa | Yearsley et al., 2011 |
| | | | | | |
| Snail parasitism | Mollusk snail (*Evalea tenuisculpta*) | Visible snails growing on shell; burrowed shell with thick mucus; thickened shell edge; | *H. rufescens* | California, USA | (Maguire and Rogers-Bennett, 2013) |
| | | | | | |

Maintaining a healthy and balanced microbiota is one of the effective directions for reducing the microbial infections and diseases, and the intestinal microbiota of abalone and other shellfish offers a great opportunity to examine the effectiveness of microbial homeostasis on pathogen and parasite control,

as aquatic animal diseases are frequently associated with shifts in the intestinal microbiome (Ghosh et al., 2025). For abalone, their intestinal microbiota has a unique relationship between the host and pathogens, with options to inhibit the pathogen, compete for nutrients, offer resistance to colonization or interact with host factors (Wu et al., 2024). The intestinal microbial communities of the gastropods produce antimicrobial compounds against marine pathogens, which supports immune regulation (Offret et al., 2019, Li et al., 2025). Moreover, the abalone gut microbiota is known to influence the modulation of immune responses by affecting the expression of immune-related genes in the host, impacting the activation and proliferation of immune cells as a self-defense mechanism (Cicala et al., 2018, Nam et al., 2018). Hereby, abalone gut environment acts as a critical interface where immune and microbial components interact to establish a state of equilibrium against microbial invasions (Okumura and Takeda, 2017).

Utilizing therapeutic treatments (e.g., antibiotics) is another way to mitigate microbial infections and diseases in abalone stocks; however, such treatments could potentially influence the overall gut microbiota. For instance, white abalone *H. sorenseni* treated with oxytetracycline showed effective antimicrobial activities against *Candidatus Xenohaliotis californiensis* that is responsible for withering syndrome, but several bacterial strains, such as *Fusobacteria,* that were normally a part of the core gastrointestinal microbiota were also absent (Parker-Graham et al., 2020). Similarly, Yu et al. (2022a) reported that microbial diversity decreased in abalone fed with artificial feeds formulated with antibiotics, and the reduction in gut microbial diversity also contributed to a lower feed efficiency in the abalone stocks. These findings suggest that certain therapeutic measures might adversely affect abalone's gut microbiota as well as highlight the complexity of microbiome-disease interactions. More experimental trials are needed towards optimizing the effectiveness of antibiotics that are customized for abalone disease control.

**2.6 Future research directions and conclusions**

Microbiome research in the digestive tracts (especially in the intestines) of wild and farmed abalone is currently at the early stage compared to that of fish and humans. The microbiota composition, in terms of microbial richness and diversity, is an initial step of exploring microbiome genetics, functions and the host-microbes' interactions. A core microbiome of overall abalone species has been established relating to abalone dominant taxa including *Mycoplasma, Psychrilyobacter*, and *Vibrio.* Still China is leading the microbiota composition of global abalone populations using *H. discus hannai* and hybrid variants, with information on other species, with different dietary needs and environmental challenges falling behind.

Hybridization of abalone species has also proven useful to enhance animal traits offering improve stress resilience, growth rates, food conversion etc. (You et al., 2019), which in-effect provides beneficial functioning of the microbiome, such as greater metabolic diversity and suppression of pathogenic taxa. Advances in microbiome research in hybrid fish species have demonstrated significant potential (Cui et al., 2022) and can be translated to abalone to explore similar host–microbe interactions and performance benefits. A next point of concern is the small sample sizes used in microbiome research, as this is unlikely to show scientific significance towards characterizing microbial communities (Chen et al., 2022). To accurately characterize microbiomes and draw ecologically and biologically relevant conclusions, adequately powered studies with larger, well-replicated sample sets are essential, especially in systems with complex host–environment interactions such as aquaculture species. Alongside the inclusion of sufficient sample numbers, studies should expand to the inclusion of environmental samples, such as those from surrounding water, seaweed, and sediment for microbial assessment. As an illustration, a microbiome study on *H. iris* showed that habitats or specific nutritional profiles of the seaweed that were digested, were associated with direct microbial transfer towards the abalone's digestive microbiome (Guo et al., 2025). Notably, early life microbiomes affect host health at later stages, hence longitudinal studies, tracking microbial changes over developmental stages can help to assess functional biomarkers associated with health, stress resilience, and growth performance. For example, a study on crabs outlined stage-specific microbiomes where the use of probiotics helped to increase growth performance during early life stages to ensure effective larvae culture (Fu et al., 2024). Bearing in mind that abalone settlement remains a bottleneck on abalone farms (Hannon et al., 2013) the use of microbiomics might be useful in assessing abalone larvae microbiomes. Studying dietary nutritional influences on abalone gut microbiome, utilizing controlled laboratory settings can help to outline the role of bacteria in digestion and growth (Danckert et al., 2021). Experimental results can contribute to diet-formulation optimizations based on various quantifiable physiological parameters (e.g., growth rate, meat quality/nutritional values, gut microbial diversity estimator, microbial infection/disease prevalence, etc.). Interestingly, in fish it was found that bacterial DNA present in the feed itself, ensured a feed microbiome carry over effect, finding that the gut microbial profiles from the distal intestinal digesta differed from feed microbiomes (Karlsen et al., 2022). Indeed an avenue that warrants investigation in abalone, considering that abalone aquaculture largely depends on formulated feeds (Li et al., 2024). In light of changing environmental and oceanographic conditions, changes in the gut microbial communities among wild abalone stocks should be monitored and tracked overtime. Moreover, the functions of associated microbial genera should be elucidated further to determine when species are beneficial or pathogenic (Danckert et al., 2021). To achieve such outcomes additional methodologies will have to be implemented as 16S rRNA gene sequencing is not able to distinguish between beneficial and pathogenic strains. Yet the use of multi-omics approaches, such as

metabolomics for example, allows a deeper understanding of the physiological response following exposure to a stressor (Alfaro and Young, 2018). Data on the metabolites produced by microbiota can help to infer function thereby ensuring an integrative framework for analysing microbial communities (Chen et al., 2022). Additionally, bioinformatic analyses such as co-occurrence network modeling and functional annotation can be employed to map ecological interactions and infer microbial contributions to host physiology (Codello et al., 2023). Together, these data-driven insights can guide evidence-based management strategies aimed at optimizing abalone health and promoting sustainable aquaculture practices.

In conclusion, abalone are benthic marine herbivores of significant ecological and economic importance globally. Ensuring the growth and health of both wild and farmed abalone stocks is essential for sustaining their productivity and long-term viability. The gut microbiome plays a critical role in host health by mediating key microbial functions such as digestion, immune modulation, and antimicrobial activity. With advancements of high-throughput sequencing technologies our ability to characterize abalone microbial richness and diversity is growing, with data documented for 14 abalone species. Accurately interpreting and applying microbiome data remains challenging, as defining the functional roles of the abalone core microbiome will likely depend on the integration of advanced technologies. The application of multi-omics approaches allows an opportunity to maximize microbiome outputs and better predict the productivity of abalone aquaculture and fisheries. The overarching economic, cultural, and ecological importance of abalone allows for continued research into the abalone microbiome.

## Chapter 3: New Zealand abalone (*Haliotis iris*) digestive regions: bacterial microbiome composition and functional potentials

**Prelude**: Abalone has a complete digestive system, and each section of the digestive tract has different morphology and biochemical characteristics. The microbiomes along the entire digestive tract may also be different. In addition, the surrounding environments where abalone dwell in also contain microbial communities, and the resemblance between the microbiomes of abalone's digestive system and the environmental microbiomes is unclear in *H. iris*. Therefore, this chapter aims to investigate the microbiomes at three digestive regions and compare the microbiome of the hosts to that of the environmental samples (seaweed and sediment).

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### Abstract

Abalone are valuable commercial marine gastropods, supporting both aquaculture and fishery markets. An important ecological aspect of their survival and physiological performance in a given habitat is a complex and balanced symbiotic relationship with microbes in their digestive system. 16S rRNA Illumina MiSeq sequencing was used to investigate the microbial composition of New Zealand abalone (*Haliotis iris*) digestive regions (buccal cavity, foregut, and hindgut), seaweed, and sediment samples from the Cook Strait, New Zealand. The findings revealed an overlap in the microbial communities in the foregut and hindgut samples which differed from buccal cavity samples. The foregut and hindgut were dominated by Fusobacteria, Firmicutes and Proteobacteria. Proteobacteria were abundant in the buccal cavity, seaweed, and sediment samples. Despite distinct overall microbial compositions in the abalone digestive tract and environmental samples (seaweed and sediment), observed overlaps in bacterial richness and diversity suggest that surrounding habitats may serve as significant reservoirs for the abalone gut microbiome. This study highlights baseline data of the bacterial community composition in wild *Haliotis iris* gut regions for future investigations of environmental drivers shaping abalone gut microbiota.

**Keywords:** Abalone, buccal cavity, foregut, hindgut, fisheries, *Haliotis iris*, microbiome, seaweed, sediment

### 3.1 Introduction

The New Zealand (NZ) black-foot abalone, *Haliotis iris*, inhabits rocky intertidal and subtidal reefs where they graze mainly on drifting seaweed (Copedo et al. 2024). This species is harvested from wild stocks to support recreational, customary, and commercial entities (Ryder et al. 2023). Various factors, such as environmental stress (Morash and Alter 2016), genetics (van der Merwe et al. 2011), diet and nutrition (Bullon et al. 2023), diseases (Moore 2023), substrate and habitat (Aguirre and McNaught 2011) have been reported to affect the growth and development of abalone. Growing evidence has also shown a close interaction between the microbes in the digestive tract and the host growth performance (Fan et al. 2019). These microbes, particularly bacteria, colonise the digestive tract of abalone (Nel et al. 2018) and support nutrient digestion, host health, metabolism, immune activity and neural development (Yu et al. 2022). Depicting the composition and diversity of the bacterial communities in the digestive tract is a prerequisite for evaluating the gut microbial functions regarding the nutrient digestion and other physiological parameters.

Abalone has a complete digestive system that can be broadly divided into four regions: buccal cavity, foregut, midgut, and hindgut, each with its own physical and biochemical functions (Harris et al. 1998a). The buccal cavity mainly consists of the salivary gland and an elaborate odontophoral apparatus, including a ribbon-like structure called the radula and connective tissues (Voltzow 2023). The foregut refers to the esophagus pouch with additional glands. The midgut includes the stomach and digestive gland, which contain the enzymes for the digestion of food particles and conversion into macronutrients such as lipids, polysaccharides, and proteins. Lastly, the hindgut includes the intestine and anus, where most of the nutrient digestion and absorption take place (Johnston et al. 2005, Lobo-da-Cunha 2019). Since the buccal cavity and foregut are closer to the outer environments compared to the midgut and hindgut, they are mostly responsible for physically breaking down and hydrolysing food particles, and as such the bacterial profiles of these two regions are hypothesised to be similar to the ambient environments (e.g., seawater, sediment, hard substrate surfaces, seaweed, etc.). The midgut and hindgut, on the other hand, are primarily involved in food digestion (e.g., macroalgae and formulated feeds), and absorption of nutrients. Moreover, the microenvironmental conditions (e.g., temperature, pH, dissolved oxygen concentration) of a specific region of the digestive system varies along the digestive tract and can influence the microbial composition and diversity (Escamilla-Montes et al. 2015). Hereby, the microbiota (the taxonomic composition and diversity of the entire microbial community) of the midgut and hindgut are expected to be different from those of the buccal cavity and foregut. The abundance and diversity of the microorganisms observed in the digestive system correspond to the microenvironment that provides optimal biochemical conditions to the bacteria.

The differences in the bacterial composition and diversity along the digestive system regions have been described in different molluscan species. Like the bacterial profiles of the stomach and digestive gland of the Mediterranean mussel (*Mytilus galloprovincialis*) (Musella et al. 2020), the stomach and intestine of the Eastern oyster (*Crassostrea virginica*) were observed to have different community structure (King et al. 2012). In abalone, intestinal samples collected from *Haliotis midae* had a higher level of bacterial abundance and diversity compared to the post-esophagus-stomach region (Erasmus et al. 1997, Harris et al. 1998b), and the bacterial composition and diversity were also different among the buccal cavity, stomach, and intestinal samples collected in California red abalone *H. rufescens* under the same macroalgal diet (Guo 2017). Evaluation of the similarities or differences in the microbiota of different digestive tract regions has not been adequately documented, especially across the entire digestive system and specifically in *H. iris*. Since the digestive system of abalone is mainly responsible for food digestion and nutrient absorption, investigating the bacterial profiles in the buccal cavity, foregut, and hindgut of abalone will initiate an opportunity to investigate their gut microbiota and their potential role modulating digestion of dietary nutrients. The potential shifts in the microbial profiles along the digestive tract can be valuable indications of potential microenvironmental, dietary, or health impacts, which will contribute to the stock management of this ecologically and commercially important natural resource.

In addition to the microenvironmental influences, macroenvironmental substances such as seawater, seaweed, and sediment in the habitats of aquatic invertebrates contain diverse bacterial compositions and micronutrients (e.g., minerals) that can be microbial sources to host animals. Seaweed- and sediment-associated bacterial communities can have high bacterial concentrations and are primarily composed of Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes, Actinobacteria, and Verrucomicrobia (Cragg et al. 1999; Selvarajan et al. 2019). Some of these bacterial taxa including *Psychrilyobacter haliotis*, *Vibrio midae*, and *Bacillus* spp. are known probiotics and were tested to show high efficiency in protein and monosaccharide digestion (Liu et al. 2023) and with antimicrobial potentials (Santiago and Mabuhay-Omar 2019) in molluscs. The trace metals (e.g., iron, zinc, magnesium, cobalt, etc.) that play an important role in the immune defense of *H. diversicolour* against pathogenic invasions were also observed in aquatic environments such as marine sediment (Marchetti et al. 2020). These natural reservoirs of probiotics and minerals can be bioactive compounds that could potentially benefit the gut health of molluscs and other invertebrates (Zhou et al. 2021; Salloum et al. 2025). Wild abalone are intertidal and subtidal algal grazers and constantly interact with the surrounding seawater, sediment, and seaweed. Consequently, the environmental substances could supply microorganisms to the digestive system of the gastropods and alter their bacterial profiles. Understanding the resemblance of the

microbiota between the abalone digestive system and the environments can be helpful to identify potential sources of certain bacterial species, especially pathogenic strains that can cause shellfish infections and diseases.

Microbiota comparisons between the digestive systems of many aquatic invertebrates and the ambient environment have been previously documented (Danckert et al. 2021). Several studies have indicated that the microbiomes of hosts' digestive systems were similar to those of environmental samples (e.g., seawater, seaweed, sediment). Sponges can acquire bacteria horizontally from ambient seawater (Turon et al. 2018); while sediment played an important role in shaping the gut microbial communities in clams (Bernardini et al. 2023). However, there is evidence suggesting that the bacterial composition and diversity in hosts' digestive tracts are distinct from those of the seawater and sediment samples. An investigation on nudibranchial species demonstrated that the gastropods' gut-associated bacterial profiles were different from those of the seawater and sediment (Stuij et al. 2023), and the bacterial community of freshwater mussels (*Pleurobema cordatum*) was less diverse than that of the surrounding river water and sediment samples (Aceves et al. 2020). Compared to their molluscan relatives, the association between the microbiomes of abalone's digestive tract and their surrounding environments has not been well documented. Investigating the wild abalone (*H. iris*) digestive tract will help understand the association between the microbial composition and diversity, and the environmental microbial communities. Such explorations will contribute to future experimental designs which will aided in understanding how wild abalone's gut microbiota can be influenced by the macroalgae.

Gut microbiome investigations often utilize amplicon sequencing technologies targeting the 16S ribosomal RNA (rRNA). The sequencing data is processed and analysed to generate amplicon sequence variants (ASVs) as an approximate to the "sub-species" level to semi-quantify the microbial communities. At times operational taxonomic units (OTUs), centroids of ASV groups at certain similarity thresholds, are also used. While ASVs and OTUs are both used, ASVs are slightly more favourable as they can reflect a more comprehensive richness compared to OTUs (Fasolo et al., 2024). Using the 16S rRNA amplicon-based Illumina sequencing system, the present study the present study aimed to conduct a baseline microbiome survey to 1) define the bacterial community composition across the different regions of the digestive system (buccal cavity, foregut, and hindgut) in wild *H. iris* from the Cook Strait, New Zealand, and 2) compare the host's digestive microbiome with seaweed and sediment which are possible gut microbiome sources.

## 3.2 Materials and methods

### *3.2.1 Abalone collection and dissection*

Wild abalone samples (n=20) were collected from Cook Strait (41° 11' 31.956" S, 174° 20' 39.48" E) with average sea surface temperature of 14.8°C at the collection site by commercial divers under special permit (720, client number 9791209) issued by Fisheries New Zealand, Ministry of Primary Industries. Abalone samples were removed from rocks in the subtidal area with a blunt shucking knife and placed in polystyrene containers covered with wet hessian bags. Drifting brown seaweed (n = 5) and sea sediment (n = 5) samples were collected at the same collection site. All collected samples were airfreighted to Auckland and were weighed [wet weight = 281.3 ± 14.9 g (mean ± SE)] and measured [shell length = 123.2 ± 2.2 cm (mean ± SE)]. Abalone were shucked, and sex was determined based on the colour of the gonad. The animals were dissected, and buccal cavity sampled, and the gut content obtained by extruding the material from the oesophagus pouch (foregut, n = 20) and intestine (hindgut, n = 20; Supplementary Figure 3.1). All tissues were placed into individual sterile 2 mL cryo-vials, followed by snap freezing in liquid nitrogen and storing at -80°C for downstream molecular applications.

### *3.2.2 Amplicon sequencing*

Genomic DNA (gDNA) of the animal gut and environmental (seaweed and sediment) samples was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), and standardised to 3 nanograms/microliter (ng/µL) for two-step polymerase chain reaction (PCR) assays. The first-step PCR amplification was conducted in triplicates with a pair of customized 16S ribosomal RNA (rRNA) primers (forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAGG-3'; reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The total volume of each PCR reaction was 25 µL containing 12.5 µL of KAPA2G Robust HotStart ReadyMix polymerase mixture with dye (KAPA Biosystems, USA), 1 µL of each of the forward and reverse primers (10 µM), 8.5 µL of PCR-grade nuclease-free water, and 2 µL of each normalized gDNA template. Thermal conditions for the first-round PCR amplification were 94°C for 3 minutes followed by 25 cycles of 94°C for 45 seconds/60°C for 60 seconds/72°C for 90 seconds and a final extension period of 10 minutes at 72°C. The first-round PCR product was visually examined on 2% agarose gels that were mixed with 1X TBE buffer and stained with the SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA). Successfully amplified sample triplicates were pooled, purified with a customised magnetic-bead-based purifying reagent, quantified with the Qubit assays, and normalised to 3 ng/µL for the second-step PCR amplification.

The second PCR amplification utilized the Illumina indexed primers, or "barcodes", for distinguishing the 16S rRNA amplicon produced in the first-round PCR amplification. The reagent master mix for each indexing PCR was also in 25 µL containing 12.5 µL of the same KAPA enzyme reagent, 1.5 µL of each of the forward and reverse Illumina-adapted indexing primers (10 µM), 7.5 µL of PCR-grade nuclease-free water, and 2 µL of the purified 16S rRNA first-round PCR product. Thermal conditions for the indexing PCR amplification were 94°C for 3 minutes followed by 8 cycles of 94°C for 60 seconds/55°C for 60 seconds/72°C for 90 seconds and a final extension period of 10 minutes at 72°C. The barcoded PCR amplicon was purified with the same customised purification reagent used in the post-PCR cleaning after the first-round PCR. The DNA concentration of the purified, indexed PCR product was quantified with the same Qubit assays and normalised to 10 nanomolars (nM). Lastly, 5 µL of each sample was pooled into one 2-mL microcentrifuge tube as a collection of sample libraries. The libraries were purified one more time using the customised purification reagent, quantified through the Qubit assays, and diluted to 2.5 ng/µL. Moreover, the diluted libraries were quantified with the Bioanalyser High Sensitivity DNA Kit (Agilent, USA) for quality control. Finally, the quantified libraries were sequenced on an Illumina Mi*Seq* platform using a v3 (600-cycle) sequencing kit (Illumina, USA) following the manufacturer's protocol at the University of Auckland (Auckland, New Zealand).

### *3.2.3 Data processing and statistical analyses*

Illumina sequences were processed through a modified DADA2 data processing pipeline (Archer et al. 2020) in R (version 4.2.2) to generate 16S rRNA amplicon sequence variants (ASVs), construct an ASV abundance table, and assign taxonomic information to the representative ASVs. ASV taxonomic assignments were based on SILVA high-quality ribosomal RNA database (Version 138) (Quast et al. 2012). Chloroplast ASVs were removed from the seaweed samples to account for the molecular interference from the macroalgal cells.

The microbial composition and diversity at the genus level across the different regions of the digestive tract and between the gut content and environmental samples were compared. Briefly, rare ASVs whose prevalences were less than 0.1% across all sample were removed. The read abundance data were not rarefied but normalized using the total sum scaling method to retain all biological information. The relative abundance of the most abundant microbial genera with at least 5% by read abundance in each sample group (i.e., buccal cavity, foregut, hindgut, sediment, and seaweed) was aggregated and plotted across the sample types in Excel to illustrate the general bacterial composition among the digestive tract, seaweed, and sediment samples. Alpha-diversity estimators (e.g., rarefaction curves, observed richness, chao1 richness, evenness, and Shannon's diversity index) were calculated using the MicrobiomeAnalyst

portal (Lu et al. 2023), with paired *t*-tests for each pair of the digestive tract regions and Kruskal-Wallis statistical tests across the hindgut, seaweed, and sediment samples at the ASV level were conducted in R (version 4.2.2) using the "vegan" package (Oksanen et al. 2013). To illustrate beta-diversity among the digestive tract regions and the environmental samples, the ASV abundance data at the genus level were normalised through the total-sum scaling (TSS) method and converted to a Bray-Curtis dissimilarity matrix. The "betadisper" tests using the "vegan" package were conducted in R to confirm the inter-group dispersions were not significant. Next, bacterial composition among the sample groups were visualized in non-metric multidimensional scaling (nMDS) plots, and permutational multivariate analysis of variance (PERMANOVA) tests were conducted using the Plymouth Routines in Multivariate Ecological Research (PRIMER) software (Version 7) (Clarke and Gorley 2015) to detect statistical significance. To further explore the similarities and differences of the bacterial profiles across all sample types, a heatmap showing the core microbiome at the prokaryotic genus level of all samples with a sample prevalence of at least 20% and a detection threshold of at least 0.1% was constructed on the MicrobiomeAnalyst portal. A cluster heatmap (using Minkovski distances and the complete clustering algorithm) was also generated on MicrobiomeAnalyst to identify hierarchical clusters of the most abundant bacterial genera observed among all samples, along with a differential abundance analysis based on linear discriminant analysis (LDA) effect size (LEfSe) (Peng et al. 2018) to detect essential bacterial genera that differentiated the microbiota among the groups. The statistical significance of the LDA analysis was determined using the Kruskal-Wallis rank test (adjusted *p*-value cut-off = 0.01), with the log LDA score value set to 2.0. Lastly, microbial functions were predicted, based on the 16S rRNA amplicon sequences, using the "Tax4Fun2" function (Wemheuer et al. 2020) in MicrobiomeAnalyst. The functional composition was predicted from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2012). The "Tax4Fun2" method was used because its functional prediction workflow and the ASV taxonomic assignments were based on the SILVA 16S reference database, and were treated as microbial function predictions and not measured genes. More details about the alpha- and beta-diversity statistical analysis can be found in the supplementary material.

### 3.3 Results

The total of 9,739,864 pre-quality check reads was generated from the different regions of the digestive tract, seaweed, and sediment samples. After passing through the DADA2 bioinformatic pipeline and data curation, 8,673,449 quality reads were produced, with 5,837 representative 16S rRNA ASVs observed from all samples (Table 3.1). While the sediment samples had the highest number of ASVs, the hindgut and sediment samples showed the highest observed unique ASV richness, and there were only 16 ASVs shared among all sample groups (Supplementary Figure 3.3). The prokaryotic ASVs were assigned to 96

classes, 220 orders, 370 families, and 676 genera. The sequencing depths were adequate for all sample groups to carry out the subsequent analyses except for the sediment samples (Supplementary Figure 3.2). Alpha-diversity estimators were based on the ASV level, and comparisons of the microbial composition, beta-diversity, and predicted microbial functions were conducted at the bacterial genus level.

**Table 3.1: Summary of the 16S rRNA genetic amplicon reads and the observed ASV richness following the DADA2 bioinformatic pipeline.**

| Sample Type | Sample Size | Post-quality-check Reads | ASVs |
|---|---|---|---|
| **All samples** | 70 | 8,673,449 | 5,837 |
| **Buccal cavity** | 20 | 2,734,445 | 1,065 |
| **Foregut** | 20 | 2,276,557 | 1,023 |
| **Hindgut** | 20 | 3,304,348 | 1,179 |
| **Seaweed** | 5 | 158,617 | 217 |
| **Sediment** | 5 | 199,482 | 3,657 |

### *3.3.1 Microbial composition across samples*

At the ASV level, the alpha-diversity matrices revealed that the microbial communities within samples were similar across the abalone digestive tract and environmental samples (Figure 3.1). However, the variances in the ASV evenness among the digestive tract samples were relatively larger, indicating possible differences among the abalone individuals (Figure 3.1C-D).

