PhD Thesis

Development and Optimisation of a Sustainable Feed Formulation for the New Zealand Farmed Abalone (*Haliotis iris*)

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ABSTRACT

The expansion of aquaculture depends on the availability and sustainability of aquafeeds. The development of aquafeeds is hampered by the lack of more eco-friendly ingredients that promote animal growth and contribute to animal welfare simultaneously. The common ingredients of aquafeeds (e.g., fishmeal and plant meals) are highly nutritious due to a well-balanced amino acid profile, fatty acid, and micronutrients. However, these ingredients are considered unsustainable due to the utilisation of wild fish stocks and extensive land and energy use for their cultivation. Alternative ingredients such as insect meals and waste-by products are of interest due to their high nutritional profile and contribution to a more circular economy. *Tenebrio molitor* (mealworm) is one species of insect with high digestibility and high levels of polyunsaturated fatty acids. Grape marc is a waste-by product from the wine production industry which disposal is becoming problematic. Grape marc is rich in antioxidants and is a potential prebiotic source. The aim of this thesis is to evaluate the inclusion of two alternative ingredients, insect meal and grape marc, on somatic growth, nutritional profile, health, shell colour and flavour of the New Zealand farmed abalone (*Haliotis iris*).

Initially, the nutritional profile and metabolomic variations were assessed in a farm in Northland (Ruakaka) over a year (Chapter 3). Data collected from this chapter showed that the nutritional profile of juvenile abalone is subject to age and seasonal variations although feed remained the same. The protein levels were the most affected during the year of grow-out whereas carbohydrate, ash, and moisture did not show extreme variations. The metabolite profiles corroborated the presence of some amino acids related to flavour and tenderness, and fatty acids related to temperature regulation showing higher levels in summer compared to winter months. The study of the nutritional profile of the commercial feed allowed us to develop four experimental diets which included fishmeal at some extent and included insect meal and grape marc to reduce the nitrogen and phosphorus load in the uneaten feed while improving physical and chemical characteristics (Chapter 4). The experimental diets were encapsulated in alginate beads to promote better seawater stability. The physical and chemical properties of encapsulated feeds revealed improved seawater stability compared to commercial feed and advantages in their frozen delivery form in terms of the texture profile. Encapsulated diets with grape marc inclusion resulted in an improved resistance to abrasion, and the encapsulation with the inclusion of both, insect meal and grape marc, significantly reduced the phosphorus and nitrogen waste in uneaten feed. In

addition, the inclusion of insect meal and grape marc showed growth promotion and comparable feed intake to commercial feed in 14-days.

After acceptable feed intake of the experimental diets in a 14-day feeding trial, the encapsulated diets were trialled in an abalone farm in Southland (Bluff, Invercargill) over 165 days. The nutritional profile and nutrient digestibility were assessed on the experimental diets, and growth parameters, proximate composition, amino acids, and fatty acids were assessed in juvenile abalone tissue (Chapter 5). Results showed that the inclusion of insect meal and grape marc did not affect the growth of abalone after 165 days compared to diets without the inclusion. However, the inclusion of grape marc reduced the digestibility of the diets significantly, while insect meal did not. The fatty acid profile of abalone was significantly affected mainly in the α-linolenic acid, arachidonic acid, and eicosapentaenoic acid content, while the amino acid profile was not significantly modified by the inclusion of insect meal nor grape marc.

The flavour-volatile compounds and shell coloration were also assessed after six months of feeding (Chapter 6). The results showed that the inclusion of insect meal did not significantly affect the flavour volatile profile, whereas the inclusion of grape marc significantly reduced the production of most of the volatile compounds. This reduction indicated an antioxidant effect of grape marc in the meat of abalone, suggesting a reduced oxidation of lipids. In addition, the inclusion of both insect meal and grape marc affected did not affect the lightness, redness/greenness, and yellowness/blueness coloration of the shells.

The study also evaluated the effect of insect meal and grape marc inclusion on the gut health and muscle metabolome of abalone (Chapter 7). The results showed that the inclusion of insect meal and grape marc did not significantly affect the gut microbial diversity nor the intestinal morphology, indicating a capacity of abalone to adapt to those ingredients maintaining a good intestinal condition. In addition, the relative abundance of gut bacteria fluctuated with the dietary inclusion of insect meal and grape marc, suggesting a possible increase of beneficial species. This study also corroborated the relevant role of fatty acids in abalone metabolism and growth as described in Chapter 5.