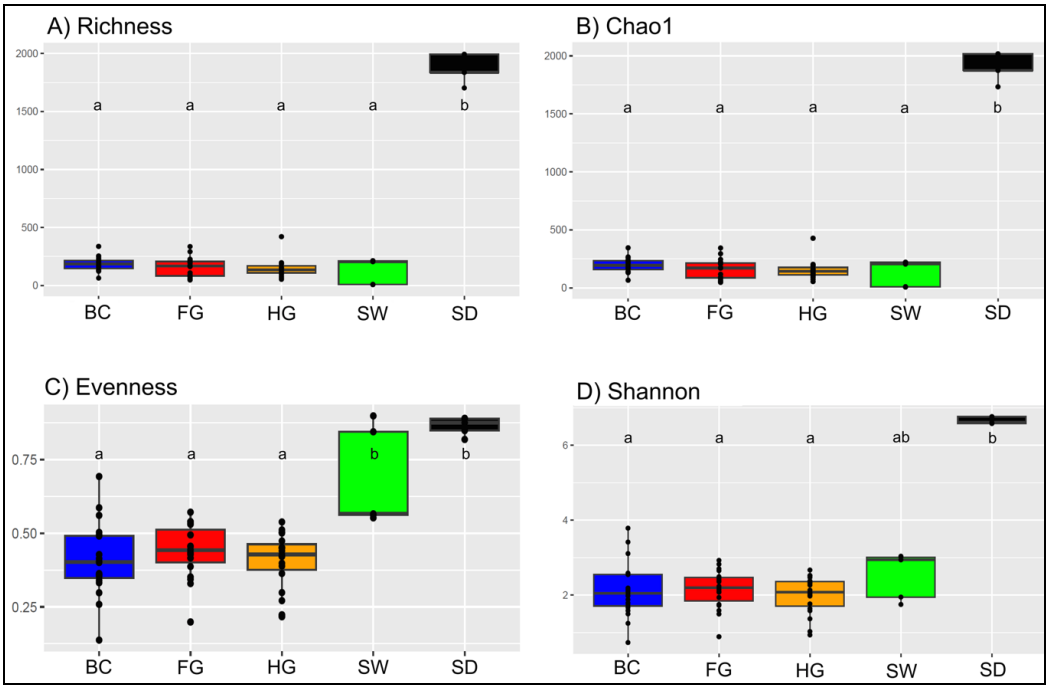


**Figure 3.1: Alpha diversity metrics showing the observed A) richness, B) Chao1 richness, C) evenness, and D) Shannon's indices (D) of the prokaryotic ASVs across all sample types: buccal cavity (BC, n=20), foregut (FG, n=20), hindgut (HG), seaweed (SW), and sediment (SD). Letters indicate statistical significance based on the Dunn's tests.**

The bacterial compositions differed among the three digestive tract regions (Figure 3.2). The foregut and hindgut shared a similar bacterial composition, with *Psychrilyobacter* as the most abundant genus. Whilst the buccal cavity bacterial composition was significantly different from the gut samples (Table 3.2 and 3.3) with *Profundimonas* observed to be more abundant, and *Mycoplasma* and *Vibrio* were less abundant compared with the gut samples (Figure 3.2).

**Table 3.2: PERMANOVA test results of the multivariate microbial diversity analysis across the sample types (buccal cavity, foregut, hindgut, seaweed, and sediment) collected in Cook Strait in May 2021. The sequencing data were normalized and transformed to Bray-Curtis dissimilarities.**

| Microbial Taxonomic Level | df | Pseudo-*F* Statistics | p(permutation) | Unique Permutations | Betadisper *p*-value |
|---|---|---|---|---|---|
| **Phylum** | 15 | 23.88 | 0.001 | 997 | 0.47 |
| **Class** | 15 | 21.39 | 0.001 | 997 | 0.40 |
| **Order** | 15 | 17.27 | 0.001 | 999 | 0.40 |
| **Family** | 15 | 18.25 | 0.001 | 999 | 0.37 |
| **Genus** | 15 | 17.89 | 0.001 | 998 | 0.31 |

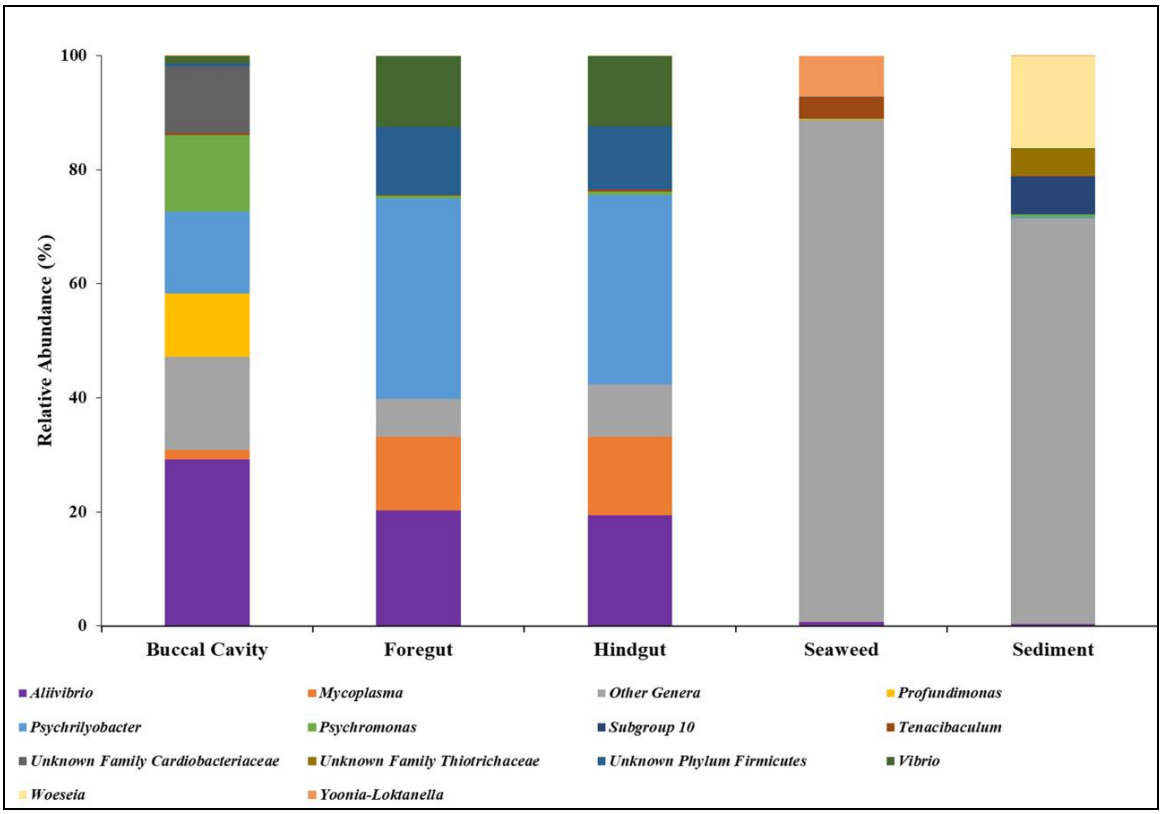



**Figure 3.2: Relative abundance of the bacterial genera with at least 5% prevalence across the five sample groups: buccal cavity (n=20), foregut (n=20), hindgut (n=20), seaweed (n=5), and sediment (n=5).**

The alpha-diversity comparisons showed significant difference in observed ASV richness (Figure 3.1A; Kruskal-Wallis test, $X^2_{(4, 67)} = 18.05$, $p<0.01$), chao1 richness (Figure 3.1B; Kruskal-Wallis test, $X^2_{(4, 67)} = 19.04$, $p<0.01$), evenness (Figure 3.1C; Kruskal-Wallis test, $X^2_{(4, 67)} = 25.01$, $p<0.01$), and Shannon's diversity index (Figure 3.1D; Kruskal-Wallis test, $X^2_{(4, 67)} = 16.03$, $p<0.01$) between the digestive tract samples and the seaweed and sediment samples, and the Dunn's tests indicated that the sediment samples were significantly different from all abalone digestive tract samples (Figure 3.1). In contrast, the alpha-diversity estimators of the seaweed samples were not significantly different from those of the digestive tract samples except for the evenness (Figure 3.1C).

The microbial composition of the seaweed and sediment samples was significantly different from that of the digestive tract samples (Table 3.2 and 3.3), as clearly indicated in the clustering of samples in the nMDS plots at different taxonomic level (Figure 3.3). However, approximately 80% of the observed bacteria recovered from the seaweed and sediment samples were assigned to unclassified taxa (Figure 3.2).

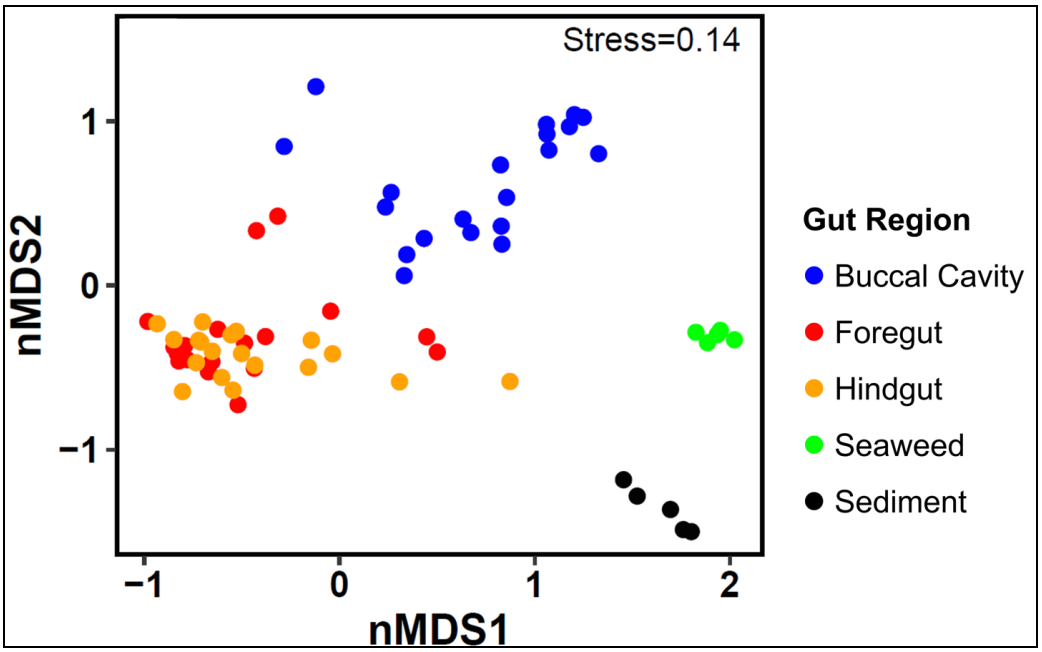



**Figure 3.3: Non-metric multidimensional scaling (nMDS) plot of the bacterial composition at genus level of the buccal cavity, foregut, hindgut, seaweed, and sediment samples. Data were normalised using the total sum scaling method and converted to Bray-Curtis dissimilarity matrices.**

**Table 3.3: Pairwise PERMANOVA test results of the multivariate bacterial diversity analysis across the sample types (buccal cavity, foregut, hindgut, seaweed, and sediment) collected in Cook Strait in May 2021. The sequencing data were normalized and transformed to Bray-Curtis dissimilarities. Asterisk indicates the Monte Carlo *p*-values were used due to low number of permutations.**

| Pairwise Group | Phylum | Class |
|---|---|---|
| **Hindgut, Foregut** | $t_{(1, 39)}$= 0.42, p (perm) = 0.001, 998 permutations | $t_{(1, 39)}$= 0.64, p (perm) = 0.76, 999 permutations |
| **Hindgut, Buccal Cavity** | $t_{(1, 39)}$= 7.05, p (perm) = 0.001, 999 permutations | $t_{(1, 39)}$= 6.60, p (perm) = 0.001, 997 permutations |
| **Hindgut, Sediment** | $t_{(1, 24)}$= 5.75, p (perm) = 0.001, 982 permutations | $t_{(1, 24)}$= 5.61, p (perm) = 0.001, 987 permutations |
| **Hindgut, Seaweed** | $t_{(1, 24)}$= 3.87, p (perm) = 0.001, 984 permutations | $t_{(1, 24)}$= 3.41, p (perm) = 0.001, 995 permutations |
| **Foregut, Buccal Cavity** | $t_{(1, 39)}$= 6.08, p (perm) = 0.001, 999 permutations | $t_{(1, 39)}$= 5.77, p (perm) = 0.001, 999 permutations |
| **Foregut, Sediment** | $t_{(1, 24)}$= 5.04, p (perm) = 0.001, 984 permutations | $t_{(1, 24)}$= 5.07, p (perm) = 0.001, 984 permutations |
| **Foregut, Seaweed** | $t_{(1, 24)}$= 3.32, p (perm) = 0.003, 980 permutations | $t_{(1, 24)}$= 3.08, p (perm) = 0.001, 983 permutations |
| **Buccal Cavity, Sediment** | $t_{(1, 24)}$= 6.87, p (perm) = 0.001, 982 permutations | $t_{(1, 24)}$= 7.62, p (perm) = 0.001, 990 permutations |
| **Buccal Cavity, Seaweed** | $t_{(1, 24)}$= 2.89, p (perm) = 0.001, 981 permutations | $t_{(1, 24)}$= 3.14, p (perm) = 0.001, 991 permutations |
| **Sediment, Seaweed*** | $t_{(1, 9)}$= 4.37, p (MC) = 0.003 | $t_{(1, 9)}$= 2.40, p (MC) = 0.016 |

| Pairwise Group | Order | Family |
|---|---|---|
| **Hindgut, Foregut** | $t_{(1, 39)}$= 0.63, p (perm) = 0.815, 999 permutations | $t_{(1, 39)}$= 0.60, p (perm) = 0.878, 997 permutations |
| **Hindgut, Buccal Cavity** | $t_{(1, 39)}$= 5.84, p (perm) = 0.001, 999 permutations | $t_{(1, 39)}$= 5.98, p (perm) = 0.001, 998 permutations |
| **Hindgut, Sediment** | $t_{(1, 24)}$= 5.77, p (perm) = 0.001, 986 permutations | $t_{(1, 24)}$= 5.59, p (perm) = 0.001, 983 permutations |
| **Hindgut, Seaweed** | $t_{(1, 24)}$= 3.18, p (perm) = 0.001, 988 permutations | $t_{(1, 24)}$= 3.04, p (perm) = 0.002, 983 permutations |
| **Foregut, Buccal Cavity** | $t_{(1, 39)}$= 5.31, p (perm) = 0.001, 999 permutations | $t_{(1, 39)}$= 5.51, p (perm) = 0.001, 998 permutations |
| **Foregut, Sediment** | $t_{(1, 24)}$= 5.38, p (perm) = 0.001, 991 permutations | $t_{(1, 24)}$= 5.11, p (perm) = 0.001, 988 permutations |
| **Foregut, Seaweed** | $t_{(1, 24)}$= 2.91, p (perm) = 0.001, 981 permutations | $t_{(1, 24)}$= 2.76, p (perm) = 0.001, 985 permutations |
| **Buccal Cavity, Sediment** | $t_{(1, 24)}$= 6.51, p (perm) = 0.001, 988 permutations | $t_{(1, 24)}$= 5.49, p (perm) = 0.001, 992 permutations |
| **Buccal Cavity, Seaweed** | $t_{(1, 24)}$= 2.76, p (perm) = 0.001, 987 permutations | $t_{(1, 24)}$= 2.67, p (perm) = 0.002, 988 permutations |
| **Sediment, Seaweed*** | $t_{(1, 9)}$= 2.35, p (MC) = 0.01 | $t_{(1, 9)}$= 2.33, p (MC) = 0.019 |

| Pairwise Group | Genus |
|---|---|
| **Hindgut, Foregut** | $t_{(1, 39)}$= 0.89, p (perm) = 0.542, 999 permutations |
| **Hindgut, Buccal Cavity** | $t_{(1, 39)}$= 5.87, p (perm) = 0.001, 999 permutations |
| **Hindgut, Sediment** | $t_{(1, 24)}$= 5.32, p (perm) = 0.001, 989 permutations |
| **Hindgut, Seaweed** | $t_{(1, 24)}$= 3.25, p (perm) = 0.001, 978 permutations |
| **Foregut, Buccal Cavity** | $t_{(1, 39)}$= 5.18, p (perm) =0.001 , 999 permutations |
| **Foregut, Sediment** | $t_{(1, 24)}$= 4.64, p (perm) = 0.001, 985 permutations |
| **Foregut, Seaweed** | $t_{(1, 24)}$= 2.78, p (perm) = 0.001, 985 permutations |
| **Buccal Cavity, Sediment** | $t_{(1, 24)}$= 5.34, p (perm) = 0.001, 989 permutations |
| **Buccal Cavity, Seaweed** | $t_{(1, 24)}$= 2.73, p (perm) = 0.001, 989 permutations |
| **Sediment, Seaweed*** | $t_{(1, 9)}$= 2.37, p (MC) = 0.021 |

### *3.3.2 Core Microbiome across samples*

The core microbiome of all samples collected included *Psychrilyobacter, Mycoplasma, Vibrio, Aliivibrio, Psychromonas*, and other unassigned bacterial genera (Figure 3.6). The samples collected from different

regions of the digestive system, seaweed, and sediment also showed distinctive bacterial profiles: bacteria identified as *Psychromonas* and *Profundimonas* were mostly observed in the buccal cavity samples; the foregut and hindgut samples had a similar bacterial profile of which *Psychrilyobacter, Mycoplasma*, and *Vibrio* were relatively more abundant; and the seaweed and sediment samples differed from the digestive tract samples with *Blastopirellula, Propionigenium, Sphingomonas*, and *Photobacterium* being more abundant (Figure 3.4). A further linear discriminant analysis (LDA) supported that the listed bacterial genera were major taxa that contributed to the partition of the sample types (Figure 3.7).

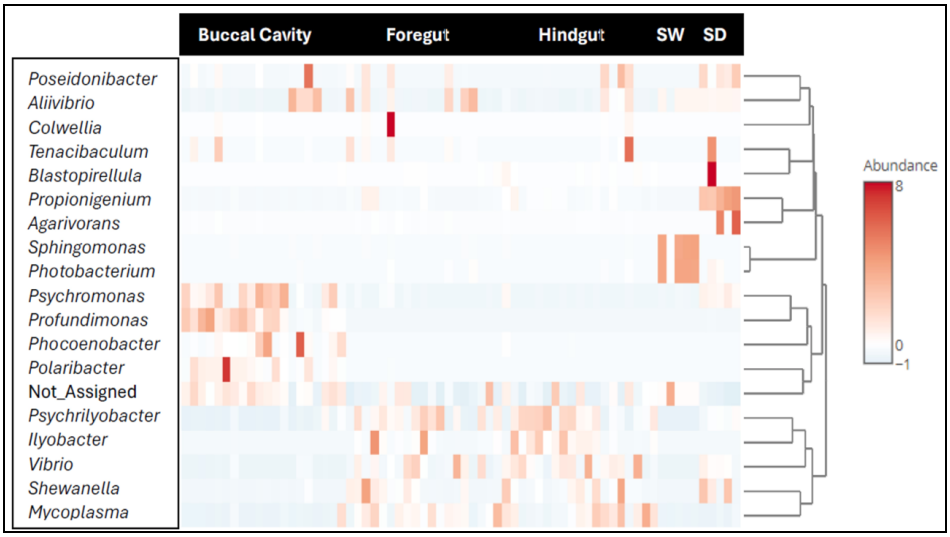



**Figure 3.4: Cluster heatmap showing the hierarchical clusters of the most abundant bacterial genera observed among all samples. The bacterial clusters were generated using the Minkovski distances and complete clustering algorithm in MicrobiomeAnalyst. "SW" denotes "seaweed" and "SD" denotes "sediment".**

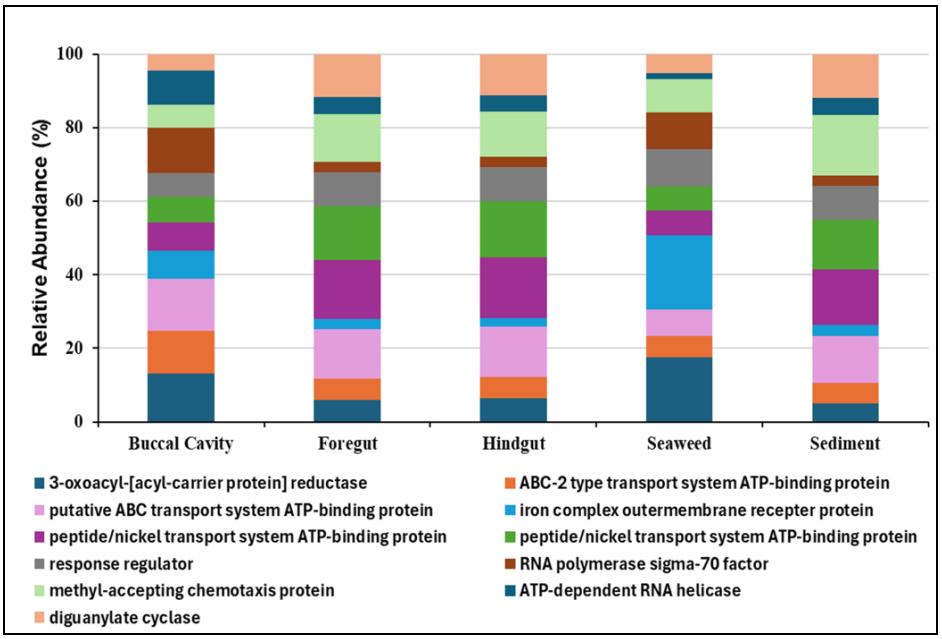



**Figure 3.5 The predicted KEGG specific functions across the five sample groups: buccal cavity (n=20), foregut (n=20), hindgut (n=20), seaweed (n=5), and sediment (n=5). The predicted bacterial functions of the samples collected from the digestive tract (buccal cavity, foregut, and hindgut) of wild *Haliotis iris*, seaweed, and sediment samples were represented as relative abundance of the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology.**

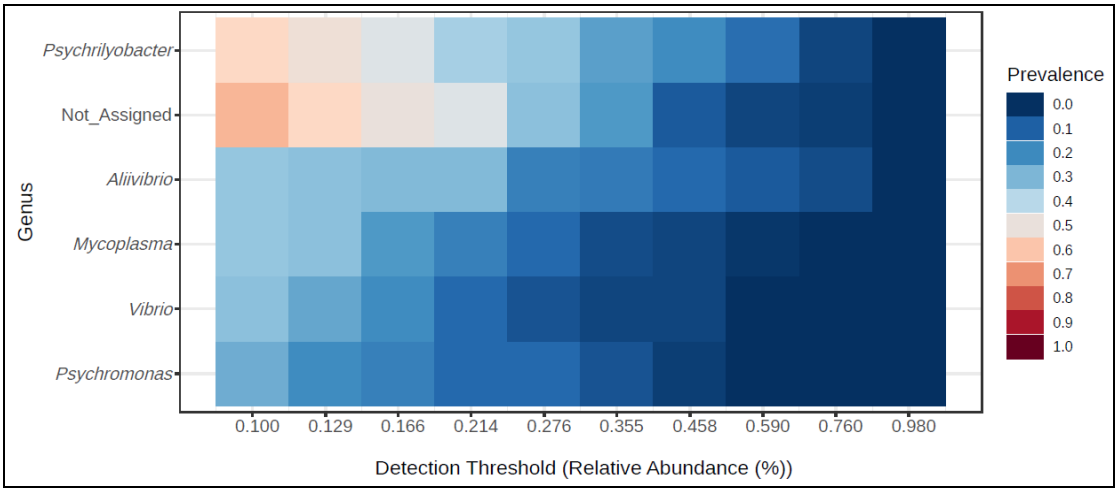



**Figure 3.6: Heat map of the bacterial core microbiome at the genus level. The sample prevalence and detection threshold were set to 20% and 0.1%, respectively.**

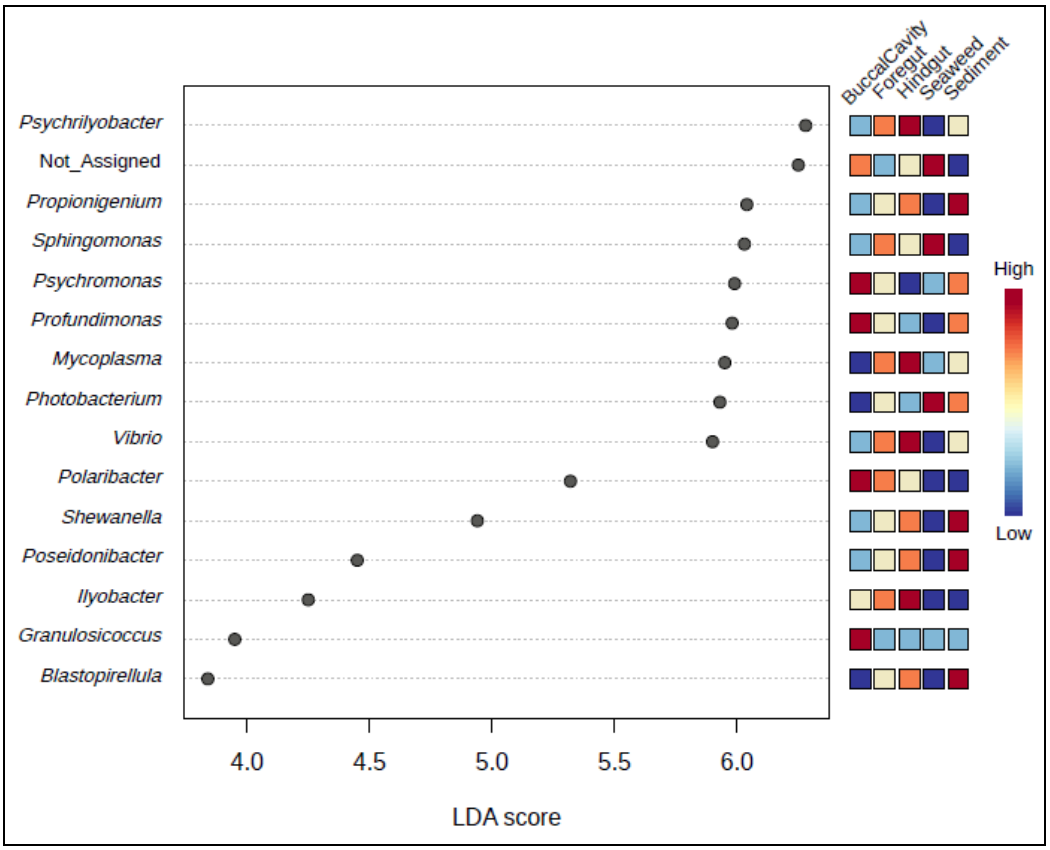



**Figure 3.7: Linear discriminant analysis Effect Size (LEfSe) identifying the top bacterial genera explaining the differences across the sample types (i.e., buccal cavity, foregut, hindgut, seaweed, and sediment). The Linear Discriminate Analysis (LDA) threshold was set to 2.0 with an adjusted p-value cut-off of 0.01 in the Kruskal-Wallis rank test.**

The predicted microbial functions across all sample types were classified into the categories of: metabolism, brite hierarchies, cellular processes, environmental information processing, and genetic information processing. Among the top specific microbial functions identified based on the KO (KEGG Orthology) abundance data, the bacteria observed in the buccal cavity samples were mostly involved in synthesising 3-oxoacyl-[acyl-carrier protein] reductase, putative adenosine triphosphate (ATP)-binding-cassette (ABC) transport system ATP-binding protein, and RNA polymerase sigma-70 factor. The bacteria observed among the seaweed samples mostly participated in the processing of 3-oxoacyl-[acyl-carrier protein] reductase and iron complex outer membrane receptor protein. The microorganisms observed in the foregut, hindgut, and sediment samples had a similar predicted functional profile, and they were mostly involved in the processing of protein profiles such as putative ABC transport system ATP-binding protein, peptide/nickel transport system ATP-binding protein, methyl-accepting chemotaxis protein and diguanylate cyclase (Figure 3.5).

## 3.4 Discussion

### *3.4.1 Microbiome comparisons across the digestive system regions and seaweed*

The results from the microbiomes of the buccal cavity, foregut, and hindgut samples of wild *H. iris* showed that the bacterial composition and the between-group microbial diversity were significantly different in the buccal cavity and gut samples. However, in *H. rufesens,* these digestive regions showed similar microbial diversity (Guo 2017). In mussel and oyster gut bacterial compositions and diversity differed from gill tissue where food particles were initially collected and ingested in bivalves (King et al. 2020, Li et al. 2022). Since abalone's buccal cavity opens to external environments and their hindgut primarily functions to digest food (therefore has different microenvironmental conditions from the buccal cavity), it was expected that the microbial profiles between the two regions would be different.

Interestingly, similar bacterial profiles, both compositionally and functionally, between the foregut and hindgut samples were observed in the present study. The foregut samples were collected from the lower esophagus pouch, while the hindgut samples were collected from the lower intestine. The observed microbiome similarities indicate that both digestive tract regions might share similar microenvironmental conditions, which is not commonly noted in molluscs (Escamilla-Montes et al. 2015). It was reported that the microenvironmental conditions, such as pH and dissolved oxygen levels, in the foregut and hindgut regions of *H. rubra* were differentiated and, therefore, shaped the bacterial profiles at these two regions (Johnston et al. 2005). The unexpected similar microbiome profile between the foregut and hindgut regions, the differences between the gut and buccal cavity samples, and the observed within-group bacterial diversity variations indicate the bacterial profiles might be influenced by certain cryptic influencers, such as habitat ambient macroenvironmental conditions or the digested seaweed.

Coastal aquatic environmental conditions can have a substantial influence on molluscan physiology, including abalone's gut microbiome. This is mostly because the intertidal and subtidal habitats where the host molluscs dwell have fluctuating seawater temperatures, salinity levels, and dissolved oxygen concentrations. Previous studies have demonstrated that macroenvironmental fluctuations can have a significant impact on the general physiology of abalone, such as, impeding larval settlement (Naylor and McShane 2001, Onitsuka et al. 2008), reducing growth rates (Cummings et al. 2019), inhibiting shell formation (Auzoux-Bordenave et al. 2020) and modifying haemocyte proteomic profiles (Wessel et al. 2018).

Another potential explanation for the observed difference in microbiome across the digestive tract is the seaweed being ingested and digested. It is clear from this study that the microbiome compositions and diversity were significantly different between the hindgut and seaweed samples, indicating that the seaweed bacterial communities did not influence the host's gut microbiota. Therefore, it is the specific digested food type (seaweed in this case) that could be an influential factor on the bacterial communities in the digestive system, as the main function of abalone's digestive system is for food digestion and nutrient absorption. In fact, previous studies have documented that different digested seaweed types can influence abalone's gut microbiome. For example, alginate-degrading bacteria such as *Psychrilyobacter, Mycoplasma*, and *Vibrio* are prevalent in the gut of abalone fed with red macroalgae (Tanaka et al. 2016, Gobet et al. 2018), whereas *Formosa* and *Clostridia* were commonly found in abalone's gut under brown algal diets in addition to the common bacteria associated with red seaweed (Tanaka et al. 2015, Guo 2017, Nel et al. 2018). While *Psychrilyobacter, Mycoplasma*, and *Vibrio* were part of the core microbiome observed in the present study, they were also among the discriminative bacterial genera that partitioned the microbial profiles across the digestive tract regions, indicating the observed bacterial communities along the digestive tract of *H. iris* might have resulted from the ingested seaweed that was rich in alginate. These findings also provide a baseline for further investigations on how wild abalone's gut microbiome would respond to different macroalgal diets.

### *3.4.2 Microbiome and predicted functions in the hindgut, seaweed, and sediment*

The present study revealed differentiated bacterial composition and significantly high bacterial diversity in the sediment samples compared to the other sample groups at different taxonomic levels. These findings are in line with previous microbiome studies conducted in other molluscs, including *Crassostrea virginica* and *Mytilus edulis* (Pierce and Ward 2019), *Ruditapes philippinarum* (Offret et al. 2020), and *Littorina* spp. (Maltseva et al., 2021). The difference in the bacterial composition and diversity observed between the hindgut and the surrounding substrate (mostly sediment) indicates that bacterial taxa are unlikely to be directly transported to the digestive tract of molluscs including abalone.

Although there was a significant difference in the bacterial composition and diversity between the hindgut and sediment samples, it is interesting to discover that both sample types shared similar predicted microbial functions. Our results of the microbial functional predictions indicated that bacteria found in the foregut, hindgut, and sediment samples were likely involved in synthesising signaling proteins that bind and transport various physical and chemical substances like peptides and nickel. There are three implications resulting from these findings. First, the microbial compositional and functional resemblance between the hindgut and sediment samples supports the concept of "functional redundancy" among

microbial communities, where taxonomically unrelated bacteria could perform similar ecological functions (Xenophontos et al. 2021). This suggests that the microbiome investigations in abalone and other shellfish hosts could focus more on the microbial functions, which can provide ecologically significant information. Second, the presence of peptide- and nickle-transporter-synthesising bacteria in the hindgut samples indicates that the bacterial communities in abalone's hindgut are likely to be selected by the nutrient metabolism needs of the hosts, which supports the idea that the foregut and hindgut microbiome of the wild abalone in this study might be affected by dietary nutrients which needs to be further investigated. Lastly, the predicted microbial functions from the 16S rRNA genetic amplicons suggest that bacteria found in the digestive tract of wild *H. iris* were involved in diverse functions other than nutrient metabolism. While the predicted microbial functions were solely based on one genetic marker, future investigations should utilise more advanced technologies, such as metagenomic shotgun sequencing, to make the microbial functional predictions more robust.

In addition to the microbial functions predicted in the host's digestive system and sediment, the present study also revealed that bacteria observed in the buccal cavity and seaweed samples participated in synthesising membrane receptor proteins for several metallic micronutrient transportation. For instance, iron is a limiting micronutrient in the open oceans (Sharada et al. 2020, Schallenberg et al. 2022) and helps for macronutrient metabolisms in shellfish (Andrews 2000). Copper is a vital component of haemoglobin for oxygen transportation (Ragg and Watts, 2015). The presence of the iron-receptor-synthesising bacteria in the seaweed samples, along with the nickel-receptor-synthesising bacteria observed in the gut and sediment samples, indicates that the habitat of wild *H. iris* in the Cook Strait may contain loads of trace metals that could be essential to promote food digestion and gut health in the local abalone populations. New Zealand as a geothermally active country, would have freshwater run-offs that constantly bring trace metals from chemically weathered volcanic rocks to nearby coastal embayment (Carey et al. 2002). Trace metals like iron (in the form of ferritin), zinc, and magnesium were observed to induce immunological responses in abalone *H. diversicolour* during pathogenic challenges, suggesting a role in immune defense against pathogenic invasions (Marchetti et al. 2020). Therefore, a significant implication of this study is that it draws our attention to the non-host environments, like sediment, in wild abalone's natural habitats where nutritionally and immunologically important micronutrients can be discovered and potentially incorporated into formulated feeds.

## Chapter 4: Gut microbiome of New Zealand abalone (*Haliotis iris*): A Chatham Islands case study

**Prelude**: In the previous chapter, we found the gut microbial composition and diversity were very similar between the foregut (esophageal pouch) and hindgut (intestine), and it is unlikely that the wild *H. iris* collected in Cook Strait acquired gut microorganisms from the environment. This chapter only utilised the hindgut samples for the gut microbiome comparison at different locations in the Chatham Islands. We also included the gut content algal composition analysis to explore whether the gut microbiomes of wild *H. iris* in the islands were associated with the seaweed type and availability found in their surrounding habitats.

*This chapter manuscript has been finalized for submission to one of the following journals, Marine Biology, New Zealand Journal of Marine and Freshwater Research, Molluscan Research, and Journal of Shellfish.*

**Abstract**

The New Zealand black-footed abalone, *Haliotis iris*, is of substantial ecological, economic, and cultural value. The Chatham Islands abalone fishery contributes significantly to the national catch but exhibits variability in growth rates across populations. To investigate the potential of microbiome-linked growth variability, sub-adult, and adult abalone from four populations were collected and analysed via morphometric comparisons and 16S rRNA Illumina MiSeq sequencing. Adults from Ascots Beach and Wharekauri (historically fast-growing sites) were heavier, longer, and had larger tissue areas than those from Owenga Harbour and Point Durham (historically slow-growing sites), with no size difference observed in sub-adult animals. Gut content analysis revealed that abalone from the fast-growing sites consumed more red algae and less green algae than the slow-growing sites' abalone, while brown algae dominated across all sites. Alpha-diversity was not significantly different among sites or age groups, except at Point Durham where only Shannon's diversity index differed between adults and sub-adults. Beta-diversity significantly differed by study site and age group. *Psychrilyobacter*, *Mycoplasma*, *Vibrio*, *Propionigenium*, unassigned Bacilli, and *Blastopirellula* were core bacteria in all study sites. The site- and age-associated gut microbial differentiations may reflect the availability and nutritional characteristics of consumed seaweed, which requires further targeted feeding studies. This study provides a baseline for future gut microbiota research on wild *H. iris* in the Chatham Islands. Understanding gut microbiota variation in relation to seaweed composition offers insight into algal nutrients and gut-bacteria-mediated digestion in supporting abalone growth and helps examine difference between fast- and slow-growing wild abalone stocks.

**Keywords:** Abalone, *Haliotis iris*, Gut microbiota, Seaweed, 16S rRNA, Illumina sequencing, Chatham Islands, New Zealand fishery

### 4.1 Introduction

The commercial abalone fishery in New Zealand (NZ) is managed under the quota management system based on the 1996 Fisheries Act. The total allowable commercial catch is set within seven main regional quota management areas. The Chatham Islands pāua fishery (PAU4), based mainly on *Haliotis iris* and secondly *H. australis*, contributes a significant proportion (35.5%) of the national catch. Even though there are limited data on the status of PAU4, it is classified as a productive and abundant fishery (Fisheries`New`Zealand 2021). Despite the $50-million (NZ dollars) revenue contributed by this economic sector annually (Stenton-Dozey et al. 2021), the growth and physiological performance of the wild abalone are not well documented. Abalone populations in NZ exhibit high variability in growth rates, manifested in significant demographic variability across different spatial scales (McShane et al. 1994b; Naylor et al. 2006). Various factors, such as water temperature (Naylor et al. 2006), food availability, wave exposure and habitat structure (McShane et al. 1994b; McShane and Naylor 1995) are known environmental drivers influencing the growth rates of abalone. Histopathological analyses of *H. iris* indicated that the differentiated growth rates could be associated with poor nutritional status among the local abalone populations (Copedo et al. 2024). In addition, metabolic pathway analysis showed that the fast-growing adult abalone utilized carbohydrates as the main energy source (Venter et al. 2022). Considering the influence of nutrition on growth differences in abalone, it is hypothesized that the bacterial composition and diversity in the digestive tract of adult abalone may also contribute to abalone population differences around the Chatham Islands.

The digestive-tract-associated microbiota, contribute to nutrient digestion and absorption (Poore 1972) and development of mucosa (Villasante et al. 2020). Moreover, the microbiota of abalone's digestive system contains abundant and diverse microorganisms, primarily bacteria, essential to nutrient digestion of the hosts (Erasmus et al. 1997). Since most of the biochemical digestion occurs in the gastrointestinal region (gut) of the host's digestive system, it is useful to focus on the gut bacterial communities for compositional profiling and prediction of potential driving factors that may influence the bacterial communities. Unlike in oysters where parental influences have been observed in the gut microbiota of offsprings (Schei et al. 2017; Unzueta-Martínez et al. 2022), wild abalone gut bacterial profiles are mostly prone to changes due to environmental parameters, such as seawater temperature (Wang et al. 2020), heavy metal concentrations (Yao et al. 2024), and diet (Bullon et al. 2023). Living in coastal intertidal and subtidal regions with fluctuating ambient oceanographic and habitat conditions makes wild abalone more

susceptible to anthropogenic disturbances. In particular, the seaweed type consumed by abalone contribute to the shaping of the gut bacterial composition and diversity (Hur et al. 2023). For example, the Pacific abalone (*H. discus hannai*) in Japan and South Korea fed with brown macroalgae were observed to have abundant bacterial strains in genera *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Psychrilobacter*, *Vibrio*, and *Mycoplasma* in their intestine (Sawabe et al. 1995; An et al. 2013; Hur et al. 2023). Abalone that grazed on red macroalgae tend to have significantly high amounts of *Bacillus*, *Faecalibacterium*, *Escherichia*, and *Shigella* in their digestive tract (Hehemann et al. 2012; Gerasimidis et al. 2020; Corino et al. 2021; De Vos et al. 2022). Since the gut bacteria in abalone often specialize in seaweed digestion, evaluating the gut bacterial composition and diversity can effectively reflect the type of algae processed through the digestive system, whether sufficient nutrient-digesting bacteria were involved in the metabolism of the consumed seaweed, and whether the hosts underwent fasting or malnutrition states, which could lead to differentiated growth performance.

The aim of this study was to characterise the microbiome of sub-adult and adult New Zealand black-footed abalone (*Haliotis iris*) from slow-growing (‘stunted’ or partially fished) and fast-growing (intensively fished) collection sites, around the Chatham Islands and to quantitatively evaluate the algal gut composition. Revealing the gut microbial community of the wild abalone populations in the Chatham Islands can provide valuable information about the health and nutritional status of abalone and assist examining the differences between the fast- and slow-growing abalone populations towards better the local commercial abalone fisheries.

## 4.2 Materials and Methods

### *4.2.1 Site descriptions and sample collections*

Wild abalone were collected in March 2020 by commercial divers from four sites around the Chatham Islands (Figure 4.1): Ascots Beach (44°00'59.0"S 176°23'11.7"W; site 1), Wharekauri (43°42'18.9"S 176°35'04.7"W; site 2), Owenga Harbour (44°01’28” S 176°21’56” W; site 3), and Point Durham (44°00'24.8"S 176°40'54.2"W; site 4). Based on historic catch data, sites 1 and 2 (Ascots Beach and Wharekauri) are classified as high-fished areas (fast-growing), while sites 3 and 4 (Owenga Harbour and Point Durham) are classified as low recovery areas that are not fished often (slow-growing) (Naylor and Fu 2016). At each collection site, 10 adult, and 10 sub-adult (sexually mature with a shell length of < 100 millimeters) *Haliotis iris* individuals were randomly collected by the diver and placed in a net bag. The animals were transported to a nearby tank holding facility in a bucket of fresh seawater and released into a flow-through system for interim housing, while sampling was being completed. Animals were collected under special permit (720, client number 9791209) issued by Fisheries New Zealand.

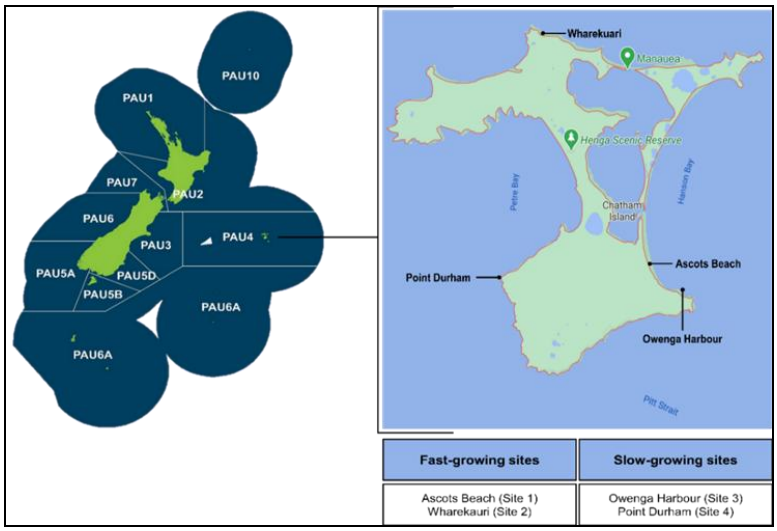



**Figure 4.1: Abalone quota management areas around New Zealand with the PAU4 illustrating the Chatham Islands where abalone were collected from site 1 - Ascots Beach, site 2 - Wharekauri, (fast-growing abalone sites), site 3 - Owenga Harbour and site 4 - Point Durham (slow-growing abalone sites).**

### *4.2.2 Abalone Processing*

Animals in both age groups were randomly collected on the same day at each sampling site. Abalone were removed from the tanks using a chipping technique where a plastic blade was slid beneath the animal to prevent clamping. Abalone were weighed to the nearest 0.01 gram (g) and the shell lengths, widths, and heights were measured to the nearest 0.10 millimeter (mm) using calipers. Gender annotations were made after shucking based on gonad colour (green in females and white/cream in males). Additionally, images were collected for soft tissue measurements. Before gut content with the lining tissue was retrieved from the digestive tract, the area was rinsed with 70% ethanol and phosphate-buffered saline (3 times). Using sterile forceps, up to 5 g of gut material was obtained by carefully extruding the posterior intestine material from the anus and homogenized. Four grams of the homogenised sub-sample was directly utilised for the algal content microscopic analysis (see Section 4.2.4), and the remaining one gram of the gut homogenate was placed into individual sterile 2 mL cryo-vials with 200 µL of RNAprotect tissue reagent followed by snap freezing in liquid nitrogen for the gut microbiome processing (see Section 4.2.5). The gut content sub-samples were processed in separate laboratory settings to avoid cross contamination between the two analytical pipelines, and the samples for the gut microbiome processing were stored at -80 °C until DNA extraction was carried out at Auckland University of Technology.

### *4.2.3 Soft tissue imaging*

Soft tissue measurements of the abalone gonad, adductor muscle, and foot muscle were made from photographs collected during the sampling procedure. These images were used to determine the tissue area using ImageJ® (version 1.53t 24), a Java-based image processing program.

### *4.2.4 Gut content composition*

Four grams of the gut content was collected from the posterior intestine of each of the 40 abalone adults (N=10 per site) and fixed in formalin for algal scoring assessments. Due to the abalone nature of feeding and algal deterioration within the gut, it was impossible to determine each specific species of seaweed. However, based on visual and morphological characteristics unique to each of the classes of seaweed, the algal content within the gut was divided into the three main classes of seaweed (green, red, and brown). Green seaweed was characterised by thin blades and green hue, red seaweed was characterised by thin blades and reddish hue, and brown seaweed was characterised by thick blades and brown hue. The gut content was emptied into petri dishes and was gently agitated to ensure a single layer of gut content lay flat on the dish. The photographs were taken and scored by visually observing the proportion of organic fragments and sand occupying the petri dish. Values per seaweed colour were represented as follows: 0: 0% cover; 1: 1-20% cover; 2: 21-40% cover; 3: 41-60% cover; 4: 61-80% cover; 5: 81-100% cover.

### *4.2.5 16S Ribosomal RNA (rRNA) amplicon sequencing*

Genomic deoxyribonucleic acid (gDNA) of the animal gut (10 adults and 10 sub-adults per site) samples (used for the gut content compositional analysis) were extracted using the DNeasy Power Soil Pro Kit following the manufacturer's protocols (Qiagen, Catalog No. 47014), quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Catalog No. Q32854), and normalized to 3 nanograms/microliter (ng/μL) for two-step polymerase chain reaction (PCR) assays. The amplicon library preparation and Illumina sequencing laboratory workflow followed the protocol written by Guo et al. (2025). Briefly, the first-round PCR amplification was conducted in triplicates with a pair of customized 16S rRNA primers (forward: 5'-CCTACGGGNGGCWGCAGG-3'; reverse: 5'-GACTACHVGGGTATCTAATCC-3'), and amplicon products were pooled by sample, purified with a customized magnetic-bead-based purification reagent, quantified with the Qubit assays, and normalized to 3 ng/μL for the second-step PCR amplification. The second-step PCR amplification utilized Illumina indexed primers to distinguish the 16S rRNA amplicons per sample. The barcoded PCR amplicon was purified and quantified. The libraries were normalised to 10 nanomolars (nM) and pooled. Bioanalyser High Sensitivity DNA Kit (Agilent, USA) was used for the final quality check of the amplicon libraries

before sequencing on an Illumina *MiSeq* platform using a v3 (600-cycle) sequencing kit (Illumina, USA) following the manufacturer's protocol at the University of Auckland (Auckland, New Zealand).

### *4.2.6 Data processing and statistical analyses*

***Morphometrics:*** Statistical analyses of animal weights, shell length, shell height, and shell width measurements were carried out in RStudio (version 1.4.1103). All data were checked for normality and heterogeneity. Two-way ANOVAs were used to analyse morphometric data, with weight, shell length, shell width, and shell height as the response variable, and life stage (two levels: adult and sub-adult) and growth type (two levels: fast- and slow-growing) as factors. Weight, shell length, shell width, and shell height data were square-root-transformed, when necessary, to meet assumptions of normality and homogeneity of variances. Data were also partitioned into four 'growth type' categories (adult fast-growing, adult slow-growing, sub-adult fast-growing, sub-adult slow-growing) and one-way ANOVAs with site as a factor were used to examine statistical differences in weight, shell length, shell width, and shell height in animals from different sites. Soft tissue measures of the adductor muscle, foot muscle and gonad were analysed using two-way ANOVAs, with collection site (four levels: Ascots Beach, Wharekauri, Owenga Harbour, Point Durham) and life stage (two levels: adult and sub-adult) as factors. For all analyses, Tukey pairwise comparisons were used to examine differences between factor levels and $\alpha$ was set at 0.05.

***Algal composition in the gut:*** Abalone gut content ranking scores were analysed using two-way ANOVAs, with site (four levels: Ascots Beach, Owenga Harbour, Point Durham and Wharekauri) and growth type (slow-growing and fast-growing) as factors. For all analyses, Tukey pairwise comparisons were used to examine differences between factor levels and $\alpha$ was set at 0.05.

***16S rRNA amplicon sequencing data:*** DNA sequence data were processed through a modified DADA2 data processing pipeline (Archer et al. 2020) in R (version 4.2.2) to generate 16S rRNA amplicon sequence variants (ASVs), construct an ASV abundance table, and assign taxonomic information to the representative ASVs. Briefly, the 16S rRNA primers were removed from the forward and reverse pair-ended sequences using the "Cutadapt" command (Martin 2011), followed by filtering out erroneous sequences and removing chimeras. Contaminant sequences in the DNA extraction and PCR negative control samples were removed using the "decontam" R package (Davis et al. 2017). The pair-ended, quality-filtered sequences were merged to form representative ASVs after being dereplicated. Microbial taxonomic information was assigned to the ASVs using the SILVA high-quality ribosomal RNA database

(Version 138) (Quast et al. 2012). Chloroplast ASVs were removed to reduce the molecular interference from the macroalgal cells.

Statistical analysis focused on microbiota comparisons at the prokaryotic genus levels between the adult and sub-adult gut samples at all sampling sites. Briefly, rare ASVs whose prevalences were less than 0.1% across all sample were removed. The microbial genus abundance data was normalised through total sum scaling method to account for the slightly different sequencing depths across all groups. Alpha-diversity estimators (chao1 richness and Shannon's diversity index) were calculated and plotted using the "vegan" package (Oksanen et al. 2013) in R (version 4.2.2) and the MicrobiomeAnalyst portal (Lu et al. 2023). Relative abundance bar charts of the most abundant microbial genera were presented to illustrate the gut microbial compositions of the abalone gut samples at each site. Furthermore, the microbial data were transformed into a Bray-Curtis dissimilarity matrix to compare beta-diversity patterns. Principal coordinate analysis (PCoA) plots were generated using the "vegan" R package to illustrate microbial compositional similarities between the age groups at each study site. Permutation MANOVA (PERMANOVA) and dispersion tests were conducted to detect statistical significance of microbial diversity. Lastly, a core microbiome of all samples was revealed in a heatmap with a detection threshold of 10% and 20% sample prevalence.

## 4.3 Results

### *4.3.1 Morphometrics*

The collective morphometric data are presented as principal component analysis (PCA) score plots showing the animal weight, shell length, shell width, shell height, adductor muscle area, foot muscle area and gonad area (as determined from imaging) from adult (42.5% were females and 57.5% were males) and sub-adult (22.5% females, 25.0% males, and 52.5% undetermined) abalone collected across the four collection sites (Figure 4.2). A clear separation between adult and sub-adult abalone is seen when considering PC1 (99.63%), which is mainly attributed to the animal weight, shell length, shell height, shell width, foot area, and adductor muscle area measurements of abalone. Adult abalone collected from the fast-growing sites (Ascots Beach and Wharekauri) showed the largest variation among the measured morphometrics attributed to individual differences. Conversely, adult abalone from the slow-growing sites (Owenga Harbour and Point Durham) showed closer clustering of individuals, suggesting similarity within the population.

While the sub-adult abalone showed no significant differences in all measured morphometric parameters across all study sites, the adult abalone showed a greater variation among the collection sites

(Supplementary Table 4.1). In general, adult abalone collected from fast growing sites (Wharekauri and Ascots Beach) were significantly heavier (ANOVA, $F_{(1,19)}$=5.77, $p$<0.001), with longer (ANOVA, $F_{(1,19)}$=5.28, $p$<0.001), wider (ANOVA, $F_{(1,19)}$=4.97, $p$<0.001), and higher (ANOVA, $F_{(1,19)}$=4.33, $p$<0.001) shells than those from the slow-growing sites (Owenga Harbour and Point Durham). Within the adult abalone, pairwise comparisons between collection sites showed that Wharekauri had the largest and heaviest individuals, while Owenga Harbour had the smallest and lightest adult population. There were no significant differences amongst the weights (ANOVA, $F_{(1,19)}$=1.03, $p$=0.92), shell length (ANOVA, $F_{(1,19)}$=0.96, $p$=0.85), shell height (ANOVA, $F_{(1,19)}$=1.09, $p$=0.95) of adult abalone collected from Ascots Beach and Point Durham.