In conclusion, this thesis has successfully performed a holistic evaluation of the effects of insect meal and grape marc inclusion in feeds for abalone aquaculture. The different angles of animal nutrition described in this thesis go beyond traditional growth evaluation, which has been considered as the gold standard. The insights from this thesis

contribute to the development of more sustainable aquafeeds to be used in the abalone industry to promote growth and animal welfare, improve the quality of the meat, reduce environmental impact, has adequate physical properties for commercialization and represents a steppingstone for the development of a blue aquaculture in New Zealand.

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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CO-AUTHORED WORKS

Chapter	Authors	Contribution	Total	Signature
1. General introduction	Natalia Bullon	-Concept & structure -Writing	90	
	Andrea C. Alfaro	-Review/edit	8	
	Ali Seyfoddin	-Review/edit	2	
2. The role of aquafeeds in abalone nutrition and health: a comprehensive review	Natalia Bullon	-Concept & structure -Writing	90	
	Andrea C. Alfaro	-Review/edit	7	
	Ali Seyfoddin	-Review/edit	3	
3. Seasonal, nutritional and metabolomic changes of juvenile farmed abalone (*Haliotis Iris*) in New Zealand	Natalia Bullon	-Experimental design -Sample analysis -Data processing and analysis -Writing	85	
	Andrea C. Alfaro	-Review/edit	8	
	Ali Seyfoddin	-Review/edit	5	
	Sara Masoomi	-Sample analysis	1	
	Tim Young	- Data processing and analysis	1	
4. Sustainable aquafeed formulations containing insect larval meal and grape marc for the New Zealand farmed abalone	Natalia Bullon	-Experimental design -Sample analysis -Data processing and analysis -Writing	90	
	Andrea C. Alfaro	-Experimental design -Review/edit	7	
	Ali Seyfoddin	-Experimental design -Review/edit	2	
	Sara Masoomi	-Sample analysis	1	

	Moganakumaar Manivannan	-Sample collection	1
5. Effect of insect meal and grape marc in the nutritional profile, growth and digestibility of juvenile New Zealand farmed abalone	Natalia Bullon	-Experimental design -Sample analysis -Data processing and analysis -Writing	86
	Andrea C. Alfaro	-Experimental design -Review/edit	7
	Ali Seyfoddin	-Experimental design -Review/edit	4
	Nazimah Hamid	-Review/edit	2
	Moganakumaar Manivannan	-Sample collection	1
6. Effect of dietary insect meal and grape marc inclusion on flavour volatile compounds and shell colour of juvenile abalone *Haliotis iris*	Natalia Bullon	-Experimental design -Sample analysis -Data processing and analysis -Writing	90
	Andrea C. Alfaro	-Experimental design -Review/edit	5
	Ali Seyfoddin	-Experimental design -Review/edit	2
	Nazimah Hamid	-Review/edit	2
	Sara Masoomi	-Sample analysis	1
7. Expanding the menu for the New Zealand farmed abalone: dietary inclusion of insect meal and grape marc (effects on gastrointestinal microbiome, digestive morphology and muscle metabolome)	Natalia Bullon	-Experimental design -Sample analysis -Data processing and analysis -Writing	86
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Chapter

1 Introduction and general thesis framework

1.1 INTRODUCTION

1.1.1 Molluscan aquaculture in the world

The aquaculture sector continues to dominate the aquatic food production chain globally with 87.5 million tonnes (Mt) of aquatic animals and 35.1 Mt of algae for human consumption and industrial utilization. The whole sector increased their production from 9.9 kg in 1960's to 20.2 kg per capita in 2020 (FAO, 2022). This was the highest recorded increase in history, due to a spike in demand for high quality protein. Currently, molluscan aquaculture produces 17.8 Mt (mostly bivalves), which surpasses marine capture by three times (FAO, 2022). Molluscan aquaculture represents almost 14.5% of the total aquaculture sector. Out of the 88 molluscan species recognized for farming, the most commercialised are oysters, scallops, and mussels. Geographically, these species are mostly cultivated in Asian countries followed by the Americas and Europe. Most of the mollusc production comes from marine and costal aquaculture with almost 80% compared to inland aquaculture with less than 10%. China is by far the largest producer of molluscan species with 15 Mt, followed by the Republic of Korea with 415 thousand tonnes, and Chile with 406 thousand tonnes. Other main molluscan producers include Japan, Vietnam, Spain, Thailand, USA, France, and New Zealand. China produces 15 Mt. of all molluscs compared to the rest of the world, which produces only 3 Mt.