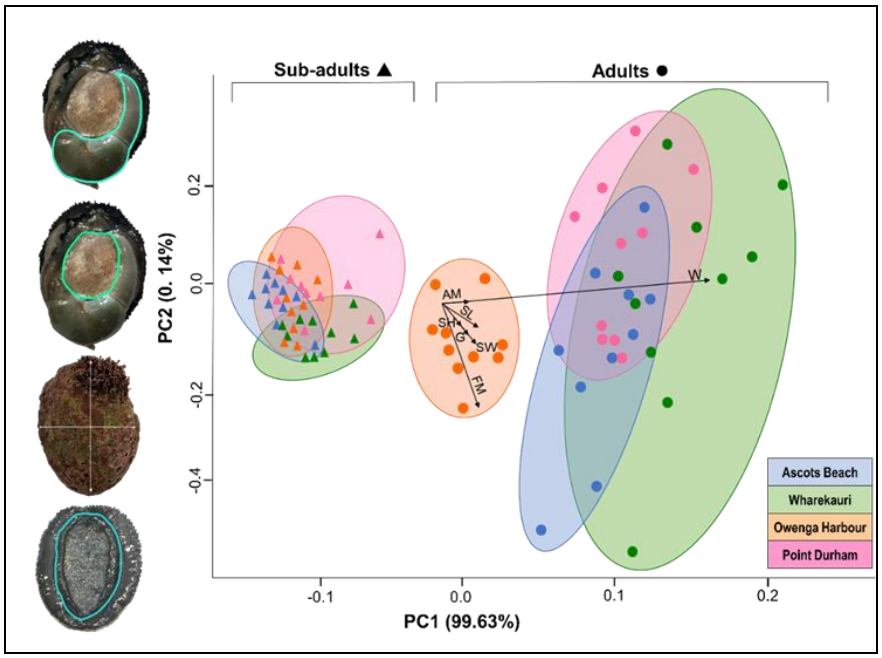



**Figure 4.2: PCA plot of adult (●) and sub-adult abalone (▲) collected from four sites (Ascots Beach, Wharekauri, Owenga Harbour and Point Durham) based on measured morphological features [shell length (SL), shell height (SH), shell width (SW), weight (W), adductor muscle (AM) area, gonad (G) area, and foot muscle (FM) area]. Groupings based on collection sites and life stage of the abalone. PC1 indicates abalone size measurements, and PC2 indicates abalone soft tissue areas.**

Soft tissue imaging (Figure 4.2) showed the significant differences between adductor muscle, foot muscle, and gonad tissue areas between adult and sub-adult abalone (ANOVA, $F_{(1,19)}$=3.88, $p$<0.001). As expected, adult populations from all collection sites had larger adductor muscle, foot muscle, and gonad tissue compared to sub-adult from all sites. Amongst the sub-adult abalone, there were no significant differences in all the measured parameters (ANOVA, $F_{(1,3)}$=2.89, $p$=0.95 for adductor muscle, $F_{(1,3)}$=2.78,

$p$=0.89 for foot muscle and $F_{(1,3)}$=2.56, $p$=0.90 for gonad area). Within the adult samples, adductor muscle, foot muscle, and gonad tissue areas were significantly different (ANOVA, $F_{(1,19)}$=2.63, $p$<0.001 for adductor muscle, $F_{(1,19)}$=2.77, $p$<0.001 for foot muscle and $F_{(1,19)}$=3.04, $p$<0.001 for gonad area) between fast-growing and slow-growing sites. Pairwise comparisons between collection sites showed adult individuals collected at Wharekauri (fast-growing site) had the largest adductor muscle, foot muscle, and gonads, whereas adult individuals collected at Owenga Harbour (slow-growing site) had the smallest tissue areas.

### *4.3.2 Gut content composition*

Assessment of the preserved gut content (consisting largely of algal debris and sand) from adult abalone revealed significant differences between the abundance of red (ANOVA, $F_{(1,39)}$=11.25, $p$=0.005) and green algae (ANOVA, $F_{(1,39)}$=11.33, $p$=0.03). Pairwise comparisons showed that the composition of red algae in abalone from fast-growing sites (Ascots Beach and Wharekauri) was between 1.27 and 3.39 units higher than the slow-growing sites (Owenga Harbour and Point Durham). In contrast, the composition of green algae at fast-growing sites was between 0.16 and 2.62 units lower than at slow-growing sites. There were no significant differences observed within brown algae (ANOVA, $F_{(1,39)}$=11.57, $p$=0.7) and composition of sand (ANOVA, $F_{(1,39)}$=11.49, $p$=0.9) amongst abalone from the different collection sites (Figure 4.3).

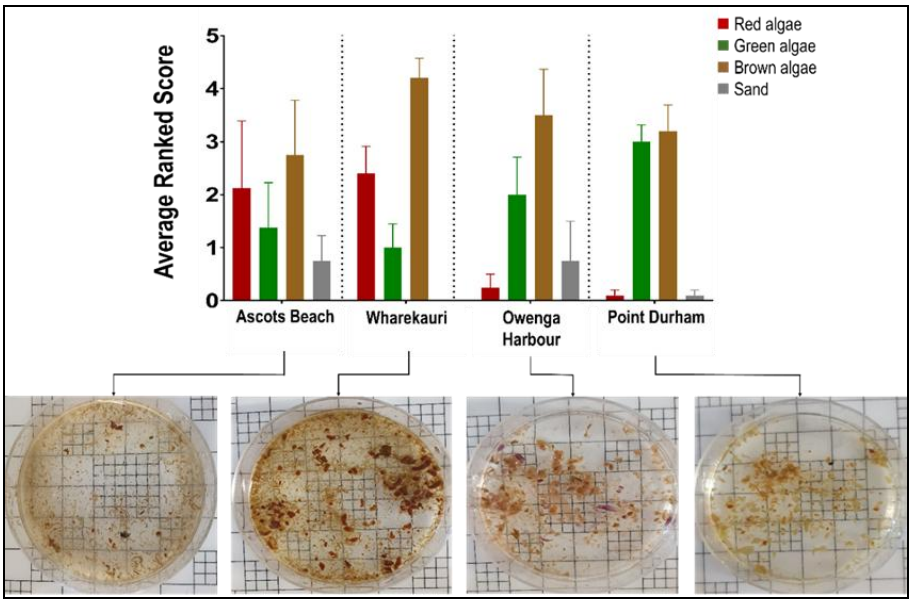



**Figure 4.3: Gut content scoring (average ± SE) by algae type (red, green and brown algae) and sand in adult abalone collected from Ascots Beach, Wharekauri, Owenga Harbour and Point Durham.**

### *4.3.3 Gut Bacterial Microbiota*

***General bioinformatics***

More than 11 million 16S rRNA sequence reads were generated from the 40 adult (~5.6 million reads) and 40 sub-adult (~5.5 million reads) abalone gut sampled across the four sampling sites (10 individuals per site). 5.4 million reads were merged and passed the quality filtering steps (Supplementary Table 4.2). There were 823 amplicon sequence variants (ASVs) identified from all gut samples. The observed 16S rRNA ASVs were taxonomically assigned to 28 phyla, 45 classes, 102 orders, 129 families, and 200 genera. The core microbiome (detection threshold of 0.1% and sample prevalence of 20%) in the samples consisted of *Psychrilyobacter*, *Mycoplasma*, *Vibrio*, *Propionigenium*, Bacilli, SAR324 clade (Marine group B), and *Blastopirellula* (Supplementary Figure 4.1).

***Gut Microbial Alpha-diversity Comparisons***

Gut microbial alpha-diversity estimators were compared at the microbial genus level between the adult and sub-adult gut samples collected across the four sites. There was no significant difference in chao1 estimator across the sampling sites as well as between the adults and sub-adults (Figure 4.4; Table 4.1). Shannon's diversity index also showed no significant differences across the four sites and between the age groups except at Point Durham where Shannon's diversity indices were significantly different between the adults and sub-adults (Figure 4.4; Table 4.1; Table 4.2).

**Table 4.1: Two-way ANOVA test results main factors of site and sample type on chao1 richness and Shannon's diversity estimators of the gut samples collected from the four study sites (Ascots Beach, Wharekauri, Owenga Harbour, and Point Durham). The non-significant interaction term was removed. The alpha-diversity estimators were calculated at the microbial genus level.**

| | Chao1 Richness | | | | | Shannon's Diversity | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **df** | **Sum of Square** | **Mean of Square** | ***F*-statistic** | ***p*-value** | **df** | **Sum of Square** | **Mean of Square** | ***F*-statistic** | ***p*-value** |
| **Site** | 3 | 271 | 90.5 | 0.28 | 0.840 | 3 | 0.718 | 0.239 | 2.572 | 0.06 |
| **Age** | 1 | 265 | 265.0 | 0.82 | 0.368 | 1 | 0.844 | 0.844 | 9.069 | 0.03 |
| **Residuals** | 75 | 24,243 | 323.2 | | | 75 | 6.977 | 0.093 | | |
| **Total** | 79 | 49,834 | | | | 79 | 8.667 | | | |

**Table 4.2: Pairwise ANOVA test results of the effects of sample type on the chao1 richness and Shannon's diversity estimators of abalone gut and seaweed samples collected from the four study**

**sites (Ascots Beach, Wharekauri, Owenga Harbour, and Point Durham). The alpha-diversity estimators were calculated at the 16S rRNA ASV level.**

| | Ascots Beach | | | Wharekauri | | | Owenga Harbour | | | Point Durham | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | *F*-statistic | *p*-value (FDR) | *df* | *F*-statistic | *p*-value (FDR) | *df* | *F*-statistic | *p*-value (FDR) | *df* | *F*-statistic | *p*-value (FDR) |
| **Chao1 Richness Adult vs. Sub-adult** | 19 | 1.54 | 0.141 | 19 | 1.11 | 0.282 | 19 | 0.586 | 0.566 | 19 | 1.61 | 0.126 |
| **Shannon's Diversity Adult vs. Sub-adult** | 19 | 1.88 | 0.077 | 19 | 1.37 | 0.186 | 19 | 6.63 | 0.118 | 19 | 5.51 | 0.02 |

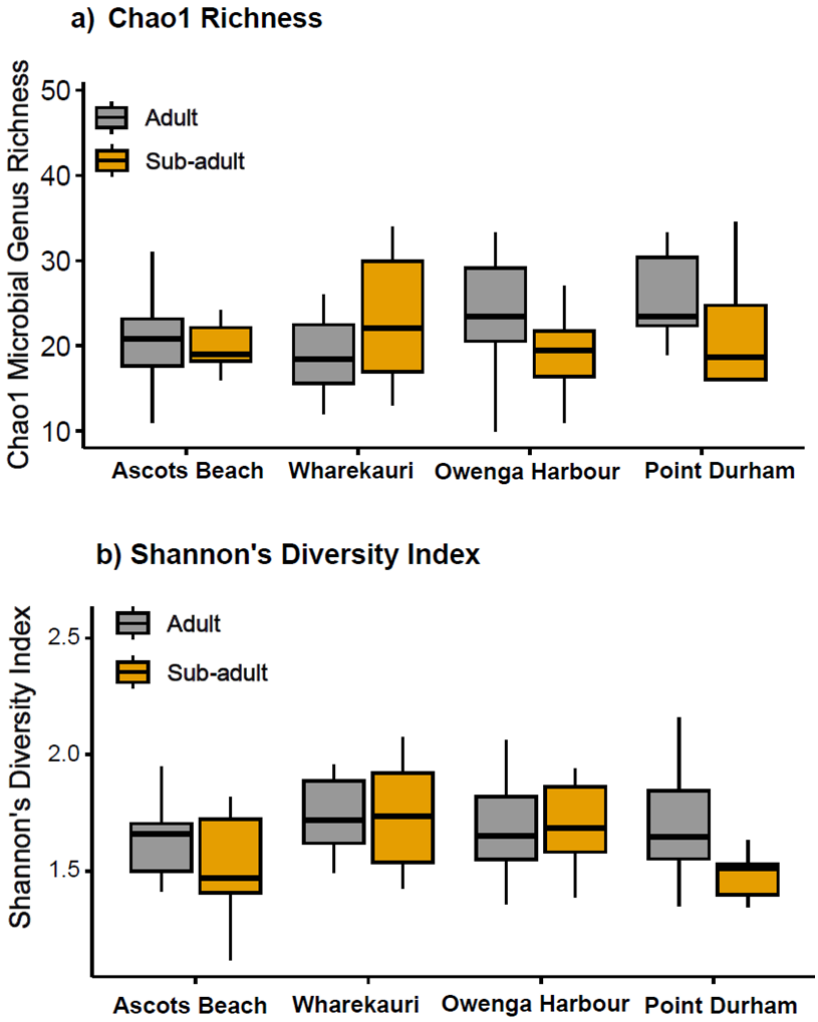



**Figure 4.4: Box plots of chao1 richness (a) and Shannon's diversity index (b) between the sub-adult and adult abalone across the four study sites (Ascots Beach; Wharekauri; Owenga Harbour; Point Durham). The alpha-diversity estimators were calculated at the microbial genus level.**

***Gut Microbial Composition and Beta-diversity Comparisons***

Abalone gut microbial composition and beta-diversity were evaluated at the prokaryotic genus level. The gut microbial composition of abalone was different at each study site. While *Psychrilyobacter*, *Mycoplasma*, and *Vibrio*, were the most abundant bacterial genera observed at all study sites within the adults and sub-adults, less abundant bacterial genera were different across the sites (Figure 4.5). For example, *Propionigenium* was seen to be more abundant at Wharekauri and Owenga Harbour in both adult and sub-adult abalone, while unassigned Bacilli were absent at Owenga Harbour, yet found at all the other sites, at both life stages. Furthermore, bacteria classified as unassigned SAR324 clade (Marine group B) and *Blastopirellua* were more evident in adult abalone and the sub-adults showed higher levels of *Vibrio*. Interestingly, *Psychromonas* were absent in the adult and sub-adult abalone collected at Ascots Beach, and in the adults from Point Durham.

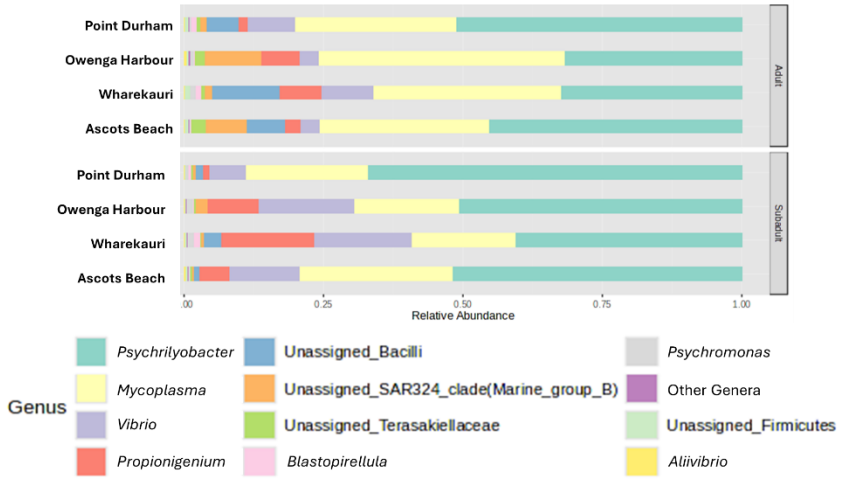



**Figure 4.5: Relative abundance of 11 most abundant microbial genera observed from the gut samples collected from the four study sites Ascots Beach; Wharekauri; Owenga Harbour; Point Durham) and between the adults and sub-adults.**

Regarding the beta-diversity comparisons at the prokaryotic genus level, abalone gut samples showed 84% and 83% similarities between the fast-growing sites (Ascots Beach and Wharekauri) and the slow-growing sites (Owenga Harbour and Point Durham), respectively. The observed beta-diversity was noticeably different between the adult and sub-adult abalone at each site (Figure 4.6). Moreover, the differentiated microbial compositions by site and age group were statistically significant (Table 4.3) but not due to between-group variations (Table 4.4), and such gut microbial compositional differentiation across the study sites and between the age groups at individual sites were also indicated by clustering heatmaps (Supplementary Figure 4.1).

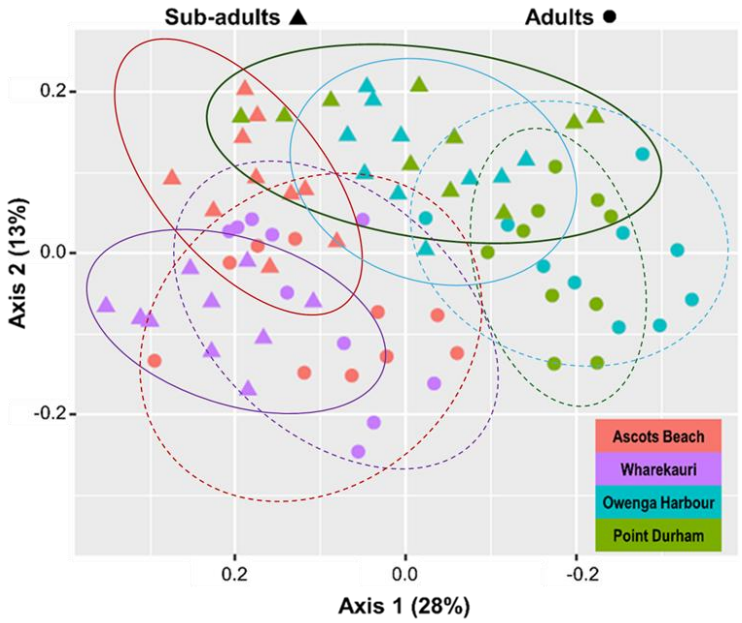



**Figure 4.6: Principal coordinate analysis (PCoA) plots showing the microbial compositions of the adult and sub-adult abalone gut samples collected the four study sites. Solid and dashed similarity eclipses are for the sub-adults and adults, respectively. Read abundance data at the microbial genus level were normalised and converted to a Bray-Curtis dissimilarity matrix.**

**Table 4.3: Permutational MANOVA (PERMANOVA) test results of the main factors of site and age group on microbial composition of the gut samples collected from the four study sites (Ascots Beach, Wharekauri, Owenga Harbour, and Point Durham). The non-significant interaction term was removed. Read abundance data at the microbial genus level were normalised and converted to a Bray-Curtis dissimilarity matrix, and the PERMANOVA test was conducted using 999 permutations.**

| | df | Sum of Square | *F* Statistics | *p*-value |
|---|---|---|---|---|
| **Site** | 3 | 2.25 | 7.70 | 0.005 |
| **Age Group** | 1 | 0.80 | 8.24 | 0.009 |
| **Residual** | 75 | 7.02 | | |
| **Total** | 79 | 10.73 | | |

**Table 4.4: Permutational dispersion (PERMDISP) test results of the main factors of site and age on the microbial composition of abalone gut samples collected from the four study sites (Ascots Beach, Wharekauri, Owenga Harbour, and Point Durham) in the Chatham Islands in March 2020. Read abundance data at the microbial genus level were normalised and converted to a Bray-Curtis dissimilarity matrix, and the between-group dispersion test was conducted using 999 permutations.**

| | Site | Residuals | Age | Residuals |
|---|---|---|---|---|
| **df** | 3 | 76 | 1 | 78 |

| | | | | |
|---|---|---|---|---|
| **Sum of Square** | 0.004 | 0.75 | 0.002 | 0.72 |
| **Mean of Square** | 0.001 | 0.007 | 0.002 | 0.009 |
| ***F* Statistics** | 0.19 | | 0.19 | |
| **Number of Perm.** | 999 | | 999 | |
| ***p*-value** | 0.443 | | 0.658 | |

***Core Gut Microbiota***

Bacteria in the genera *Psychrilyobacter*, *Mycoplasma*, *Vibrio*, *Propionigenium*, unassigned Bacilli, unassigned SAR324_clade, and *Blastopirellula* formed the core gut microbiome of all abalone gut samples collected from the four study sites (Figure 4.7) based on the set detection threshold (10%) and sequencing read prevalence (20%).

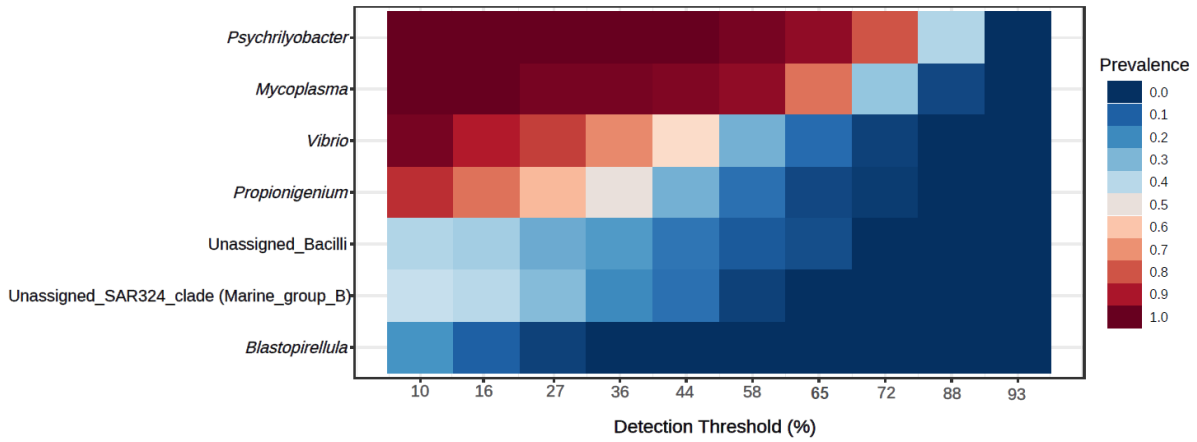



**Figure 4.7: Core microbiota of all gut samples collected from the four study sites (Ascots Beach, Wharekauri, Owenga Harbour, and Point Durham).**

## 4.4 Discussion

Molluscan gut microbiota performs important functions (e.g., assisting host food digestion) and can be influenced by various genetic and environmental factors (e.g., wave action and seawater temperature) including diet type (Kang and Kim 2015). While abalone gut microbiome research is expanding, there is still limited knowledge pertaining to the dietary effects on gut microbial composition in wild *Haliotis iris* populations with different growth backgrounds. This study provides the first exploratory analysis into the gut microbiome of adult and sub-adult abalone collected from four Chatham Islands sites with varying animal growth and seaweed backgrounds. We characterized the gut microbial composition and diversity of the local wild abalone populations in connection with algal gut content and hypothesised that seaweed type could influence the host's gut microbiota of the abalone populations, which would ultimately

differentiate the growth and morphometric measurements. The morphometric measurements (i.e., sizes of adductor and foot muscles and gonad tissues) among the adult abalone collected from the fast-growing and slow-growing sites in the present study was also previously observed among wild *H. iris* populations in New Zealand (McShane et al. 1994a; Naylor et al. 2006). Abalone in New Zealand are known to have varying growth rates, and this can be mostly associated with food availability and the nutrients absorbed from abalone's diets (Nguyen et al. 2023). The microbial community dwelling in the gut of abalone is an important component to efficiently assist with food digestion (Erasmus et al. 1997). Therefore, understanding the changes in abalone's gut microbial composition and diversity can provide valuable information on how gut microbial communities could affect the efficiency of food digestion and ultimately the growth and health of these economically important shellfish.

### *4.4.1 Differentiated Abalone Gut Microbiota and Study Site Characteristics*

The present study revealed similar gut microbial diversity but different bacterial composition across the four study sites. The observed gut microbial differences by site could be related to some site characteristics. For example, Ascots Beach and Owenga Harbour are both located on the same side of the island within proximity to each other (Figure 4.1), and a noticeable phenomenon discovered from the gut content microscopic analysis was that abalone collected from these two sites showed relatively high levels of sand. The ingestion of sand is a natural phenomenon in abalone due to their grazing feeding mode (Harris et al. 1998), and previous research by Schiel (1993) showed that some post-settlement *H. iris* populations on this stretch of sandy habitats along the east coast of the Chatham Islands were completely buried in sand. Abalone that were found to consume a high amount of sand at Ascots Beach and Owenga Harbour showed a high proportion of *Propionigenium* bacteria (Figure 4.5) which is often found in sediment of marine habitats (Munkongwongsiri et al. 2022). Since sand has little nutritional value compared to seaweed, consuming a high proportion of sand would lead to abalone malnutrition, reduce the efficiency of bacterial gut function in digesting seaweeds, and lower the overall gut microbial diversity. Collectively, the sandy site characteristic highlights a potential factor influencing the gut microbiota and impacting the growth and health conditions of the local *H. iris* populations.

### *4.4.2 Differentiated Abalone Gut Microbiota and Seaweed Type*

Adult and sub-adult abalone from the slow and fast-growing sites in this study showed differentiated gut microbial compositions despite having similar alpha-diversity estimators across the sites. Evidence in other abalone species also suggested differentiated gut microbial richness and diversity in different age groups. Adult *H. diversicolour* (Huang et al. 2010) and *H. midae* (Nel et al. 2018) exhibited higher intestinal microbial diversity than the juvenile samples. The differentiated abalone gut microbiota could

be related to the type of seaweed (i.e., brown, red, and green seaweed) that is consumed by wild abalone species like *H. discus hannai* (Tanaka et al. 2003), *H. midae* (Nel et al. 2018) and *H. tuberculata* (Gobet et al. 2018). For example, mmicroorganisms in the gut of abalone that fed on brown seaweed such as kelp (*Ecklonia maxima* and *Sargassum horneri*) are often dominated by *Mycoplasma*, fermentative *Clostridia* bacteria, *Psychrilobacter*, and *Vibrio* (Nel et al. 2017). While the proliferation of beneficial bacterial strains like *Mycoplasma sp*. and *Vibrio halioticoli*, were found to be associated with the addition of red seaweed into abalone diets (Iehata et al. 2014). Moreover the inclusion of *Ulva* spp. in abalone diets has been linked to an increase of beneficial *Vibrio* and *Mycoplasma* strains (Guo 2017). The gut algal content analysis in the present study showed that wild *H. iris* consumed different proportions of mixed seaweed types at all study sites, which could explain why some of the core gut bacterial genera such as *Psychrilobacter*, *Mycoplasma*, and *Vibrio* had different relative abundance across the study sites. In fact, researchers have previously documented that *H. iris* prefers to eat drifting brown algae over red and green algae, but they ultimately consume different groups of algae depending on their availability and accessibility (Cornwall et al. 2009). Comparatively, on a short-term basis (24 hours), *H. diversicolour* were reported to consume more leathery brown algae than red and green, while on a long-term basis (20 days), the consumption of more green algae was documented (Alcantara and Noro 2005). Literature suggests that abalone digests preferred algal species within 24 hours of food consumption, while less digestible algal species remain identifiable in abalone gut for more than 48 hours after food consumption (Britz et al. 1996). As such, seaweed digestibility in abalone might be related to the nutritional characteristics (e.g., carbohydrates) of each seaweed type, which could influence the abalone's gut microbial composition and diversity.