1.1.2 Molluscan aquaculture in New Zealand

Aquaculture is a relatively small, but economically important in New Zealand as it provides protein for human consumption, rural employments, and business opportunities. The industry has grown relatively fast in the last 40 years with annual sales of around NZD 650 million (Ministry for Primary Industries, 2022). The mostly recognized species are the New Zealand GreenShell™ mussels (*Perna canaliculus*), Pacific oysters (*Crassostrea gigas*), and King/Chinook salmon (*Oncorhynchus tshawytscha*). Other cultivated species with growing production includes abalone (*Haliotis iris*), and flat oysters (*Ostrea chilensis*). Potential candidates for a growing industry in New Zealand include geoduck (*Panopea zelandica),* yellowtail kingfish (*Seriola lalandi lalandi*), gropers (*Polyprion oxygeneios*), and eels (*Anguilla australis*, *Anguilla dieffenbachia*) (NIWA, 2006).

A total of 86.9% of the aquaculture production in New Zealand is attributed to marine molluscan production (FAO, 2022). For this reason, the New Zealand government seeks to develop this industry towards NZD 3 billion in annual sales by 2035 (New Zealand

Government, 2012). The New Zealand growth pathway does not only include revenues in sales and exports, but also generating a more financial sustainable operation, inclined to provide more regional benefits, capable of adapting to the current of climate change scenarios, and which brings jobs and social inclusion (Ministry for Primary Industries, 2022).

1.1.3 Abalone (*Haliotis iris*) and abalone aquaculture in New Zealand

Abalone are a widespread group of gastropods, from the genus *Haliotis*, inhabiting both tropical and temperate waters around the world. Approximately 90 species of abalone are found worldwide, and about 15 species are cultured in Australia, China, Japan, Korea, New Zealand, Philippines, South Africa, and Taiwan (Sales & Janssens, 2004). Although abalone production is relatively small, it is one of the most highly prized delicacies globally, especially in Asia (Cook, 2014).

In New Zealand, the three more important species of abalone are *Haliotis iris*, *Haliotis australis*, and *Haliotis virginea*. All of them are called Pāua, the Māori indigenous term. *Haliotis iris* is known as black-foot pāua, *Haliotis australis* as yellow-foot pāua. The most commercial species is *Haliotis iris* given that it is bigger compared to *Haliotis australis* and easily found in the wild. Abalone are recognised for having a very sedentary life, moving when required specially at night when drifting seaweed is limited or clamping together to avoid predation. They reach maturity at a length of 93–133 mm in the wild, and some can grow up to 150–180mm (Paua Industry Council LTD, 2022). Pāua production in New Zealand mostly comes from wild fisheries from the 11 areas regulated by the Ministry for Primary Industries (Ministry for Primary Industries, 2021). Pāua are caught based on their population size, health of stock and fishing pressure and the region of the Chatham Island has one with the highest reported catch reaching 209 098 Kg in 2022 (Fisheries New Zealand, 2023). Pāua aquaculture used to be circumscribed to 12 farms along New Zealand. However, there are currently two major farms in operation.

1.1.4 Fishmeal and alternative ingredients in aquaculture feeds

According to FAO (2020), almost 10% of the global fish production is processed into fishmeal (FM) and fish oil (FO), of which 69% of fishmeal and 75% of fish oil production are used to feed farmed fish. From the global fish production, 88% is destined for human consumption and 12% for non-food purposes. Of the latter, 80% is reduced to fishmeal and fish oil, which is mainly destined for feed in aquaculture and livestock (FAO, 2020). Fishmeal use in aquafeeds has substantially increased since 1960 (Jannathulla et al., 2019) and the

aquaculture sector is responsible for the use of almost 75% of global fishmeal production compared to the pig and chicken feed sector, which represent 25% together (Shepherd & Jackson, 2013). Within the aquaculture sector, fishmeal is the main ingredient of compounded feeds for carnivorous and herbivorous species. However, carnivorous species mainly dominate fishmeal consumption. There are three main consumers of fishmeal: crustaceans (29%), salmonids (24%) and marine finfishes (23%).