### *4.4.3 Effects of Seaweed Carbohydrates on Abalone Gut Microbiota*

Each of the three types of seaweed (i.e., brown, red, and green) has specific carbohydrate (mostly polysaccharides) profiles that often require specific bacterial taxa to break down. As a result, abalone gut microbiota could be shaped by the seaweed polysaccharides. Brown seaweed contains a high amount of alginate, laminarin, and fucoidan, and the *Mycoplasma*, fermentative *Clostridia*, *Psychrilobacter*, and *Vibrio* bacteria found in the gut of the Pacific abalone (*H. discus hannai*) fed on *Ecklonia maxima* and *Sargassum horneri* were biochemically tested to be able to hydrolyze these brown algal polysaccharides (Hur et al. 2023). Similarly, bacterial strains capable of digesting fucoidan-rich seaweed (e.g., *Ecklonia maxima*) were notably abundant in the gut of the South African abalone *H. midae* (Barkai and Griffiths 1986). In addition to the most abundant bacterial strains, some minor bacterial genera, such as the unassigned marine group B SAR324 clade, also play an important role in seaweed polysaccharide digestion which were previously seen to be dominant in the intestines of *Haliotis diversicolour* and *H.*

*tuberculata* (Won et al., 2010; Gobet et al. 2018; Huang et al. 2021). Genome-centric metagenomics suggested that some SAR324 bacteria strains possess adenosine triphosphate-binding cassette transporters for carbohydrate metabolism (Huang et al. 2021). Such metabolomic characteristics are in line with previous findings where certain bacteria, including the SAR324 group, have been described as key taxa in the degradation of complex polysaccharides such as alginate, which is a major component of brown algae (Hyun et al. 2013; Tanaka et al. 2016). Since the gut content composition analysis showed that brown seaweed was the most abundant seaweed type identified in the adult abalone in this study, it is reasonable to observe alginate-digesting bacteria, including the SAR324 strains, in the gut of the local wild abalone.

Red seaweed contains a high amount of agar, carrageenan, and alginate which provide a more diverse and stable gut microbial environment in abalone (Iehata et al. 2014; Gobet et al. 2018). A study by Kang and Kim (2015) showed that certain *Bacillus* strains could metabolise red seaweed agar and carrageenan to produce reduced sugars, critical to mollusks as energy source. In the present study, abalone from the fast-growing sites showed higher levels of red seaweed in the gut content compared to their slow-growing counterparts. The presence of relatively large proportion of unassigned Bacilli in the gut of adult and sub-adult abalone at Ascots Beach and Wharekarui (fast-growing sites) could be associated with the consumption of red seaweed. In addition to Bacilli bacteria, *Psychromonas*, a genus of psychrophilic bacteria, was mostly found in the gut samples of abalone that were observed to consume a high volume of red seaweed in the present study. *Psychromonas* mainly contribute to the degradation of algal polysaccharides and has been associated with accelerated transportation of nutrients and the promotion of host digestion (Wu et al. 2023). The differentiated *Psychromonas* abundance observed across the study sites and between the age groups could be due to dietary changes from abalone's early life stages to juveniles and eventually adults, which can also lead to variations in the abundance and diversity of other bacterial species (Jiang et al. 2017; Hur et al. 2023).

Green seaweed, particularly from the genus *Ulva* spp., is another food source that can have a significant impact on the gut microbiota and microbial functionality in abalone and other mollusks (Bansemer et al. 2016). Many green seaweed species like *Ulva* spp. are rich in amino acids, fatty acids, polyphenols, and unique polysaccharides (Hagan and Fungwe 2023). These macronutrients can positively modulate the gut microbiota by promoting the growth of beneficial bacteria and enhancing short-chain fatty acid production (Shannon et al. 2021; Hagan and Fungwe 2023; Xu et al. 2023). Sub-adult abalone from all the collection sites in this study showed higher levels of *Vibrio* when compared to the adults. Several *Vibrio* strains (e.g., *V. midae*) are seen as facultative anaerobes and can either be pathogens that trigger shellfish diseases or probiotics, as seen in the gastrointestinal tract of *H. midae* that support digestive tract

functions and fermentation processes (Danckert et al. 2021). There could be two main reasons why the relative abundance of *Vibrio* bacteria could be differentiated in the life stages of abalone. First, different seaweed types can lead to variations in the availability of nutrients and substrates that support the growth of *Vibrio* spp., and this is mostly observed during dietary shifts between developmental stages (Cicala et al. 2018; Gobet et al. 2018). Second, the immune response of abalone at different developmental stages can also influence the abundance of *Vibrio* bacteria. For example, juvenile abalone have been observed to exhibit different immune responses compared to adults, which could affect their susceptibility to *Vibrio* infections and consequently alter the relative abundance of these bacteria in their gut (Huang et al. 2010; Wang et al. 2015).

**4.5 Future Research Directions**

The present study combined the gut content microscopy and Illumina amplicon sequencing technologies to reveal the interplay between diet (seaweed) and wild abalone gut microbiota on Chatham Islands. While our results highlighted the connections between dietary seaweed and the local abalone's gut microbial composition and diversity, it would be beneficial to conduct long-term gut microbiota investigations using the same methods at the same study sites for data validation. In addition, research activities in at least three other directions are needed to further test the hypothesis that seaweed type can significantly tailor the gut microbiota of wild *H. iris* to select microbial communities that specifically digest the seaweed nutrients. First, biological and ecological surveys on local seaweed composition, diversity, and morphology in abalone habitats around the Chatham Islands are recommended to understand seaweed availability and accessibility to the wild abalone populations. The health and diversity of seaweed communities are important for abalone growth and health. Studies have shown that abalone populations recover when kelp restorations take place (Eger et al. 2022), with the opposite also being true where a decline in kelp forests triggers abalone mortality (Rogers-Bennett and Catton 2019). Second, targeted seaweed feeding experiments under controlled laboratory conditions are needed to specifically explore how abalone's gut microbial communities would respond to each type or species of seaweed. Lastly, field experimental translocations of wild abalone populations with different but known seaweed habitat characteristics could also be useful to investigate the dietary seaweed effects on wild *H. iris* that have the same genetic backgrounds. The growth differences in this study were largely documented in adult animals with little differences seen between sub-adults from the same collection sites. Moreover, the gut microbial compositions of the sub-adult and adult abalone among the study sites were noticeably different. These findings indicate that *H. iris* could be housed at slow-growing sites until they reach the juvenile and sub-adult stages and then translocated to fast-growing sites to improve growth in adulthood. To some extent, the slow-growing sites could be considered as spawning sites where the

adults settled there will serve as breeding stock, while offspring can be moved to other areas. It has been suggested that juveniles of around 30 mm are the best size to be released as part of stock enhancement programs, while enhancement by free-swimming larvae were classified as less successful (Cook 2023). The translocation survival of the Roe's abalone (*H. roei*) were much higher in adult than juvenile populations (Strain et al. 2019). Also, translocation of adult pink abalone (*H. corrugata*) resulted in successful dense aggregations of new populations (Taniguchi et al. 2013). Data collected from such translocation efforts will be constructive to determine if such initiatives will be valuable for the Chatham Islands fishery.

Abalone support valuable commercial, recreational, and customary fisheries in New Zealand and an understanding of the processes driving variation within different abalone fishing sites is crucial to ensure long-term conservation and responsible management of *H. iris*. While it has been noticed that anthropogenic, biological, and climate-related stressors could disproportionately impact wild abalone habitats and abalone fishery stocks (Rogers-Bennett and Catton 2022), it is essential to continuously monitor the gut microbial responses of this ecologically and economically valuable shellfish species to seaweed and other environmental factors in New Zealand. A clear understanding of abalone gut microbial communities can be used to optimize feeding strategies, enhance growth and disease resistance, and ultimately benefit abalone fishery management and support sustainable aquaculture operations.

### 4.6 Conclusions

This study provides novel insight into sub-adult and adult abalone morphometrics, gut microbiota, and digested seaweed proportional comparisons across four locations on Chatham Islands. Abalone from fast-growing sites (Ascots Beach and Wharekauri) had more red algae and less green algae present in the gut compared to the slow-growing sites (Owenga Harbour and Point Durham), and the corresponding microbes that digest these types of seaweeds might support early digestion, which could aid eating and growth parameters over time. *Psychrilyobacter, Mycoplasma*, and *Vibrio* were the dominant bacterial genera that formed the core microbiota of all gut samples, with variations in the gut microbial composition present between the adult and sub-adult populations at all study sites, potentially driven by size-related biological differences which are commonly observed in other abalone species. Differentiated growth and microbiome could be related to seaweed types that were digested in the gut of the wild *H. iris*, alongside environmental parameters, suggesting that subsequent feeding experiments with specific seaweed species and environmental conditions (temperature and sediment changes) are recommended to clarify the effects of diet and the environment on the gut microbiota and how these effects or supports abalone growth.

**Chapter 5: *Haliotis iris* gut microbial dynamics: A longitudinal study in the Chatham Islandss**

**Prelude**: The gut microbiome of wild *H. iris* populations in the Chatham Islands and their association with seaweed type were investigated in Chapter 4. Besides seaweed type and site, the gut microbiome of wild abalone populations could also be different across different time points of the year. Therefore, this chapter aimed to explore and compare the gut microbiome of two *H. iris* populations overtime (six sampling points).

*This chapter manuscript is being finalized to be submitted to one of the following journals, New Zealand Journal of Marine and Freshwater Research, Marine Ecology or Molluscan Research. A final journal submission is estimated to be in June, 2026.*

**Abstract**

Wild abalone fisheries from the Chatham Islands contribute substantially to the commercial harvest of abalone in New Zealand. Abalone in different populations in the Chatham Islands have been observed to display varying growth rates which might require different management approaches to effectively look after the population. Assessment and monitoring of the gut microbiome can potentially serve as an indicator of ecosystem changes and support abalone population studies. This study characterized the gut microbiome of abalone (*Haliotis iris*) collected from Ascots Beach (an abalone fast-growing site) and Owenga Harbour (an abalone slow-growing site) in the Chatham Islands, across six sampling timepoints from March 2020 to April 2022. While the average Chao1 microbial genus richness and Shannon's diversity index were approximately 30 and 2.2, respectively, at both sites, beta-diversity of the gut microbial compositions was significantly different between sites. Additionally, Shannon's diversity of the abalone gut microbial communities between March and May 2021 was significantly lower than that of the remaining sampling points, indicating the changes of the gut microbiota might be associated with changes of their habitat environments. Moreover, *Psychrilyobacter*, *Mycoplasma* and *Vibrio* were substantial core microbiota components and major contributors to the observed gut microbial differentiations. This study provides for the first time a longitudinal assessment of the microbiome changes associated with abalone from the Chatham Islands. The data and results of this study can be referenced for future wild abalone gut microbiome research in New Zealand and will support the management and interventions related to the wild abalone stock monitoring and ecosystem condition.

**Keywords:** Abalone, *Haliotis iris*, Gut microbiota, Chatham Islands, Fishery, 16S rRNA, Longitudinal study

## 5.1 Introduction

New Zealand hosts one of the last viable wild harvest abalone fisheries, built primarily on *Haliotis iris*, but also includes *H. australis* (Fisheries New Zealand, 2024). Abalone are found on subtidal rocky shores throughout New Zealand, supporting economic, customary, recreational and cultural practices (Nash, 2019). Both commercial and customary fisheries adhere to stringent catch reporting within quota management areas with total allowable catch allowances (Virgin et al., 2025). The Chatham Islands are found 800 kilometers east of the New Zealand mainland and contributes more than a quarter to the national 720 tons of wild abalone that is commercially harvested (Will et al., 2011, Ellen, 2021). This region, as many other regions, show distinct differences in abalone growth rates classified as fast-growing and slow-growing individuals based on documented timeframes to a harvestable size (125 mm in shell length) (Nguyen et al., 2023). Many factors have been associated with growth variation in abalone such as wave action, food availability, topographic complexity (Copedo et al., 2024), geographic isolation affecting genetic flow (Will et al., 2015), long lifespan (Rogers-Bennett et al., 2019), and low levels of regional species endemism (Walton et al., 2023). These and other factors accumulate to a complex interplay posing challenges to the management of the Chatham Islands fishery. Despite growth difference of abalone, this fishery is classified as relatively productive and abundant and managed under a Quota Management System in association with a real-time management responses including voluntarily shelving of annual catch entitlements and harvest control measures (Nash, 2019).

Many climate-related stressors are influencing whole ecosystems, necessitating single-species fishery management plans to include ecosystem-based indicators as early detectors of future challenges to stock productivity (Rogers-Bennett et al., 2019). Moreover the tracking of multiple metrics are needed to develop an ecosystem-based conceptual model to inform management (Rogers-Bennett and Catton, 2022). Rogers-Bennett et al. (2019) suggests that monitoring of ecosystem features such as algal food, ocean temperature, distribution shifts and competitor abundances can serve as management responses to strengthen abalone fishery productivity. Moreover parameters such as sedimentation (Chew et al., 2013), sea temperature (Naylor et al., 2006), ocean acidification (Cummings et al., 2019), and weather events (Roberts et al., 2007), macroalgal species abundance, and diversity (D'Archino et al., 2019) have also been reported to impact abalone growth and development and contribute to ecosystem changes.

Another layer of data with the potential to serve as indicator of ecosystem changes is the microbiome (Ribas et al., 2023). The microbiome contains a multitude of microorganisms that reside within or on the animal, which may originate from surrounding seawater-associated cells, or they can be passed on through generations from the host (Apprill, 2017). Compared to the microbiome of other parts of

abalone's whole body, the gut microbiome has been mostly investigated due to its diverse microbial functions and connections to the gastropod's health and physiology. The abalone gut microbiome reflects the community of microorganisms dwelling in the intestine-anus section of the animal's digestive system (Huang et al., 2010), responsible for assisting food digestion, nutrient absorption, and health mitigation (Nam et al., 2018). Microbes contribute to energy production from carbon dioxide in water, with the water microbiome changing seasonally due to multiple environmental factors including temperature, salinity, geology, nutrient level, other water quality parameters, which could eventually result in changes of the host-associated microbiome (Sehnal et al., 2021).

Seasonal shifts in the gastrointestinal microbiota have been previously documented in several marine mollusks such as the eastern oyster (*Crassostrea virginica*), the blue mussel (*Mytilus edulis*; Pierce & Ward 2019), the European abalone (*Haliotis tuberculata;* Gobet et al. 2018), and the giant abalone (*Haliotis gigantea*; Tanaka et al 2015). The observed seasonal shifts could alter the gut microbial evenness and functional diversity in the host species and were corresponded to the seasonal changes in food (i.e. seaweed) type and availability and seawater temperature, indicating dietary options and environmental stimuli could have significant contributions to the seasonal gut microbiome shifts in abalone and bivalves (Akter et al. 2022). Compared to other abalone species, investigations on the potential temporal gut microbiota changes in *Haliotis iris*, especially among the wild populations, have not been previously conducted. Given the ecological and economic importance of this abalone species in New Zealand as well as worldwide, exploring whether the gut microbiome of wild *H. iris* would change through multiple seasons in their natural habitats can shed light on the broader implications for abalone growth, health, and adaptations. This will ultimately benefit the fishery and aquaculture management of this shellfish asset.

The aim of the present study was to evaluate the gut microbial diversity and composition of adult abalone (*H. iris*) overtime. Herein, abalone were characterized from two distinct sampling locations (Ascots Beach, a fast-growing site, and Owenga Harbour, a slow-growing site) in the Chatham Islands, across six sampling time points (March 2020 to April 2022). Results of this study can provide another layer of data that can be used in monitoring ecosystem functionality and health of wild abalone populations.

## 5.2 Methods

### *5.2.1 Sample Collection and Processing*

Wild pāua (*Haliotis iris*) were collected by commercial divers from two populations, Site 1 - Ascots Beach (44°00'59.0"S 176°23'11.7"W) and Site 2 - Owenga Harbour (44°01'28" S 176°21'56" W) in the

Chatham Islands (Figure 5.1). Based on historical catch data, site 1 (Ascots Beach) is classified as a high fished area with fast-growing abalone while site 2 (Owenga Harbour) is fished less often due to the slow growth of abalone at this site (Naylor and Fu, 2016). There were six sampling timepoints: T0 - 15 March 2020 (on-site, baseline at 15.01°C; Autumn), T1 - 26 November 2020, (13.43°C; Spring); T2 - 4 March 2021, (16.07°C, Autumn); T3 - 2 April 2021, (15.44°C, Autumn); T4 - 15 May 2021, (13.70°C, Autumn) and T5 - 7 April 2022. (15.48°C, Autumn). Temperatures were acquired using daily satellite information from Group for High Resolution Sea Surface Temperature (GHRSST) MUR L4 product (Copedo et al., 2025).

Twenty adult abalone were randomly collected from each study site at each sampling time point except at Ascots Beach in May 2021 when there were only 10 abalone collected due to logistical issues, with a total sample size of 230. The animals were placed in net bags and transported to holding tank facility with flow-through system until samples were shipped to Auckland University of Technology (AUT) for laboratory processing. Animals were collected under special permit (720, client number 9791209) issued by Fisheries New Zealand.

Abalone were weighed to the nearest 0.01 g, and the shell lengths, widths and heights were measured to the nearest 0.10 mm along the longest axis, using callipers. The abalone were shucked, sexed (based on gonad colour: females = green; males = white), and dissected. In instances where the colour of the gonad could not be accurately assessed, individuals were classified as immature. After removing abalone shells, the viscera was rinsed with phosphated saline buffer and 70% ethanol. The gut content with the lining tissue was aseptically retrieved from the posterior intestine of each animal, and the gut samples were transferred into individual sterile 2 mL cryo-vials filled with 200 µL of the RNAprotect tissue reagent (Qiagen, Catalog No. 76106) followed by snap freezing in liquid nitrogen and temporarily stored at -80 °C until molecular laboratory processing.

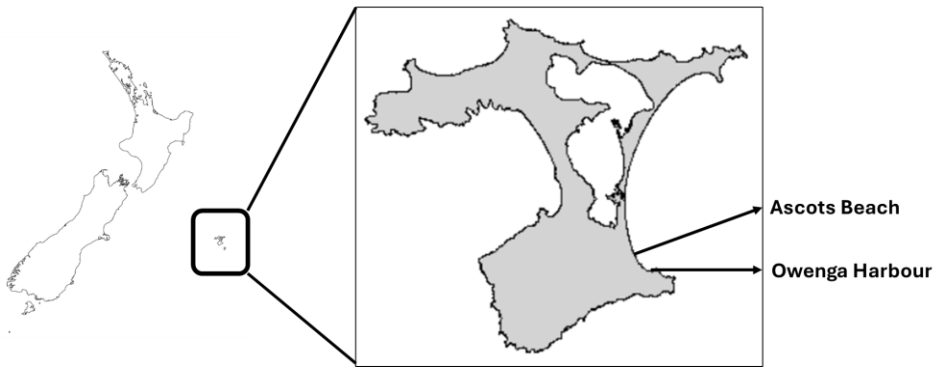


**Figure 5.1: New Zealand and the Chatham Islands map outline illustrating where abalone were collected from Ascots Beach (**fast-growing site**)and Owenga Harbour (**slow-growing site**).**

### *5.2.2 16S ribosomal RNA (rRNA) Amplicon Library Preparation*

The16S rRNA amplicon library preparation of the abalone gut samples followed the protocol described in Guo et al. (2025) and outlined in the supplementary material. Briefly, genomic deoxyribonucleic acid (gDNA) of all samples was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Catalog No. 47014, Germany), quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Catalog No. Q32854, USA), and normalised to 4 nanograms per microliter (ng/µL) with nuclease-free water for a two-step polymerase chain reaction (PCR) amplification. The first-step PCR amplification was conducted in triplicates using a customized 16S rRNA primer (forward: 5'-CCTACGGGNGGCWGCAGG-3'; reverse: 5'-GACTACHVGGGTATCTAATCC-3') targeting the V3-V4 genetic region. The first-step PCR product was pooled by sample, purified with a customized magnetic-bead-based purifying reagent, quantified using the same Qubit™ reagent. PCR products were normalized and indexed following the Illumina *MiSeq* protocol. The 16S rRNA libraries were pooled with equal molarity and sequenced on an Illumina *MiSeq* platform using the v3 (600-cycle) sequencing kit (Illumina, USA) following the manufacturer's protocol.

### *5.2.3 Bioinformatics and data analysis*

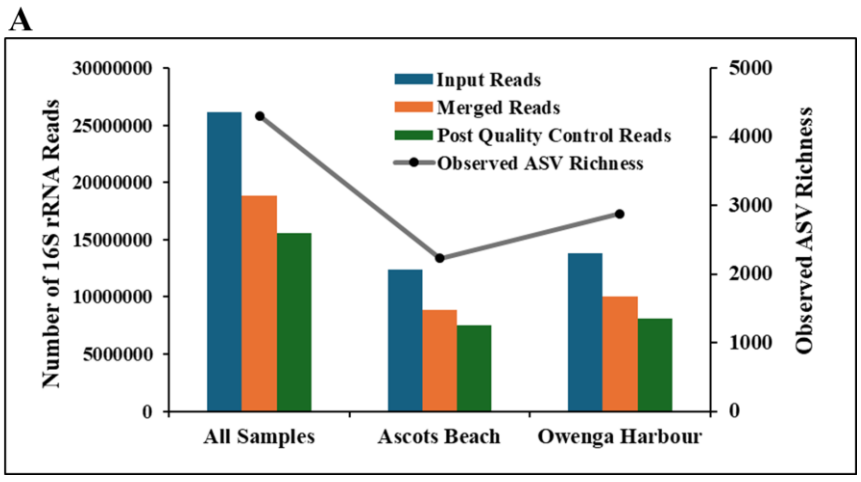
Amplicon sequence data of all the abalone gut samples were processed through a modified DADA2 data processing pipeline (Archer et al., 2020) in R (version 4.2.2; R Core Team, 2022) to generate 16S rRNA amplicon sequence variants (ASVs), construct ASV abundance table, and assign taxonomic information to the ASVs. Statistical analysis mainly focused on microbial composition and diversity comparisons at the genus level across the sampling time points (March 2020, November 2020, March-May 2021, and April 2022) at both sites (Ascots Beach and Owenga Harbour). Briefly, rare ASVs whose prevalences were less than 0.1% across all sample were removed, and rarefaction curves were generated to assess sequencing depths between the sites and across all sampling time points. Relative abundance bar charts of the top 10 most abundant microbial genera of the gut samples were plotted across the sampling time points at each site via the MicrobiomeAnalyst portal (Lu et al., 2023).Alpha-diversity estimators (chao1 richness and Shannon's diversity index) were calculated and plotted using the "vegan" package (Oksanen et al., 2013) in R (version 4.2.2), and two-way ANOVA tests (significance level=0.05) were conducted to test statistical significance. Moreover, the microbial data were normalized using the total sum scaling method and transformed to a Bray-Curtis dissimilarity matrix to compare beta-diversity patterns. Non-metric multidimensional scaling (nMDS) plots were generated using the "vegan" and "ggplot2"

(Wickham, 2016) R packages to illustrate microbial compositional similarities across the sampling points at each study site, and permutation MANOVA (PERMANOVA) and dispersion tests were conducted to detect statistical significance of the microbial diversity. Similarity percentage (SIMPER) analyses were conducted to reveal what specific microbial genera contributed to the observed compositional differences between each pair of sampling time points at each study site. Furthermore, the core microbiome (detection threshold of 0.1% and sample prevalence of 20%) and microbial clustering (using the Minkowski distance and complete clustering algorithm) heatmaps were generated to illustrate the overall gut microbial compositional similarities and differences across the sampling time points at both study sites.

## 5.3 Results

### *5.3.1 General Bioinformatics*

The total number of quality 16S rRNA sequences (reads) generated through the modified DADA2 pipeline from 230 abalone gut samples were 15,604,964 and produced 4,307 ASVs. The sequencing depths at both study sites across all sampling points were adequate (Figure 5.3). Read abundance was similar in the two sites but varied across sampling time points in both locations (Figure 5.2A). The 16S rRNA read abundance was higher at sampling points T2 and T3 (March and April 2021), while observed ASV richness was lower at T2 and T3 (Figure 5.2B-C). The observed ASVs were taxonomically assigned 281 microbial genera.