Fishmeal was originally included in the 1960's in feeds for pigs and poultry at 50% and 48% respectively. In the early stages of aquaculture (in the 1980s), fishmeal was allocated by 10% in this sector. Due to the increase in global fish consumption, fishmeal inclusion in aquafeeds raised to 73% in 2010. However, its use its expected to decrease by 2030 due to the increasing market price of small pelagic forage fish and increasing demand of forage fish for human consumption (Tacon & Metian, 2008).

The reason behind the use of fishmeal in aquaculture is the excellent palatability, high nutritional profile rich on polyunsaturated fatty acids and excellent amino acid profile. Due to these reasons, fishmeal has been used as a main protein component in the manufacture of aquaculture feeds in the last 30 years. Fishmeal is usually made up from processing species of fish not used for direct consumption, fish trimmings or from the by-products of seafood processing. Pelagic species mostly used for fishmeal production are anchovies, mackerel, and sardines. Wild-caught pelagic species accounted for 78% and trimmings (heads and backbones) for 22% of fishmeal production in 2018. In other words, wild pelagic species are caught for the purpose of fishmeal production making this industry a non-edible millionaire commodity producing 5.8 million tonnes in 2018, the highest in the last 10 years (European Comission, 2021).

Due to the elevated price of fishmeal, alternative ingredients have been tested and successfully included in aquatic feeds to replace fishmeal. Plant proteins, such as soymeal, cornmeal, barley, canola (Akiyama, 1990; Gatlin III et al., 2007), shrimp meal (Cho, 2010), terrestrial animal by-products, such as fermented feather meal, blood meal, poultry by-products, bone meal (Luthada-Raswiswi et al., 2021), insect meals (Belforti et al., 2015; Belghit et al., 2018; Ferrer et al., 2019), and single-cell proteins (Glencross et al., 2020), such as microalgae (Liu et al., 2022) and autolysed yeast biomass (Rimoldi et al., 2020) have been included with promising results. The main limitations of the inclusion of plant proteins are the presence of antinutritional factors (ANF), such as trypsin inhibitors, lectins, phytic acid, rachitogenic factors, and saponins (Yasothai, 2016) which can affect the intestinal health of animals. Similarly, the main limitation of the inclusion of insect meals is

the limited regulation on its use for aquaculture and agriculture purposes and the presence of antinutrients, such as oxalates, tannins, alkaloids, phytates, and saponins (Ojha et al., 2021).

1.1.5 Formulated feeds for abalone aquaculture

Formulated feeds in aquatic animal nutrition offer convenience in terms of a replicable nutritional provision during a long period of time at a cost that can be maintained. In a sense, a commercial diet must outperform natural diets, not only in growth rate, but in cost and quality aspects, guaranteeing less leaching of nutrients, and ease of access and storage (Haubjerg et al., 2015). In addition, it must be attractive to the species and be optimum in terms of particle size and feeding methods (Kirkendale et al., 2010).

The delivery of feeds for abalone animals is mostly provided in pellets or flakes in different sizes, depending on the age of the animals (Lebata-Ramos et al., 2021) and the grow-out system (Kirkendale et al., 2010). The extrusion process, to produce pellets, is a high-temperature, short time process where the mixture of ingredients is moistened and heated by the addition of steam and water while pressed (Bogevik et al., 2021). The flake form, compared to pellets, are easy to float and less nutritionally dense (Lebata-Ramos et al., 2021). In abalone, research literature shows the existence of formulated feeds mostly in the form of pellets, such as 3 mm long pellets (Bautista-Teruel & Millamena, 1999), 4 x 3 x 2 mm flat pellets (Bansemer et al., 2016), 5 x 5 x 2 mm flat pellets (Bates et al., 2017; Hoang et al., 2017), 1 cm^2 flakes (Ansary et al., 2019; Cho, 2010; Mai et al., 1995), pellets (Currie et al., 2019; Masoomi et al., 2021), powder (Ferreira et al., 2015), crumble, and chips (Kirkendale et al., 2010).