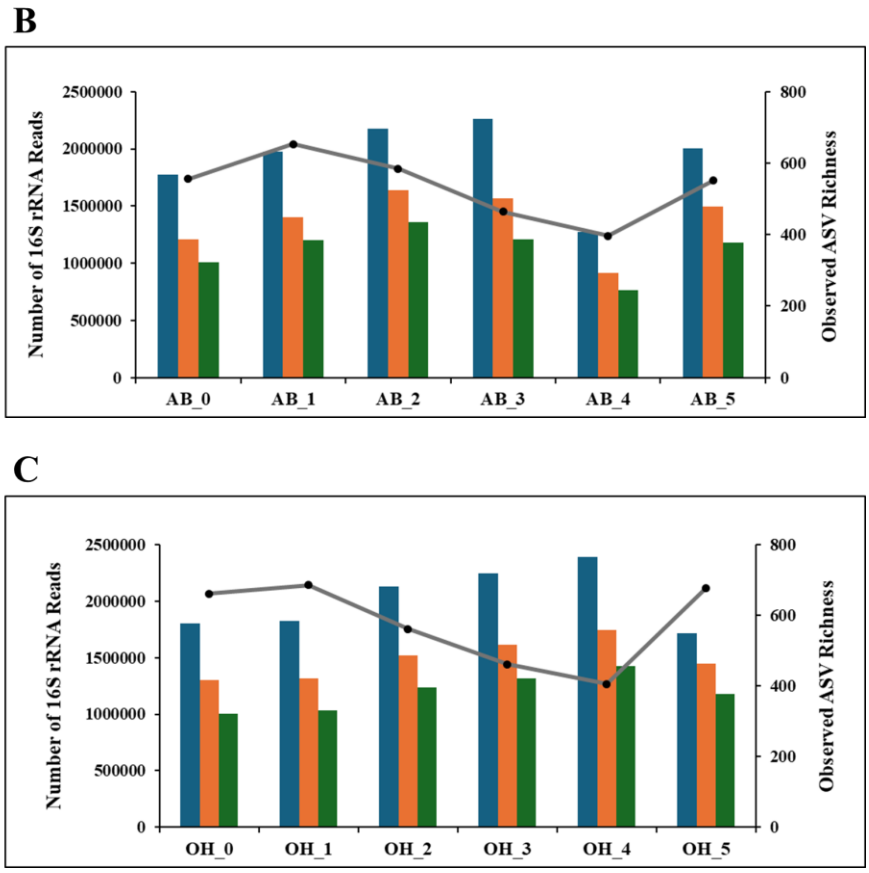



**Figure 5.2: Number of 16S rRNA reads and observed amplicon sequence variant (ASV) richness of all abalone gut samples collected at Ascots Beach (AB) and Owenga Harbour (OH, A) and across the sampling time points (0: March 2020, 1: November 2020, 2: March 2021, 3: April 2021, 4: May 2021 and 5: April 2022) at Ascots Beach (B) and Owenga Harbour (C).**

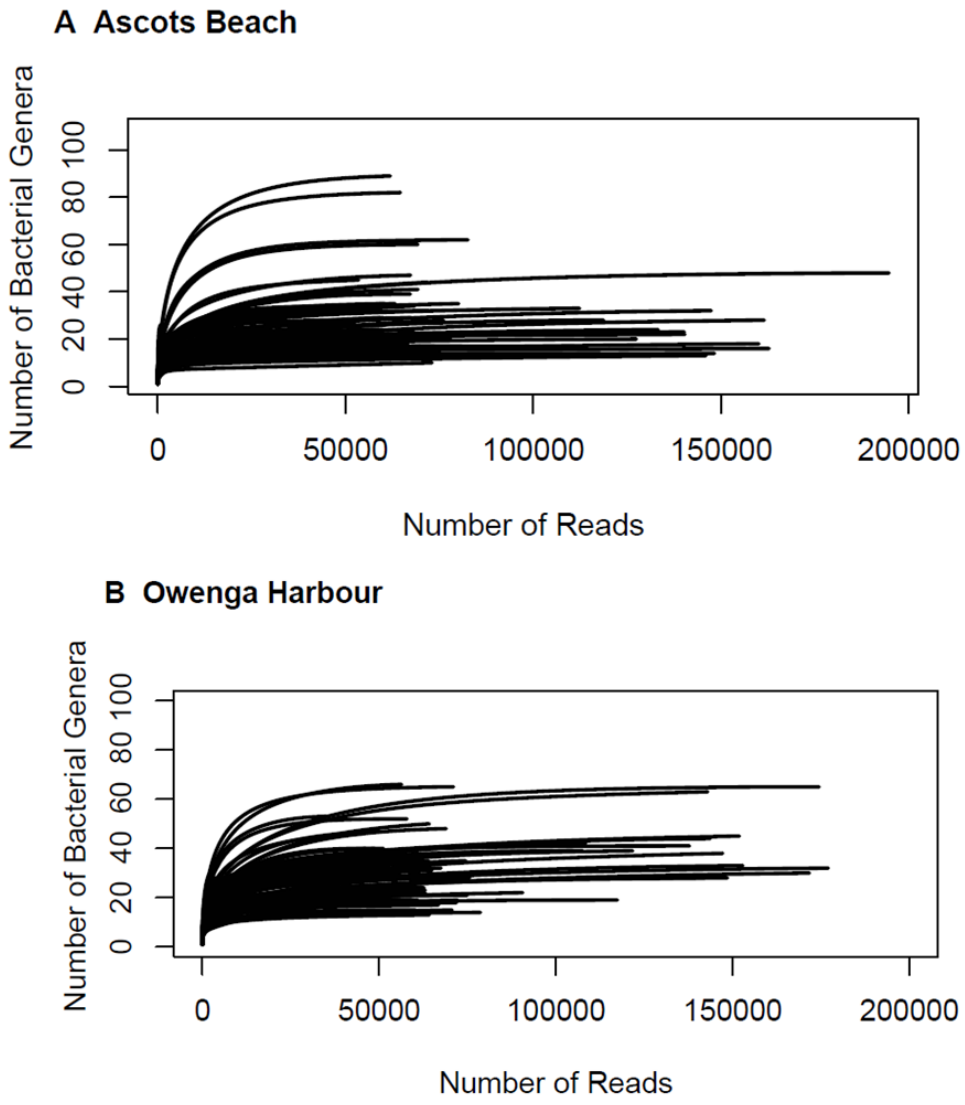



**Figure 5.3: Rarefaction curves of all abalone gut samples collected from Ascots Beach (A) and Owenga Harbour (B) in the Chatham Islands through six sampling time points (March 2020, November 2020, March-May 2021, and April 2022).**

### *5.3.2 Gut microbial richness and diversity*

The alpha-diversity estimators (Chao1 richness and Shannon's diversity index) were not statistically significant between Ascots Beach and Owenga Harbour (Table 5.1, Figure 5.4a). However, Shannon's diversity indices, calculated at the microbial genus level, of the gut samples collected between T2 and T3 were significantly lower than those at the remaining time points (T0, T1, T4 and T5) (Figure 5.4b).

Beta-diversity assessments indicated significant differences between the study sites and across the sampling time points within each site, and the observed diversity differences were not due to between-

group dispersions (Table 5.2; Figure 5.5). Abalone gut samples collected between T2, T3 and T4 showed significant differences in beta-diversity. (Table 5.3; Figure 5.5).

**Table 5.1: Two-way ANOVA test results of the microbial genus Chao1 richness and Shannon's diversity indices of abalone gut samples collected at Ascots Beach and Owenga Harbour across six sampling time points (March 2020, November 2020, March-May 2021, and April 2022). The non-significant interaction terms between the main factors were removed from the statistical models in both alpha-diversity estimators.**

| **Chao1 Richness (Microbial Genus)** | | | | | |
|---|---|---|---|---|---|
| | **df** | **Sum of Square** | **Mean of Square** | ***F*-statistics** | ***p*-value** |
| **Site** | 1 | 1062 | 1062 | 5.79 | 0.251 |
| **Sampling Time Point** | 5 | 9173 | 1843 | 8.78 | 0.173 |
| **Residuals** | 223 | 46188 | 209 | | |

| **Shannon's Index (Microbial Genus)** | | | | | |
|---|---|---|---|---|---|
| | **df** | **Sum of Square** | **Mean of Square** | ***F*-statistics** | ***p*-value** |
| **Site** | 1 | 4.24 | 4.24 | 3.11 | 0.089 |
| **Sampling Time Point** | 5 | 5.29 | 1.06 | 9.77 | <0.001 |
| **Residuals** | 223 | 23.95 | 0.108 | | |

**Table 5.2: Permutational dispersion (PERMDISP) and MANOVA (PERMANOVA) test results of the microbial composition of the abalone gut samples by site (Ascots Beach and Owenga Harbour) and sampling time points (T0: March 2020, T1: November 2020, T2-4: March-May 2021, and T5: April 2022). The gut microbial abundance data were normalized and converted to Bray-Curtis dissimilarity matrices, and the statistically non-significant interaction term between the main factors was removed from the PERMANOVA model.**

| **PERMDISP Test** | | | | | | |
|---|---|---|---|---|---|---|
| **Factor** | **df** | **Sum of Square** | **Mean of Square** | ***F*-statistics** | ***p*(permutation)** | **Number of Permutations** |
| **Site** | 1 | 0.00024 | 0.00024 | 0.013 | 0.91 | 999 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **(Site Residuals)** | 228 | 2.27 | 0.02 | | | |
| **Sampling Time Point** | 5 | 0.0034 | 0.0067 | 0.042 | 0.35 | 999 |
| **(Sampling Time Point Residuals)** | 224 | 1.83 | 0.016 | | | |

**PERMANOVA Test**

| **Factor** | **df** | **Sum of Square** | **Mean of Square** | ***F*-statistics** | **p(permutation)** | **Number of Permutations** |
|---|---|---|---|---|---|---|
| **Site** | 1 | 5,886 | 5,885.8 | 12.4 | 0.001 | 998 |
| **Sampling Time Point** | 5 | 19,339 | 3,867.7 | 8.15 | 0.001 | 999 |
| **Residues** | 223 | 51,247 | 474.51 | | | |

**Table 5.3: Pairwise permutational MANOVA (PERMANOVA) test results of the sampling time point factor on the microbial composition of the abalone gut samples collected from Ascots Beach and Owenga Harbour.**

| | **Ascots Beach** | | | **Owenga Harbour** | | |
|---|---|---|---|---|---|---|
| **Groups** | ***t*** | ***p*-value** | **Number of Permutations** | ***t*** | ***p*-value** | **Number of Permutations** |
| **March 2020-November 2020** | 2.41 | 0.077 | 993 | 2.22 | 0.061 | 995 |
| **March 2020-April 2021** | 3.69 | 0.001 | 992 | 2.22 | 0.001 | 994 |
| **March 2020-May 2021** | 4.07 | 0.001 | 992 | 3.37 | 0.001 | 990 |
| **March 2020-April 2022** | 2.65 | 0.061 | 994 | 2.29 | 0.06 | 992 |
| **March 2020-March 2021** | 2.17 | 0.042 | 993 | 2.95 | 0.001 | 995 |
| **November 2020-April 2021** | 1.76 | 0.024 | 992 | 2.10 | 0.002 | 992 |
| **November 2020-May 2021** | 1.96 | 0.01 | 992 | 1.76 | 0.021 | 993 |
| **November 2020-April 2022** | 1.95 | 0.082 | 992 | 1.87 | 0.058 | 992 |
| **November 2020-March 2021** | 1.63 | 0.031 | 995 | 2.24 | 0.004 | 994 |
| **April 2021-May 2021** | 1.76 | 0.018 | 995 | 2.35 | 0.002 | 993 |
| **April 2021-April 2022** | 2.43 | 0.051 | 995 | 2.25 | 0.001 | 997 |
| **April 2021-March 2021** | 2.16 | 0.003 | 997 | 1.42 | 0.05 | 994 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **May 2021-April 2022** | 3.04 | 0.001 | 997 | 1.99 | 0.004 | 997 |
| **May 2021-March 2021** | 2.35 | 0.005 | 994 | 2.17 | 0.001 | 991 |
| **April 2022-March 2021** | 1.78 | 0.017 | 993 | 2.00 | 0.009 | 995 |

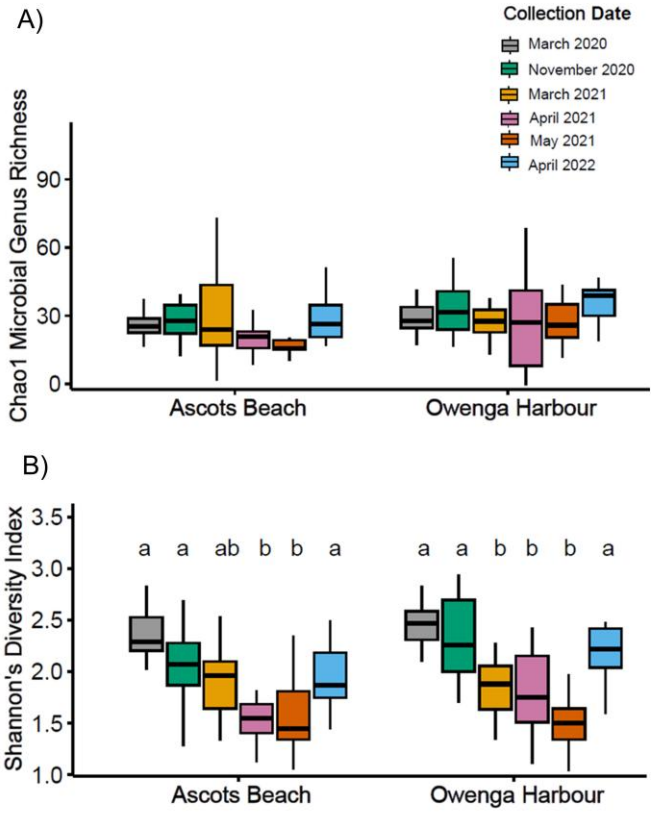



**Figure 5.4: Boxplots of A) microbial genus Chao1 richness and B) Shannon's diversity indices of abalone gut samples collected at Ascots Beach and Owenga Harbour across six sampling time points (T0-March 2020, T1-November 2020, T2-March 2021, T3-April 2021, T4-May 2021, and T5-April 2022). Letters above the coloured boxes indicate statistical significance within each study site (One-way ANOVA, $p<0.05$).**

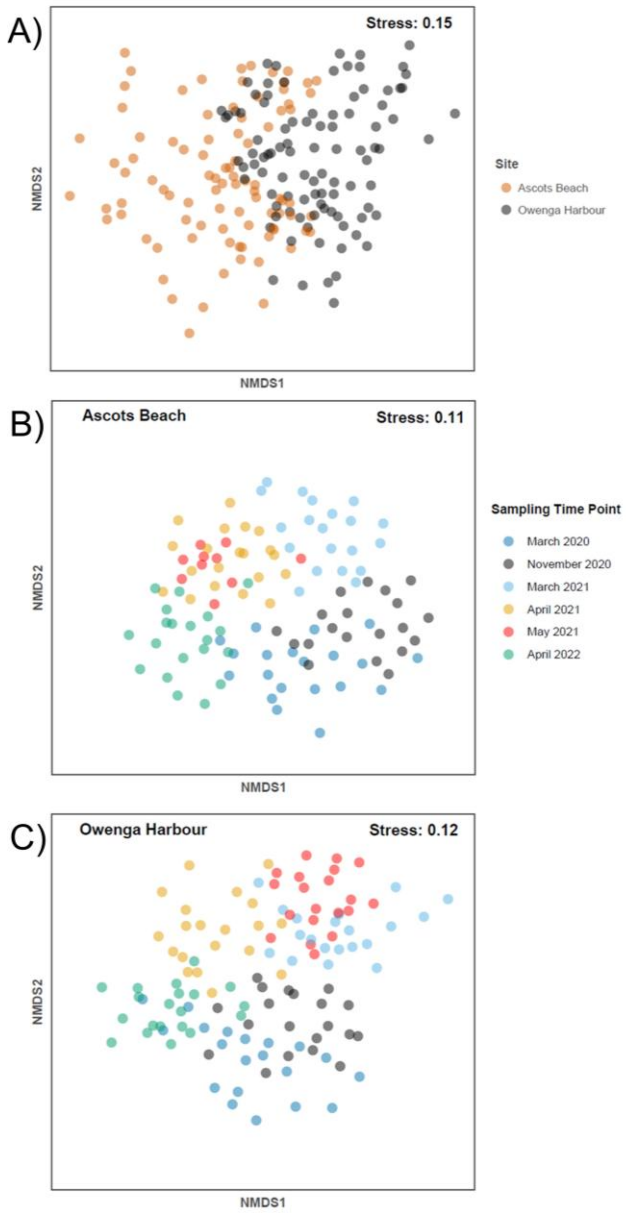
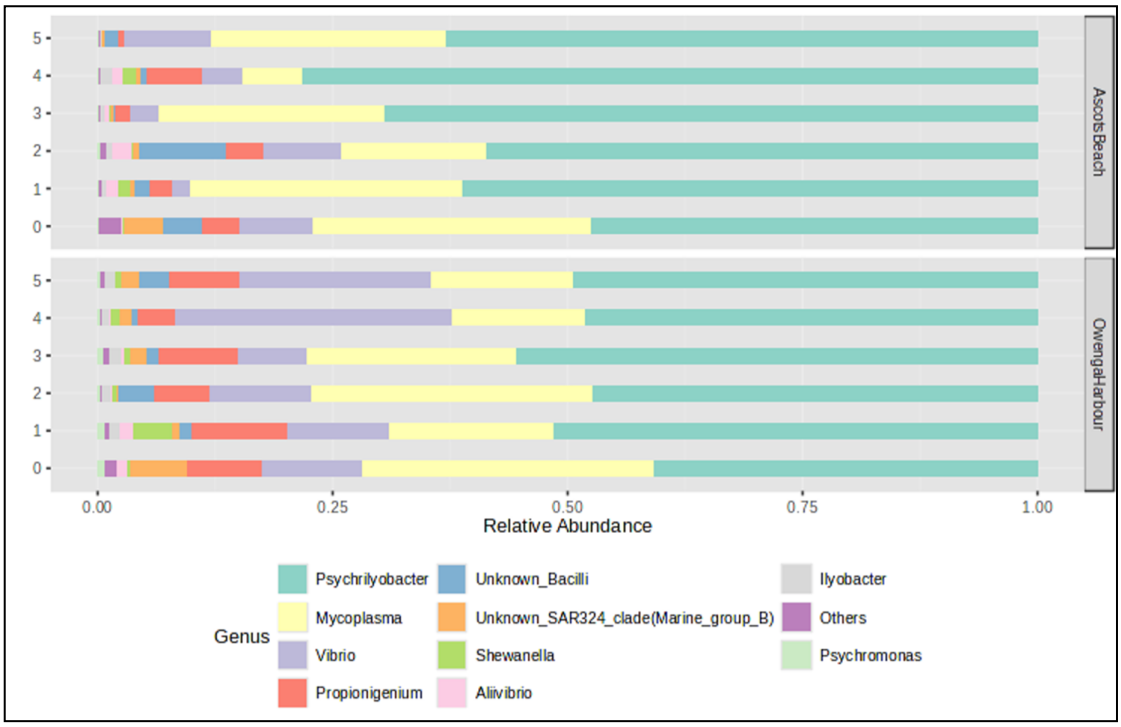



**Figure 5.5: Non-metric multidimensional scaling (nMDS) plots showing the microbial compositional similarities among the abalone gut samples collected between A) the two study sites and across six sampling time points at B) Ascots Beach (fast-growing site) and C) Owenga Harbour (slow-growing site). The gut microbial abundance data were normalized and converted to Bray-Curtis dissimilarity matrices.**

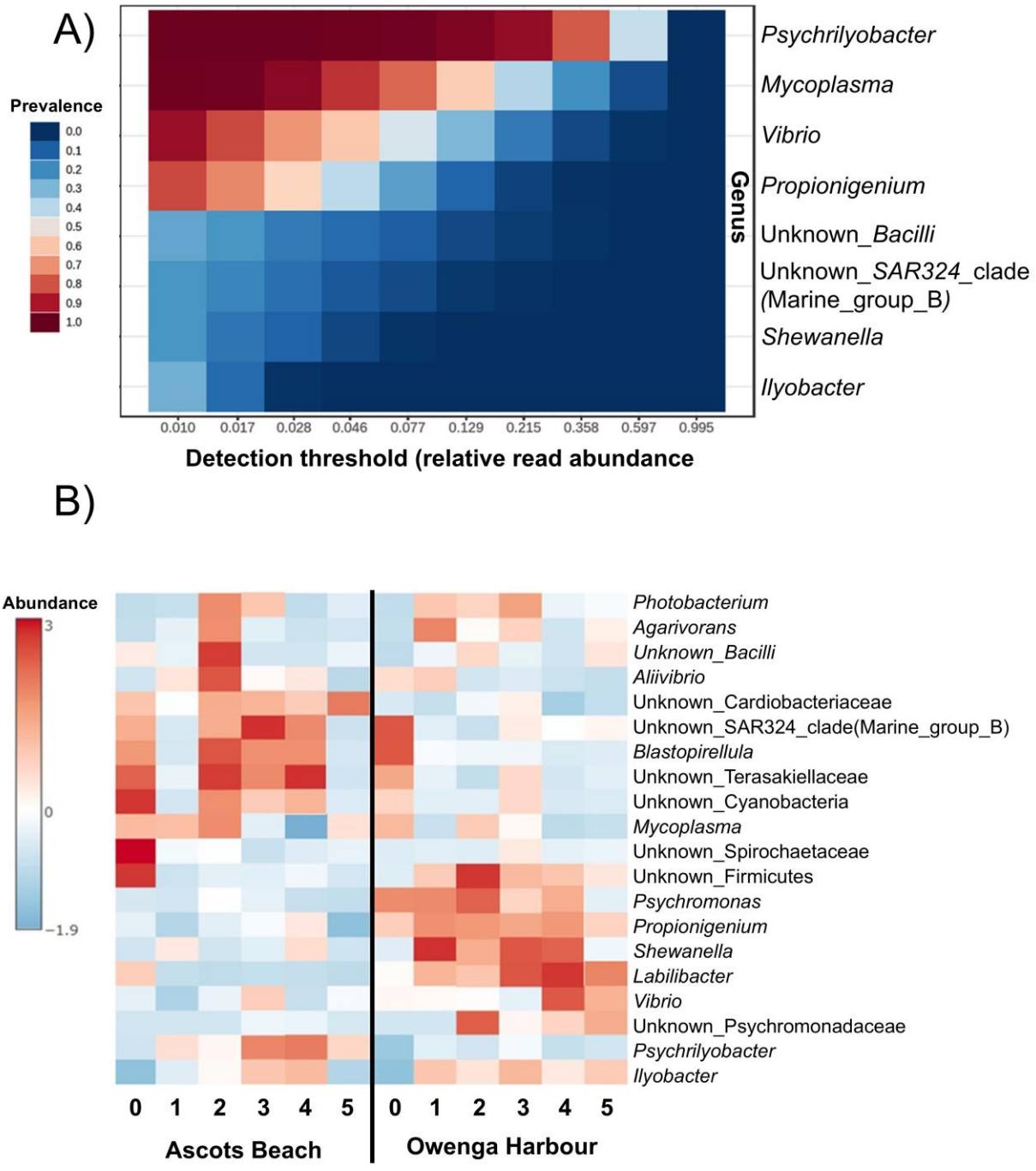
### *5.3.3 Gut microbial composition and core microbiota*

Microbial composition of abalone gut samples was dominated by *Psychrilyobacter*, *Mycoplasma* and *Vibrio* but differentiated in less abundant bacterial genera than *Psychrilyobacter*, *Mycoplasma* and *Vibrio* by study site and sampling time point (Supplementary Figure 5.1). More specifically, the Owenga

Harbour abalone gut samples showed more *Propionigenium* reads than the samples collected at Ascots Beach, where relatively more unassigned Bacilli and *Aliivibrio* bacteria were observed (Supplementary Figure 5.1). Additionally, the four bacterial genera, *Psychrilyobacter*, *Mycoplasma*, *Vibrio*, and *Propionigenium*, mentioned above formed a substantial part of the core microbiota of all samples (Supplementary Figure 5.2A), and the observed microbial composition of the abalone gut samples collected during T2 and T3 at each study site were different from that of T1, T2, T3 and T5 time points (Supplementary Figure 5.2B). Furthermore, the SIMPER analysis indicated that bacterial genera, including *Psychrilyobacter*, *Mycoplasma*, *Vibrio*, *Propionigenium*, *Aliivibrio*, *Shewanella*, Unknown Bacilli, and Unknown SAR324 clade (Marine group B) collectively contributed to at least 80% of the observed compositional differences between the sites and across the sampling time points (Table 5.4).




**Supplementary Figure 5.1: Relative abundance of the Top 10 most abundant microbial genera observed in the abalone gut samples collected at Ascots Beach and Owenga Harbour across the six sampling time points.**




**Supplementary Figure 5.2: A) Core gut microbiota and B) general gut microbial composition heatmaps of abalone gut samples collected at Ascots Beach and Owenga Harbour across the six sampling time points.**

**Table 5.4: Similarity percentage (SIMPER) results showing the top microbial genera that cumulatively contributed to the observed compositional differences of abalone gut samples between Ascots Beach and Owenga Harbour as well as between each pair of the sampling time points.**

| **Ascots Beach vs. Owenga Harbour** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
|---|---|---|---|
| ***Psychrilyobacter*** | 0.135 | 0.096 | 0.33 |
| ***Mycoplasma*** | 0.104 | 0.085 | 0.584 |
| ***Vibrio*** | 0.06 | 0.071 | 0.731 |
| ***Propionigenium*** | 0.031 | 0.039 | 0.807 |
| **Unknown Bacilli** | 0.026 | 0.048 | 0.87 |
| **Unknown SAR324 clade (Marine group B)** | 0.017 | 0.022 | 0.913 |
| **Ascots Beach** | | | |
| **March 2020 vs. November 2020** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.135 | 0.085 | 0.35 |
| ***Mycoplasma*** | 0.112 | 0.061 | 0.64 |
| **Unknown Bacilli** | 0.034 | 0.039 | 0.728 |
| **Unknown SAR324 clade (Marine group B)** | 0.026 | 0.016 | 0.794 |
| ***Vibrio*** | 0.015 | 0.013 | 0.833 |
| ***Propionigenium*** | 0.01 | 0.007 | 0.859 |
| **March 2020 vs. March 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.146 | 0.082 | 0.338 |
| ***Mycoplasma*** | 0.127 | 0.061 | 0.631 |
| **Unknown Bacilli** | 0.067 | 0.082 | 0.785 |
| **Unknown SAR324 clade (Marine group B)** | 0.025 | 0.016 | 0.843 |
| ***Vibrio*** | 0.022 | 0.018 | 0.894 |
| ***Propionigenium*** | 0.01 | 0.009 | 0.918 |
| **March 2020 vs. April 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.167 | 0.082 | 0.458 |

| | | | |
|---|---|---|---|
| ***Mycoplasma*** | 0.087 | 0.05 | 0.698 |
| **Unknown SAR324 clade (Marine group B)** | 0.028 | 0.016 | 0.775 |
| **Unknown Bacilli** | 0.027 | 0.033 | 0.849 |
| ***Vibrio*** | 0.017 | 0.014 | 0.895 |
| ***Propionigenium*** | 0.011 | 0.008 | 0.924 |
| **March 2020 vs. May 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.176 | 0.09 | 0.394 |
| ***Mycoplasma*** | 0.129 | 0.043 | 0.683 |
| **Unknown SAR324 clade (Marine group B)** | 0.028 | 0.016 | 0.745 |
| **Unknown Bacilli** | 0.027 | 0.033 | 0.806 |
| ***Propionigenium*** | 0.025 | 0.032 | 0.863 |
| ***Vibrio*** | 0.021 | 0.017 | 0.91 |
| **March 2020 vs. April 2022** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.107 | 0.075 | 0.305 |
| ***Mycoplasma*** | 0.093 | 0.082 | 0.568 |
| ***Vibrio*** | 0.049 | 0.05 | 0.708 |
| **Unknown Bacilli** | 0.029 | 0.031 | 0.79 |
| **Unknown SAR324 clade (Marine group B)** | 0.028 | 0.016 | 0.871 |
| ***Propionigenium*** | 0.013 | 0.008 | 0.906 |
| **November 2020 vs. April 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.14 | 0.114 | 0.433 |
| ***Mycoplasma*** | 0.111 | 0.098 | 0.775 |
| **Unknown Bacilli** | 0.015 | 0.036 | 0.82 |
| ***Propionigenium*** | 0.011 | 0.009 | 0.855 |
| ***Vibrio*** | 0.011 | 0.012 | 0.887 |
| ***Aliivibrio*** | 0.009 | 0.012 | 0.916 |
| **November 2020 vs. May 2021** | **Average** | **Standard** | **Cumulative Contribution** |

| | | Deviation | |
|---|---|---|---|
| ***Psychrilyobacter*** | 0.151 | 0.118 | 0.4 |
| ***Mycoplasma*** | 0.125 | 0.117 | 0.73 |
| ***Propionigenium*** | 0.026 | 0.033 | 0.8 |
| ***Vibrio*** | 0.019 | 0.02 | 0.851 |
| **Unknown Bacilli** | 0.016 | 0.036 | 0.893 |
| ***Aliivibrio*** | 0.012 | 0.014 | 0.925 |
| **November 2020 vs. April 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.139 | 0.101 | 0.346 |
| ***Mycoplasma*** | 0.134 | 0.103 | 0.679 |
| ***Vibrio*** | 0.053 | 0.056 | 0.81 |
| **Unknown Bacilli** | 0.021 | 0.036 | 0.863 |
| ***Propionigenium*** | 0.011 | 0.009 | 0.891 |
| ***Aliivibrio*** | 0.01 | 0.013 | 0.915 |
| **March 2021 vs. April 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.148 | 0.132 | 0.411 |
| ***Mycoplasma*** | 0.091 | 0.099 | 0.662 |
| **Unknown Bacilli** | 0.063 | 0.092 | 0.838 |
| ***Vibrio*** | 0.026 | 0.02 | 0.911 |
| ***Propionigenium*** | 0.011 | 0.011 | 0.941 |
| ***Aliivibrio*** | 0.01 | 0.021 | 0.969 |
| **May 2021 vs. April 2022** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.155 | 0.108 | 0.377 |
| ***Mycoplasma*** | 0.136 | 0.116 | 0.707 |
| ***Vibrio*** | 0.051 | 0.05 | 0.83 |
| ***Propionigenium*** | 0.032 | 0.035 | 0.908 |
| **Unknown Bacilli** | 0.012 | 0.019 | 0.938 |
| ***Shewanella*** | 0.008 | 0.009 | 0.957 |
| | **Owenga Harbour** | | |

| March 2020 vs. November 2020 | Average | Standard Deviation | Cumulative Contribution |
|---|---|---|---|
| ***Mycoplasma*** | 0.138 | 0.066 | 0.285 |
| ***Psychrilyobacter*** | 0.117 | 0.082 | 0.526 |
| ***Propionigenium*** | 0.057 | 0.054 | 0.644 |
| ***Vibrio*** | 0.052 | 0.051 | 0.752 |
| **Unknown SAR324 clade (Marine group B)** | 0.037 | 0.023 | 0.829 |
| ***Shewanella*** | 0.024 | 0.013 | 0.878 |
| **March 2020 vs. March 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.101 | 0.08 | 0.279 |
| ***Mycoplasma*** | 0.094 | 0.06 | 0.538 |
| ***Vibrio*** | 0.041 | 0.036 | 0.652 |
| **Unknown SAR324 clade (Marine group B)** | 0.041 | 0.025 | 0.765 |
| ***Propionigenium*** | 0.021 | 0.016 | 0.823 |
| ***Aliivibrio*** | 0.017 | 0.029 | 0.869 |
| **March 2020 vs. April 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.131 | 0.101 | 0.37 |
| ***Mycoplasma*** | 0.089 | 0.068 | 0.622 |
| **Unknown SAR324 clade (Marine group B)** | 0.034 | 0.023 | 0.719 |
| ***Propionigenium*** | 0.023 | 0.016 | 0.786 |
| ***Vibrio*** | 0.017 | 0.016 | 0.834 |
| ***Aliivibrio*** | 0.016 | 0.029 | 0.88 |
| **March 2020 vs. May 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Vibrio*** | 0.145 | 0.103 | 0.287 |
| ***Mycoplasma*** | 0.145 | 0.063 | 0.573 |
| ***Psychrilyobacter*** | 0.091 | 0.056 | 0.754 |
| **Unknown SAR324 clade (Marine group B)** | 0.036 | 0.023 | 0.825 |

| | | | |
|---|---|---|---|
| ***Propionigenium*** | 0.027 | 0.02 | 0.879 |
| ***Aliivibrio*** | 0.016 | 0.03 | 0.91 |
| **March 2020 vs. April 2022** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Mycoplasma*** | 0.12 | 0.062 | 0.258 |
| ***Vibrio*** | 0.106 | 0.075 | 0.488 |
| ***Psychrilyobacter*** | 0.072 | 0.056 | 0.643 |
| ***Propionigenium*** | 0.042 | 0.039 | 0.733 |
| **Unknown SAR324 clade (Marine group B)** | 0.041 | 0.025 | 0.822 |
| **Unknown Bacilli** | 0.027 | 0.037 | 0.881 |
| **April 2021 vs. May 2021** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Vibrio*** | 0.146 | 0.104 | 0.306 |
| ***Psychrilyobacter*** | 0.131 | 0.102 | 0.58 |
| ***Mycoplasma*** | 0.126 | 0.087 | 0.844 |
| ***Propionigenium*** | 0.022 | 0.024 | 0.89 |
| **Unknown SAR324 clade (Marine group B)** | 0.018 | 0.019 | 0.927 |
| ***Shewanella*** | 0.006 | 0.007 | 0.94 |
| **April 2021 vs. April 2022** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Psychrilyobacter*** | 0.125 | 0.095 | 0.276 |
| ***Vibrio*** | 0.108 | 0.074 | 0.515 |
| ***Mycoplasma*** | 0.104 | 0.074 | 0.744 |
| ***Propionigenium*** | 0.043 | 0.047 | 0.837 |
| **Unknown Bacilli** | 0.027 | 0.034 | 0.897 |
| **Unknown SAR324 clade (Marine group B)** | 0.021 | 0.029 | 0.944 |
| **May 2021 vs. April 2022** | **Average** | **Standard Deviation** | **Cumulative Contribution** |
| ***Vibrio*** | 0.104 | 0.083 | 0.263 |
| ***Psychrilyobacter*** | 0.098 | 0.064 | 0.513 |

| | | | |
|---|---|---|---|
| ***Mycoplasma*** | 0.076 | 0.075 | 0.706 |
| ***Propionigenium*** | 0.045 | 0.044 | 0.82 |
| **Unknown Bacilli** | 0.027 | 0.035 | 0.888 |
| **Unknown SAR324 clade (Marine group B)** | 0.021 | 0.031 | 0.942 |

**5.4 Discussion**

To optimize management strategies in the face of changing environments, it is necessary to perform overtime measures on both well-performing and sub-optimal abalone populations. The primary goal of this study was to perform a longitudinal assessment of the gut microbial diversity and composition of two wild *Haliotis iris* populations from the Chatham Islands (Ascots Beach is classified as a site with fast-growing abalone, while abalone at Owenga Harbour are seen as slow growers based on historical fishery data). In the present study, both alpha- and beta-diversity metrics of abalone's gut microbiota were significantly affected by study site and sampling time. The observed gut microbiota differences between the two study sites (Ascots Beach vs. Owenga Harbour) could potentially be associated with specific microbial functions that the gut bacteria performed and the seaweed habitat characteristics.

Abalone gut microbial communities, mostly consist of bacteria, are mainly involved in seaweed nutrient digestion and immunological health enhancement (Apprill et al. 2017; Aker et al. 2022). The present study revealed that *Psychrilyobacter*, *Mycoplasma*, and *Vibrio* were the most abundant bacterial genera in all abalone gut samples collected from both study sites. This is in line with previous findings in which these core bacterial genera were also dominant in the European abalone *H. tuberculata* (Gobet et al. 2018), the California red abalone *H. rufescens* (Guo 2017) and the Mexican blue (*H. fulgens*) and yellow (*H. corrugata*) abalone (Cicala et al. 2020). Bacteria in Genus *Psychrilyobacter* are primarily involved in fermentation processes and potential probiotic functions. For example, the *Psychrilyobacter haliotis* B1 strain isolated from *Haliotis discus hannai* and *H. diversicolour* was biochemically tested to be able to ferment monosaccharides and disaccharides present in the host's diets and synthesize short-chain fatty acids (SCFAs), which are crucial for facilitating nutrient degradation and the host's energy metabolism (Liu et al. 2023). In addition, *Psychrilyobacter* bacteria could also produce vitamins and antibiotics that are supportive to abalone growth and antipathogenic responses (Liu et al. 2022). *Mycoplasma* bacteria mostly play an important role in ammonia metabolism and gut homeostasis maintenance (Rasmussen et al. 2023). A gut microbiome driven factor investigation on six cephalopod species demonstrated that Mycoplasma could help modulate inflammation and enhance immune responses in cephalopods (Kang et

al. 2022). *Vibrio* is a diverse bacterial genus with various beneficial and detrimental properties. While several *Vibrio* species (e.g., *V. halioticoli*) possess the ability to degrade polysaccharides, such as agar and alginate, in seaweed (Tanaka et al. 2003; Ruiz et al. 2023), other *Vibrio* strains (e.g., *V. vulnificus* and *V. splendidus*) are pathogens with high virulence to trigger infections and diseases in oysters and mussels globally (Ramos et al. 2014; Dubert et al. 2016; Rincé et al. 2018).

In addition to the three core bacterial genera, the present study interestingly found *Propionigenium* is more substantial within the Owenga Harbour population while unassigned Bacilli and *Aliivibrio* bacteria were more abundant in the abalone from Ascots Beach. Similar to some *Psychrilyobacter* bacteria, *Propionigenium* is associated with the production of propionate, a type of short-chain fatty acid, through fermentation processes that break down brown seaweed polysaccharides and other metabolites (Nam et al. 2018). Bacilli bacteria (mostly beneficial bacteria), on the other hand, are mainly capable of degrading polysaccharides present in red seaweed (Abdullah et al. 2024). The site-specific abalone gut microbiota observed in this study was previously seen and could be explained by the differences in seaweed availability, quality, and composition between the sites (Cornwall et al., 2009). Ascots Beach and Owenga Harbour have been historically considered “fast-growing” and “slow-growing” sites, respectively (Naylor et al. 2006). Abalone gut content samples previously collected from Ascots Beach showed a fresh and diverse seaweed assemblage, whereas the macroalgae available for feeding at Owenga Harbour was old and tough (unpublished data). Moreover, relatively more red seaweed was found in the gastrointestinal region of the abalone samples collected at Ascots Beach than at Owenga Harbour, indicating the Ascots Beach abalone consumed more red macroalgae than their counterparts at Owenga Harbour (unpublished data). Several studies documented that abalone fed on red seaweed, especially mixed with brown seaweed, showed higher growth rates, and improved meat protein content (Naidoo et al. 2006; Guo 2017). Therefore, the availability of a mixed macroalgae, with a relatively high red seaweed proportion, at Ascots Beach might select more gut bacteria specialized in digesting red seaweed polysaccharides and promote the growth of abalone compared to the abalone populations at Owenga Harbour. However, targeted feeding experiments on the local abalone populations are required in the future to further test this hypothesis.

Besides the observed site differences in the abalone gut microbiota, we also found that the gut microbial composition and diversity were temporally different across the six sampling points. Abalone collected from both study sites showed a decrease in microbial diversity at T2-4 (March 21, April 21 and May 21) sampling points. Similar observations were previously reported in the giant abalone (*Haliotis gigantea*; Tanaka et al. 2015) and the European abalone (*Haliotis tuberculata*; Gobet et al. 2018) and other marine

invertebrates such as sea cucumbers (Deng et al., 2022), mussels (Cabezón et al., 2024), oysters (Pierce et al. 2016) and sea urchins (Rodríguez-Barreras et al. 2024). The seasonal changes of the gut microbial differences across the sampling points, especially for the lower microbial diversity in abalone sampled between March and May 2021, might be related to the potential changes in environmental factors such as seawater nutrients, temperature and oxygen content (Sunagawa et al., 2015). For example, Li et al. (2025) noticed that variations in seawater chemistry such as pH and nutrient levels could shape the gut microbial communities in *Haliotis discus hannai*. Considering the wild abalone mostly live in the intertidal zones and are exposed to the surrounding seawater with unpredictable oceanographic conditions, and the T2 - T4 sampling points (relatively lower gut microbial diversity detected) in this study were in a seasonal transition from late summer to late autumn, changes in their ambient environments, especially the seawater temperatures, are likely to seasonally change the host's gut microbial composition and diversity.

One of the direct effects of thermal changes to the gut microbiota of wild abalone could be altering the host's gut microbial profiles by proliferating certain bacterial taxa while inhibiting other bacterial groups. For example, a laboratory feeding experiment conducted on *H. discus hannai* illustrated that changes in the gut microbiota of *H. discus hannai* were highly associated with the seawater temperature changes (Wang et al. 2020). Similar results were also found in bivalves. For instance, a high temperature induction experiment conducted on a cold-water scallop, *Patinopecten yessoensis*, revealed an increased relative abundance of Bacteroidetes and decreased bacterial abundance in Proteobacteria and Firmicutes (Kong et al., 2023), and opportunistic bacterial genera such as *Vibrio* and *Arcobacter* were monopolized in *Mytilus galloprovincialis* as the rearing seawater temperatures increased (Li et al., 2018).

A potential mechanism for the thermal stress to alter the gut microbial diversity in abalone could be the thermal changes influence microenvironmental conditions (e.g., pH and dissolved oxygen levels) inside the digestive tract of the hosts. Bacteria that assist macronutrient metabolism in the digestive tract of abalone and bivalves are often involved in enzymatic activities that require optimal ranges of microenvironmental parameters (e.g., temperature, pH, dissolved oxygen, etc.) (Yu et al. 2022). The temperature changes were seen to initially increase but eventually reduce the enzymatic activities in the gastrointestinal tract of abalone (Harris et al., 1998b; Frederick et al., 2022) or could decrease the dissolved oxygen levels in seawater and eventually in the host's gut, making the conditions more favorable for the anaerobic and facultative aerobic bacterial strains and ultimately reducing the efficiency of the macronutrient metabolism (Reid et al., 2019).

In line with the proliferation of gut microorganisms, a negative and detrimental scenario would be the proliferation of pathogenic bacterial strains, such as *Vibrio* spp., under the thermal challenge, which has become a major threat to the molluscan fisheries and aquaculture. *Vibrio* was a large component of the core gut microbiota among the wild *H. iris* populations in our study, and the relative abundance of this bacteria genus was higher in April and May when seawater temperatures often oscillate. While this genus is a significant contributor to the degradation of complex dietary compounds and supporting the host's digestive processes (Gobet et al., 2018, Danckert et al., 2021), it could also be harmful to mollusks when temperatures increase in aquatic environments. For example, high mortality rates among the Pacific oysters (*Crassostrea gigas*) were also associated with the proliferation of pathogenic *Vibrio* spp. in Asia and Australia (Pathirana et al., 2022, Zhao et al., 2023). The growth and survival of copepods were also reduced as thermophilic *Vibrio* bacteria, accounting for more than 70% of the total bacterial abundance, were detected under elevated temperatures (Vu et al., 2023). One of the essential functions of diverse and balanced gut microbiota in mollusks is to support immune responses and disease resistance. High abundance of microbial pathogens triggered by thermal stress could increase microbial infection susceptibility by shrinking the intestinal lumen and increased goblet cells to further promote pathogen proliferation in a negative feedback loop (Kong et al., 2023). The dual roles of *Vibrio* can generate seasonal swings in functional capacity (e.g., polysaccharide degradation capacity peaking when alginate-rich diets are consumed) while also generating site-specific disease risk when environmental conditions favor opportunistic infections. Moreover, the polysaccharide-degrading capability of *Vibrio* could align with seasonal dietary shifts and alginate availability in abalone's gut, environmental abiotic conditions, when suitable, can shift *Vibrio* toward mutualism or pathogenicity.

Besides the direct effects on abalone's gut microbiota, the changes of environmental parameters could also influence the macroalgal communities in the natural habitats, which ultimately affects the gut microbiota in abalone. Environmental changes (e.g., elevated seawater temperatures) could disturb the nutrient cycles and contingently have negative impacts on macroalgal abundance, compositions, and distributions (Nauer et al., 2022). For instance, the bull kelp communities consisted of *Durvillaea antarctica*, *D. poha*, and *D. willana* around the South Island of New Zealand went to extinction during high seawater temperature months (Thomsen et al., 2019). In western Australia, the kelp (*Ecklonia radiata*) forests along the coastline were seen to shrink dramatically with approximately 43% of the abundance disappeared in winter 2011 when the coastal seawater temperatures were unexpectedly high (Wernberg, 2021). In the Northern Hemisphere, severe marine heatwaves were recorded in California in three consecutive years between 2014 and 2016 during which the local bull kelp (*Nereocystis luetkeana*) collapsed concurrently with several abalone mass mortalities (Rogers-Bennett et al., 2021). Given the fact

that these macroalgal species are important natural food sources for wild abalone, perturbations on these seaweeds due to the environmental shifts could lead to a regime shift from macroalgal-dominant to turf-algae-dominant, making the quality of the nutritional profiles of the seaweeds very poor to abalone and affecting the gastropod's gut microbial composition and diversity (Gurgel et al., 2020).

While seaweed nutritional profiles and environmental conditions can be important factors that influence abalone gut microbiota, the interaction between the two factors could also affect abalone gut microbiota and meat quality. For example, Wang et al. (2020) demonstrated that while the changes in the gut microbiota of *H. discus hannai* were highly associated with the seawater temperature changes, the experimental subjects were also sensitive to the dietary changes, indicating diet could be a co-factor with environmental factors for alternating abalone gut microbiota. Consequently, changes in the gut microbial profiles associated with dietary and seasonal shifts could be reflected in phenotypical differences such as the differentiated total lipid and fatty acid concentrations observed in an abalone hybrid between *H. laevigata* and *H. rubra* in Australia (Mateos et al. 2010).

Indeed, the effects of elevated temperatures, seaweed, and their interplay can influence the gut microbiota of wild *H. iris*, but how exactly each of these factors would influence the gut microbial diversity and composition still require further investigation. One way to examine such temperature and dietary effects is through targeted experiments with multiple temperature and seaweed treatments in controlled experiments to understand the potential patterns, predict the resilience of this fishery and aquaculture asset to climate change, and develop strategies to mitigate the negative impacts in New Zealand. Comprehensive analyses of spatial and temporal variation of unique abalone microbial and environmental communities are needed, along with baseline reference data, to effectively interpret microbiome changes (Ribas et al., 2023). The ability to translate changes within biological reference points with adaptive fishery management approaches is the best way to develop predictive models to support fishery, restoration, and aquaculture resilience in a changing ocean (Rogers-Bennett et al., 2019).

### 5.5 Conclusions

This was the first longitudinal study characterizing gut microbiome on wild *Haliotis iris*. The gut microbial diversity and composition of the abalone collected at Ascots Beach and Owenga Harbour were significantly different between T2– T4 (March-May 2021). Abalone gut samples showed lower microbial richness and diversity between March and May 2021 at both study sites, and the differentiated gut microbial profiles across the sampling time points were mainly explained by the different proportions of

bacteria in Genera *Psychrilyobacter*, *Mycoplasma*, unknown Bacilli, *Propionigenium,* and *Vibrio*. In addition, the abalone gut microbiota was also significantly different between the two study sites where differences of growth rates and seaweed compositional structures were previously reported, indicating dietary and nutritional factors could also contribute to the observed differentiated gut microbiota under the potential elevated temperature effects. Overall, the observed temporal and site gut microbiome differences could be related to the differences in seaweed availability and nutritional profiles as well as environmental conditions, which need to be specifically tested through further targeted experiments. Understanding whether and how wild *H. iris* gut microbiota would change seasonally, especially under the threats of global warming, will help to predict the digestion efficiency and nutritional states and prevent pathogen-related infections or diseases of this ecologically and economically important resource in this country. A diverse and healthy gut microbiome in wild *H. iris* in the Chatham Islands can provide optimal microbial functions to support growth and other physiological performance of local abalone populations and eventually benefit abalone fishery management and revenue.

# Chapter 6: General Discussions, Limitations, Future Research Directions and Overall Thesis Conclusions

## 6.1 General Discussion

In the past two decades, the abalone gut microbiome has been the focus of research, specifically the taxonomic and functional diversity of the gut microorganisms and their significance in abalone biological and physiological performances (e.g., survival, growth, digestion, immunological responses, etc.) (Daume, 2006; Zhao et al., 2018; Li et al., 2024). One of the conceptual abalone gut microbiome research workflows is to explore "who" are in the gut (i.e. species richness and diversity of the gut microbial communities), "how" the gut microorganisms got into the gut (i.e., "vertical transmission", meaning gut microorganisms are transferred from parents to offsprings vs. "horizontal transmission", meaning gut microorganisms are acquired from ambient environments), "why" are there differences in the gut microbiota (i.e., factors affecting the gut microbial communities) and ultimately "what" the microbial communities do in abalone's gut (i.e., microbial functions and host-microbe interactions). With respect to factors influencing abalone and other molluscan gut microbiomes, they are mostly categorized as "genetic", "environmental", and the interacting factors in the literature (Chalifour & Li, 2021; Scanes et al., 2021a; Salloum et al., 2025). Abalone gut microbiome research has been mostly conducted in Asia and Australia, but such comparative studies have been extremely sparse among abalone populations in the Southern Hemisphere, particularly in New Zealand. The black-footed abalone (*Haliotis iris*) are endemic to New Zealand (i.e., limited genetic interference from outside abalone species), and commercial and recreational abalone fisheries and aquaculture on *H. iris* co-exist within the country. Unlike in many Asian countries where most of the abalone production is contributed by the aquaculture sector, abalone production through commercial catch outcompetes the aquaculture production in New Zealand (Fisheries New Zealand, 2021; Stenton-Dozey et al., 2021; Ryder et al., 2023; Peters et al., 2024). Moreover, wild abalone are constantly exposed to changing coastal marine environments where seaweed food sources and oceanographic conditions are often unpredictable. With the impacts of climate change and global warming, environmental conditions in wild abalone's habitats can change more dramatically and become stressors to the gut microbial communities as well as the physiology of the abalone hosts. Therefore, investigating the gut microbiome and understanding how the gut microbiota of wild *H. iris* populations change under the influences of various conditions can provide insights of food digestion and nutrient absorption efficiency of the gastropods, which directly relates to the growth rate and nutritional state of the wild abalone populations.

Utilizing the Illumina MiSeq amplicon-sequencing technology, the overall aims of the present thesis were to explore the gut microbial composition and diversity (i.e., gut microbiota), as a fundamental and important component of the gut microbiome research, of NZ wild *H. iris* populations and to illustrate some influencing environmental factors that might potentially alter the abalone's gut microbiome. The main body of this thesis begins with a literature review (Chapter 2) to provide a general understanding of the abalone gut microbiome, relevant research methods, and environmental factors that may influence abalone gut microbial composition and diversity. The literature review specifically focuses on diets and oceanographic conditions (with an emphasis on seawater temperature changes) as major environmental factors of influencing abalone and other molluscan gut microbiomes. Chapter 3 of this thesis compares the microbial richness and diversity at three regions along the digestive tract of the wild *H. iris* samples collected in Cook Strait as well as from the surrounding sediment and seaweed samples. Results from Chapter 3 revealed that the microbial composition and diversity were similar between the foregut (esophagus pouch) and hindgut (intestine) but significantly different from that of the buccal cavity, sediment, and seaweed samples, indicating it is unlikely that those wild abalone directly acquired microorganisms present in the sediment or the surface of seaweed. Building on the finding that there were no significant differences in the microbiota between the foregut and hindgut of these wild samples as well as several farmed *H. iris* stock batches (Bullon et al., 2025), the subsequent chapters only utilized intestinal samples for the gut microbiome investigations. As previously mentioned, wild abalone populations are also found in the Chatham Islands that are distantly separated from the main NZ islands, and the wild abalone commercial fishery substantially supports the overall national abalone production. Differentiated growth rates were historically noted among the abalone populations in the Chatham Islands, but gut microbiome research and the roles of the gut microbial communities of the wild abalone populations are still underexplored. Therefore, the remaining experimental chapters were designed to fill such research gaps. In Chapter 4, the gut microbial composition and diversity of two shell length size groups among four abalone populations in the Chatham Islands were characterized and compared. There were significant gut microbiota differences observed by size groups and study sites (abalone population) with potential dietary influences (i.e., consumed seaweed types). This chapter (Chapter 4) demonstrated that the gut microbiome of wild *H. iris* from the Chatham Islands may be shaped by local seaweed availability and type (i.e., red, brown, or green seaweed) and may eventually influence the host's growth and physiology, which has direct implications for fishery management and sustainability. Using the gut microbiome data collected from the two study sites (Ascots Beach and Owenga Harbour) as a baseline reference, the final experimental chapter (Chapter 5) provides a longitudinal gut microbiome survey of the Chatham Islands study sites that were sampled between November 2020 and April 2022. The survey results illustrated gut microbial community shifts over time at both study sites, providing evidence that

the gut microbiome of wild *H. iris* populations in the Chatham Islands might change in response to the changes in oceanographic conditions over time. The core gut microbiomes in all experimental chapters always consisted of *Mycoplasma*, *Pschrilobacter*,and *Vibrio* but varied by minor microbial genera (Table 6.1).

**Table 6.1: Core gut microbial genera detected in the experimental chapters (Chapters 3-5) of the present thesis.**

| Chapter | Core Gut Microbial Genera |
|---|---|
| Chapter 3 | *Psychrilyobacter*, *Aliivibrio*, *Mycoplasma*, *Vibrio,* and *Psychromonas* |
| Chapter 4 | *Psychrilyobacter*, *Mycoplasma*, *Vibrio, Propionigenium,* Unassigned Bacilli, Unassigned SAR324 Clade (Marine group B) and *Blastopirellula* |
| Chapter 5 | *Psychrilyobacter*, *Mycoplasma*, *Vibrio, Propionigenium,* Unassigned Bacilli, Unassigned SAR324 Clade (Marine group B), *Shewanella,* and *Ilyobacter* |

Together, the literature review and the three experimental chapters present a comprehensive understanding of the general abalone gut microbial community composition and specific gut microbiome characterizations of NZ wild *H. iris* populations across spatial and temporal dimensions as well as some potential environmental factors influencing the abalone growth rate. Results presented in this thesis collectively suggest that the wild *H. iris* gut microbiome might be linked to both diet and oceanographic conditions, which has implications for sustainable fishery management and aquaculture practices on this important abalone species.

## 6.2 Suggested Improvements and Future Research Directions

All experimental chapters in the present thesis have provided an informative starting point that can be referred to as baseline studies for wild abalone gut microbiome investigations. While the present thesis provides novel insights into the gut microbial composition and diversity of NZ wild *H. iris* under environmental influences, there are several improvements that can be suggested and discussed to guide future abalone gut microbiome research.

### *6.2.1 Increase Field Sampling Resolution*

Chapter 4 of this thesis characterised the gut microbial composition and diversity of wild *H. iris* populations overtime in the Chatham Islands. While samples were collected at six time points (including the baseline survey conducted in Chapter 3) across two years (March 2020-April 2022), the sampling scheme was primarily based on elevated seawater temperature predictions and could have been increased

to reveal strong seasonal gut microbiota pattern(s), as it was illustrated in Gobet et al. (2018) where continuous abalone (*H. tuberculata*) sampling was done bimonthly. High-resolution sampling enables researchers to monitor temporal variations in the gut microbial communities potentially linked to various dietary and environmental different dietary inputs and environmental conditions. For instance, more frequent gut microbiome analysis from high-resolution sampling events could accumulate data for researchers to search potential patterns of core gut microbial ratios (e.g., *Vibrio*/*Psychrilyobacter*, *Mycoplasma*/*Vibrio*) that might be used as "bioindicators". Wild abalone often consume various seaweed that require specific gut bacteria to digest them, and the available seaweed options in abalone's natural habitats could be different from site to site and might not all be consumed by the gastropods. Therefore, the "bioindicators" could help predict what type of seaweed are digested in the gut of wild abalone to decipher where the nutrient sources might come from, which could be important when it is difficult to morphologically or genetically identify the digested seaweed species in the gut content. By the same token, the bacterial ratios could also indicate microbial infections or diseases might have affected wild abalone if certain pathogenic strains are unexpectedly more abundant.

### *6.2.2 Include More Environmental Samples*

Comparative studies on abalone gut and environmental microbial profiles in natural habitats are suggested to be routinely conducted in the future. In the experimental Chapter 3, microbial analysis on abalone's gut microbiota was compared to the microbial communities of seaweed and sediment in the abalone's natural habitats. The inclusion of seaweed and sediment samples in Chapter 3 demonstrated that the gut microbial composition and diversity of wild *H iris* in Cook Strait were significantly different from that of the seaweed and sediment samples. To verify this finding and obtain additional evidence of the microbiota comparisons between abalone's gut (as well as other regions in the digestive tract) and the surrounding environments, environmental samples such as seawater filtrates, seaweed, sediment, and boulders should also be frequently sampled and analysed. Similar to the experimental design of Chapter 3, the initial sampling plan for Chapter 4 also included seaweed sampling for microbial sequencing investigations, and the corresponding research aim was to compare the microbial profiles between abalone's gut and the seaweed samples. However, due to the unpredictable weather/oceanographic conditions and logistical difficulties (seaweed availability and accessibility), only limited seaweed samples that could not cover the entire seaweed species in the seaweed communities from each sampling site were collected. Furthermore, the seaweed quantity and extracted DNA quality recovered from the collected seaweed samples were degraded and did not pass the quality check for further downstream analyses. Consequently, we did not include any sequencing data from the collected seaweed samples. Adding such environmental microbial

information can help researchers unravel whether abalone acquire their gut microorganisms horizontally, which would also be helpful to prevent disease outbreaks.

### *6.2.3 Utilise Controlled Conditions for Targeted Rearing Experiments*

Chapters 4 and 5 of the thesis respectively showed that the seaweed type and oceanographic conditions overtime were two potential influencing factors on the gut microbiome of wild *H. iris* populations on Chatham Islands. In addition to the present thesis, the novel gut microbiome research conducted by Bullon et al. (2025) on a farmed *H. iris* stock also mentioned environmental factors, such as formulated feeds could significantly influence the gut microbial composition and diversity among cultivated abalone stocks. However, the digested seaweed type could only be categorized to the phylum level (i.e., Phaeophyta, Chlorophyta, Rhodophyta). One important reason for this limitation is that the seaweed compositions of the sampling sites were diverse and complex, and the unknown consumption and digestion times of the wild abalone resulted in differentiated seaweed degradation states (e.g., fresh, semi-digested, degraded but not fully digested) in the gut of the host, making it impossible to precisely identify and standardize the microscopic quantification of the gut seaweed content.

Similarly, specifically identifying what oceanographic parameters might have been correlated with the differentiated gut microbiome observed overtime on the Chatham Islands in Chapter 5 was not possible. Abalone are poikilotherms and naturally dwell in intertidal and subtidal marine environments; therefore, their metabolism, gut microbiome and other physiological performances are subjected to environmental changes (Guo, 2017; Wang et al., 2020). While previous gut microbiota surveys on other abalone and animal models indicated the alterations of gut microbial communities could be significantly related to elevated water temperatures (Brothers et al., 2018; Wang et al., 2020; Scanes et al., 2021b; Wu et al., 2022), such conclusion could not be drawn in the present thesis mainly due to the lack of direct seawater temperature measurements at each sampling event. Moreover, even if the seawater temperatures were changed in association with the abalone gut microbiota shifts, changes in other environmental parameters, such as salinity, pH level and/or dissolved oxygen concentration, might also influence the gut microbial composition and diversity at various degrees. Therefore, this thesis could only preliminarily reveal some environmental factors that could potentially influence the gut microbiota of wild *H. iris* populations due to the limited environmental factor explicitness.

To specifically test and evaluate how gut microbiomes of wild abalone could be influenced by seaweed and/or aquatic chemistry, it is recommended to design targeted feeding and rearing experiments under controlled conditions. Previous captive studies, including a gut microbiome survey on farmed *H. iris* by

Bullon et al. (2025), have demonstrated that aquaculture is an ideal testing ground for evaluating the dietary nutritional and environmental effects on abalone gut microbiome. One of the advantages of conducting feeding and rearing experiments in controlled environments, such as at aquaculture facilities, is that they can control certain genetic and environmental factors while evaluating the effects of targeted factors on the gut microbiome of wild abalone. Researchers can selectively breed their stocks to develop or remove specific genetic traits of interest. The host individuals reared in captivity were often fertilized from the same isogenic lines compared to the wild populations whose lineages and developmental stages are often unknown. By using genetically similar individuals in experiments, researchers can minimize the genetic variability within their study populations and attribute differences in response solely to the manipulated factor rather than genetic differences. Additionally, several studies have indicated that the physiological and ecological parameters, including the gut microbial profiles, of abalone and other mollusks were highly influenced by their ambient aqueous environments (Onitsuka et al., 2008; Auzoux-Bordenave et al., 2020; Scanes et al., 2021b; Ullah Khan et al., 2021; Li et al., 2021). The increasing frequency and intensity of climate change phenomena will add more stress and uncertainty to shellfish hosts and scientific researchers. Aquaculture facilities, however, often operate in controlled environments such as indoor tanks or ponds equipped with environmental control systems. These systems regulate the surrounding temperature, light, and water quality parameters to minimize the impact of external fluctuations on the designed experiments. Hence, aquaculture settings can reduce the confounding genetic and random environmental variables to ensure the robustness and reliability of the gut microbiome quantification results.

### *6.2.4 Utilisation of Faecal Matter as Gut Microbiome Approximation*

Faeces may be used as an effective sample source for monitoring the gut microbial profiles of abalone and sustainably evaluating the effects of multiple dietary and environmental factors of interests on the same individuals. Faeces are solid biowastes, or end products of food digestion, that are secreted through the anus of animals that typically possess a complete digestive system. Due to their proximity to the GI region of an animal's digestive tract, faeces theoretically share a lot of biochemical characteristics with the gut content and are therefore regarded as valuable sources for various gut microbiome studies. For instance, human faeces have been frequently used as key biomarkers and proxies to understand the microbial composition and functionality of the GI tract (Guan et al., 2021; Piancone et al., 2022). Moreover, in a microbial ecological study conducted on three lizard species, Kohl et al. (2017) demonstrated that the faecal microbiota was more comparable to the microbial communities sampled from the hindgut. Similarly, faecal samples collected from juvenile ostriches showed a comprehensive

bacterial composition that resembled the birds' cloacal/colon microflora, and the authors suggested using faecal samples to monitor the animals' microbial community in the hindgut (Videvall et al., 2017).

Compared to large animals such as birds, reptiles, and mammals, investigations on the microbiome resemblance between the faecal and gut samples are extremely limited in abalone and other mollusks, mainly because the molluscan faeces are relatively more difficult to collect and separate from ambient interfering factors such as seawater. Although faecal matters are directly secreted from the GI tract, their microbial communities will change over time as they are exposed to aqueous environments. As a result, the faecal microbiome may not reflect the true gut microbial profile of the host. In fact, Griffin et al. (2021) demonstrated that the faecal microbiome of the blue mussels (Mytilus edulis) was not similar to that of the intestinal samples collected from the same individuals. This might be due to sample degradation because the researchers allowed the faeces to reside in the seawater-filled experimental jars for three hours before the sample collection. Without examining the microbiome of the rearing seawater samples, the interaction between the faeces and the seawater may alter the faecal microbial profiles, leading to the conclusion that the faecal microbiome is not a valid proxy for the gut microbiome of this bivalve species. Therefore, collecting abalone faeces as instantly as possible is a key requirement to test the hypothesis that the microbiome composition and diversity of the faecal samples are not significantly different from those of the gut samples collected from the same individuals.

Since molluscan faecal samples are difficult to aseptically collect and maintain, and there are abundant mollusks in the wild and at aquaculture facilities, it is understandable that researchers can dissect mollusks and directly collect the gut content samples for the gut microbiome analysis. However, doing so poses three research limitations. First, animal dissection and gut sample collection procedures always require meticulous and aseptic techniques to reduce contamination, which are often time-consuming. Second, the feeding or challenging experiments can only evaluate the gut microbial responses to single factors because the animals need to be sacrificed for sample collections, leading researchers to find (and eventually sacrifice) a large number of host subjects to test the effects of multiple dietary or environmental factors. Lastly, the potential genetic effects, such as some intra-specific characteristics, among the testing subjects can be difficult to control, as previously discussed, if the research aim is to investigate the multifactorial effects on the gut microbiomes. Hence, it will be helpful to utilize molluscan faecal samples as a non-lethal method to monitor the hosts' gut microbial responses to dietary and/or environmental stimuli of interest in future gut microbiome studies. Doing so requires testing the hypothesis that the faecal microbial composition and diversity are not significantly different from those of the gut microbiome of the same abalone individual under the same experimental conditions.

There are several key steps to follow along the faecal microbiome resemblance hypothesis testing route and toward the execution of replacing the gut content samples with faecal samples in the molluscan gut microbiome research field. First, the defecation pattern of abalone needs to be fully understood. It is essential to know when abalone defecate during a day or a week and how much faeces the subjects can produce each time. Understanding the defecation pattern will help researchers collect the faecal samples on time to reduce the potential for faecal degradation and seawater interference and ultimately ensure the accuracy and precision of the microbiome resemblance. Second, as a pilot study at the hypothesis testing stage, the animals do need to be sacrificed for gut content sample collections, unfortunately. This will be direct method validation, which is a necessary step in the pipeline. Last but not least, any environmental samples that are associated with a specific experiment also need to be collected for microbiome analysis. For example, the rearing seawater must be filtered, and the seawater filtrate samples must be included in the downstream molecular assays as a control or comparison to the faecal samples. Similarly, if a feeding experiment is conducted or some types of substrates are added to the rearing containers, the relevant diet and substrates must be aseptically processed as control measures. In summary, the faecal microbiome may be utilized as an alternative to approximate the gut microbiome of abalone, but this approach needs to be carefully designed and thoroughly validated prior to wide applications. The replacement of the GI microbiome with the corresponding faecal microbiomes of the same abalone individuals will advance the evaluations of the effects of multiple dietary and/or environmental factors on the same hosts while controlling for the random genetic effects. This will also be a sustainable method of moving forward as it does not require animal sacrifice anymore.

### *6.2.5 Comprehensive Gut Microbiome Research via More Advanced Sequencing Technologies*

Future abalone gut microbiome research activities will benefit from using more advanced sequencing technologies so that the characterization of abalone gut microbiomes can be both taxonomically and functionally. The Illumina MiSeq amplicon-sequencing platform was utilized throughout all the experimental chapters. While this sequencing technology provides high-throughput sequencing data under a relatively cheaper unit price, and the data processing pipeline is free, mature, and widely accessible, its fundamental principle of single-genetic-marker-based amplicon laboratory and bioinformatic workflow can only generate microbial monogenetic abundance data with short sequences (~450-550 bp) with limited microbial functional information (Wen et al., 2017; Chen et al., 2023). Although certain bioinformatic pipelines exist for converting gut microbiome sequencing data, targeting prokaryotic genetic markers (e.g., 16S ribosomal RNA), into microbial functional data to predict microbial functions

(Langille et al. 2013; Lu et al., 2023), such microbial functional predictions rely on single genetic amplicon data, that might be incomplete (Kaiser et al., 2016; Du et al., 2019).

Shotgun metagenomic sequencing systems are one of the promoted sequencing techniques that can be used for future abalone gut microbiome research when financially and bioinformatically permitted. There are several advantages to utilizing this sequencing system as a technological improvement. First, shotgun metagenomic sequencing relies on multiple genetic markers to recover entire microbial genomes. Given such wide genetic ranges, this sequencing technology can taxonomically identify microorganisms to species and strain levels more confidently (Maki et al., 2021). Second, shotgun metagenomic sequencing is a more appropriate tool for microbial functional profiling, as it sequences entire genetic material of the microbiome. Researchers can infer various and specific metabolic pathways and functions of the gut microbial communities to better understand key microbial functional roles, such as nutrient digestion and antimicrobial responses inside abalone gut (Kim et al., 2011; Sim et al., 2012). Furthermore, there are multiple developed bioinformatic pipelines (e.g., MetaWARP and Sunbeam) for processing shotgun metagenomic data that fit researchers with various bioinformatic skill levels (Uritskiy et al., 2018; Clarke et al., 2019). Therefore, under the evolving and extensive laboratory and bioinformatic supports, advanced sequencing technologies like the shotgun metagenomics can substantially contribute to abalone gut microbiome research in the future by providing efficient microbial taxonomic and functional data simultaneously.

### 6.3 Overall Thesis Conclusions

In conclusion, this thesis makes several significant contributions to the emerging literature on abalone gut microbiomes. **First**, this is the first series of gut microbiome investigations on wild NZ *H. iris,* filling a critical geographic and species-specific gap in abalone microbiome research. Prior studies have largely focused on aquaculture populations or other abalone species, leaving wild *H. iris* largely unexamined. By documenting microbial diversity across multiple digestive regions, sites, and temporal scales, this work establishes a much-needed reference baseline for future ecological, aquaculture, and conservation studies. **Second**, it adds new discoveries of abalone gut microbiome to the existing knowledge. While many marine herbivores, including abalone, are often assumed to have distinct microbial functions segregated across different gut compartments, this thesis provides the novel observation of high similarity between foregut and hindgut microbiota in wild *H. iris*. This unexpected result suggests that microbial contributions to digestion may be more spatially integrated than previously thought, raising new questions about digestive physiology and microbial roles in abalone. **Third**, the study advances the literature by demonstrating that microbial community structures vary not only across sites in the Chatham Islands but

also across months, which underscores the influence of seaweed type and availability and fluctuating oceanographic conditions on gut ecology. This contribution moves the field beyond static characterizations of abalone gut microbiota toward a more dynamic, environmentally responsive framework. **Fourth**, the research positions the abalone gut microbiome as a critical interface between host physiology and environmental change. By linking microbial community composition to dietary and environmental factors, the thesis strengthens the conceptual understanding that gut microbiota act as both indicators and mediators of host performance. **Finally**, this thesis provides a roadmap for future abalone gut microbiome research by clearly suggesting methodological and experimental improvements and proposing targeted, controlled studies to test diet and aquatic environmental parameter effects. By acknowledging these gaps while providing practical next steps, this work helps shape the methodological evolution of abalone microbiome studies. From a conservation perspective, findings of this thesis establish gut microbiota references and illustrate gut microbial community shifts which can serve as early indicators of ecological stress and prevent microbial infections and diseases, helping safeguard this culturally and economically valuable species. Overall, the present thesis demonstrated that the gut microbiome of NZ wild *H. iris* is dynamic, could potentially be influenced by diets and environmental conditions and linked to abalone physiology, positioning the gut microbiome not only as an ecological marker of abalone growth and health, but also as a pathway toward more adaptive and sustainable stewardship of *H. iris* populations in NZ.

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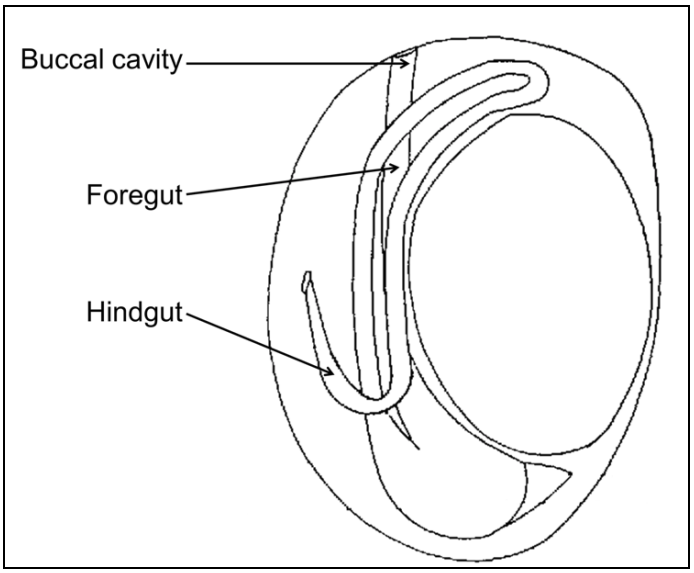
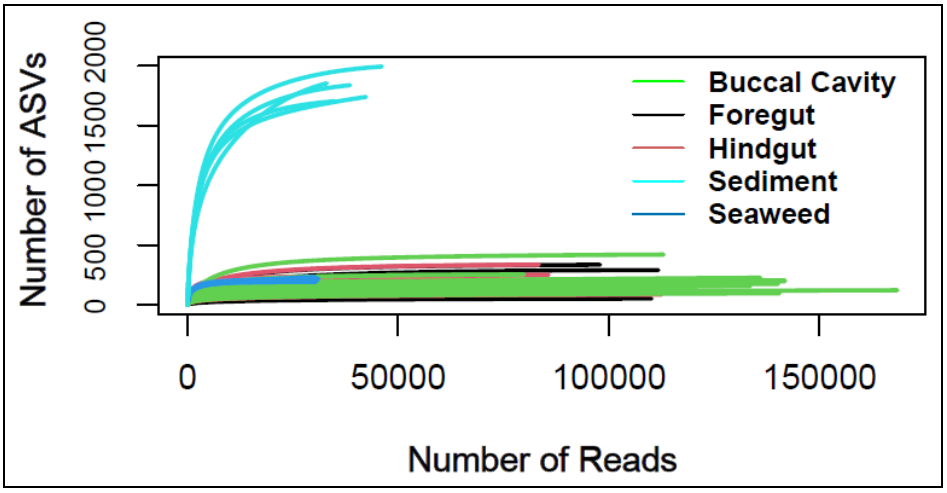
# Appendices

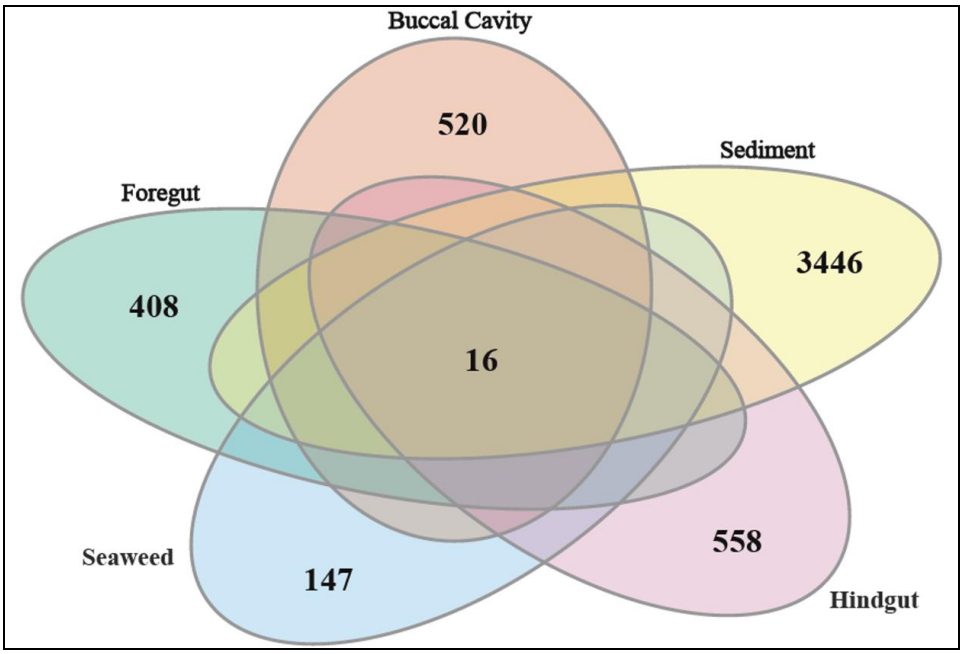
## Supplementary Materials for Chapter 3:




**Supplementary Figure 3.1: Schematic illustration of abalone's digestive tract and where the samples were collected at each digestive region.**




**Supplementary Figure 3.2: Rarefaction curves showing the number of observed ASVs per sequenced samples collected from the buccal cavity (n=20), foregut (n=20), and hindgut (n=20) samples of wild *Haliotis iris*, seaweed (n=5), and sediment (n=5) samples.**




**Supplementary Figure 3.3: Venn diagram showing the number of shared and unique 16S rRNA ASVs among the five sample groups (buccal cavity, foregut, hindgut, seaweed, and sediment) collected from Cook Strait in May 2021.**

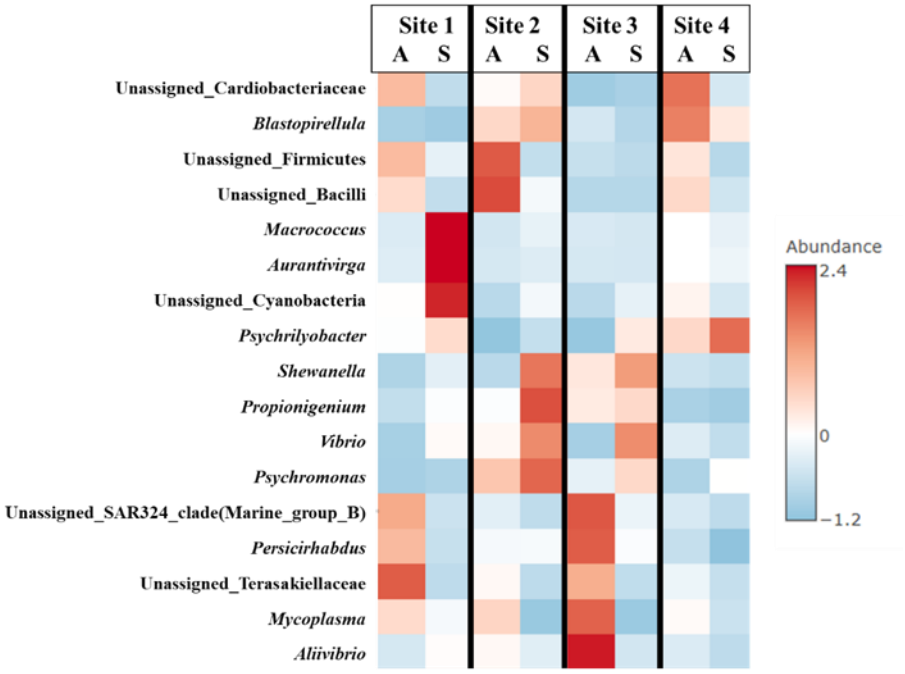
## Supplementary Materials for Chapter 4:

**Supplementary Table 4.1: Abalone weight, shell length, width, height, and soft tissue measures as an average (± SE) of 10 adult and sub-adult abalone collected from Ascots, Wharekauri, Owenga Harbour and Point Durham.**

| Measures | Life stage | Collection site | | | |
|---|---|---|---|---|---|
| | | Ascots | Wharekauri | Owenga Harbour | Point Durham |
| | Growth type | Fast-growing | | Slow-growing | |
| Weight (g) | Sub-adult | 77.4 ± 6.4 | 118.0 ± 8.9 | 91.5 ± 4.4 | 121.0 ± 13.1 |
| | Adult | 440.4 ± 13.1 | 526.1 ± 19.6 | 288.2 ± 7.9 | 460.8 ± 11.2 |
| Shell Length (mm) | Sub-adult | 79.9 ± 2.0 | 92.2 ± 2.2 | 84.6 ± 1.0 | 90.3 ± 2.0 |
| | Adult | 131.3 ± 1.4 | 143.0 ± 1.7 | 116.8 ± 1.2 | 131.2 ± 1.9 |
| Shell Width (mm) | Sub-adult | 61.0 ± 1.3 | 68.5 ± 1.2 | 60.0 ± 1.4 | 64.6 ± 1.9 |
| | Adult | 99.4 ± 1.3 | 106.6 ± 2.4 | 88.7 ± 1.9 | 97.1 ± 1.9 |
| Shell Height (mm) | Sub-adult | 23.1 ± 0.7 | 23.2 ± 0.5 | 20.6 ± 0.8 | 25.7 ± 1.5 |
| | Adult | 46.1 ± 0.8 | 39.6 ± 1.1 | 37.4 ± 1.2 | 44.8 ± 0.9 |
| Adductor muscle ($mm^2$) | Sub-adult | 5.8 ± 0.3 | 9.2 ± 0.5 | 7.5 ± 0.4 | 8.5 ± 0.6 |
| | Adult | 24.2 ± 1.1 | 27.6 ± 1.4 | 17.3 ± 0.5 | 25.8 ± 1.2 |
| Foot muscle ($mm^2$) | Sub-adult | 22.0 ± 1.3 | 29.2 ± 1.2 | 24.1 ± 1.1 | 25.0 ± 1.9 |
| | Adult | 77.7 ± 2.6 | 86.3 ± 3.9 | 51.6 ± 1.9 | 74.0 ± 2.6 |
| Gonad ($mm^2$) | Sub-adult | 7.7 ± 0.5 | 12.0 ± 1.2 | 8.5 ± 0.6 | 10.8 ± 0.9 |
| | Adult | 37.2 ± 2.2 | 40.9 ± 2.1 | 25.4 ± 1.2 | 31.3 ± 0.8 |

**Supplementary Table 4.2: DADA2 data processing summary of 16S rRNA reads and amplicon sequence variants (ASVs) generated from the gut samples collected from all wild *Haliotis iris* at four study sites (Ascots Beach, Wharekauri, Owenga Harbour, and Point Durham) on the Chatham Islandss in March 2020.**

| Site | Sample Type | Sample Size | Input Reads | Merged Reads | Chimeras-removed Reads | Number of ASVs |
|---|---|---|---|---|---|---|
| All Sites | All Samples | 80 | 11,148,409 | 5,446,756 | 5,416,849 | 823 |
| | Adults | 40 | 5,614,010 | 2,746,934 | 2,726,143 | 545 |
| | Sub-adults | 40 | 5,534,399 | 2,699,822 | 2,690,706 | 491 |
| Site 1 | Adults | 10 | 1,332,091 | 716,033 | 713,528 | 225 |
| | Sub-adults | 10 | 1,317,124 | 658,041 | 655,333 | 230 |
| Site 2 | Adults | 10 | 1,319,392 | 629,678 | 627,089 | 184 |
| | Sub-adults | 10 | 1,205,307 | 570,337 | 568,151 | 221 |
| Site 3 | Adults | 10 | 1,538,407 | 730,122 | 736,237 | 232 |
| | Sub-adults | 10 | 1,593,306 | 780,536 | 778,773 | 224 |
| Site 4 | Adults | 10 | 1,424,120 | 671,101 | 649,289 | 275 |
| | Sub-adults | 10 | 1,418,662 | 690,908 | 688,449 | 156 |




**Supplementary Figure 4.1: Clustering heatmap showing the read abundance of major microbial genera observed in all abalone gut samples between the sub-adults (S) and adults (A) across the four study sites (Site 1: Ascots Beach; Site 2: Wharekauri; Site 3: Owenga Harbour; Site 4: Point Durham).**