1.1.6 Abalone feed, feed supplementation and feed optimisation

The feed choice in abalone aquaculture settings depends on the country, mainly due to the seaweed available for feeding and its regulations. Formulated feeds are traditionally a compounded formula including carbohydrates, protein, and lipids as main macronutrients. Vitamins and minerals are included as micronutrients and their exact requirements in abalone are still not well-defined. Approximations on micronutrient requirements for abalone have been mainly based on the vitamin and mineral requirements for other species (Fleming et al., 1996), and sometimes resembling the micronutrients available in seaweeds, which form the natural diet of abalone (Bansemer et al., 2016).

1.1.6.1 Feed supplements

The list of feed supplements is extensive and their inclusion in feeds will depend on their cost and the ease of use in the final formulation. Vitamins and minerals can be considered feed supplements when they are given in excess to the nutritional requirements. As the micronutrient requirements for abalone is not well-understood, supplementation with vitamins and minerals needs further investigation. Water soluble vitamins, such as vitamin B and C have demonstrated to be important in the immune response of abalone. For instance, the addition of vitamin C at a dose of 9 g per Kg of diet has improved the antioxidant activity in *Haliotis discus hannai* but has not been shown to affect growth (Li et al., 2020). The deficiency of Vitamin B_1 or thiamine in formulated diets have reduced the growth of *Haliotis discus hannai* without causing changes in the nutritional content of protein, carbohydrate and lipid (Zhu et al., 2002). The deficiency of vitamin B_6 or pyridoxine has not produced a significant change in the shell growth of *Haliotis discus hannai*. However, the inclusion of pyridoxine at 800 mg per kg diet in formulated feeds has promoted significant more activity of cellular immune parameters, such as phagocytic activity and respiratory burst (Chen et al., 2005). Fat-soluble vitamins, such as vitamin A, D, E, and K have also demonstrated to be critical in abalone growth and health. Vitamin A and D interaction seems to have a role on abalone growth. Excessive vitamin A (1×10^6 IU/kg) antagonized actions of vitamin D and decreased the soft body calcium content and the shell growth. However, lower doses of vitamin A of 1×10^3 IU/kg along with doses of vitamin D of 5×10^3 IU/kg increased specific growth rate (SGR) (Zhang et al., 2007). Vitamin E at a dose of 50 and 5000 mg per kg of diet have positively affected the polyunsaturated fatty acids available in the body of *Haliotis discus hannai*. The supplementation of vitamin E is associated with the prevention of oxidation of lipids and therefore when vitamin E is present, essential fatty acids such as linoleic acid (C18:2n-6), α-linolenic acid (C18:3n-3), and arachidonic acid (C20:4n-6) were found to be higher in *Haliotis discus hannai* after 120 and 240 days of feeding (Fu et al., 2007).

Minerals are added to the feed formulation as they are highly present in seaweeds. Minerals, such as calcium (Ca), potassium (K), zinc (Zn), iron (Fe), cooper (Cu), and magnesium (Mg) are important for abalone (Latuihamallo et al., 2019). However, dietary doses for abalone have not been clearly determined either. Only one study by Tan and Mai (2001) has determined that *Haliotis discus hannai* need at least 16–18 mg/kg dietary zinc from ZnMet or 32–35 mg/kg from ZnSO4 to not show deficient growth or reduced activity of alkaline phosphatase, an enzyme dependent on zinc for activation. Surprisingly, the zinc

requirements for abalone were found to be higher than those reported from channel catfish, rainbow trout, carp, tilapia, and red drum (Tan & Mai, 2001). Abalone do not seem to need dietary calcium, as this mineral is absorbed from the surrounding waters (Tan et al., 2001). On the contrary, dietary phosphorus seem to play an important role in growth and doses of 0.5% have promoted growth, while higher doses of 1.7–2.2% have reduced the content of zinc and manganese and growth of abalone after 16 weeks (Tan et al., 2001).

Another category of feed supplements are nutraceuticals, which are particles that help to mitigate stress by improving the immune response of animals. Nutraceuticals derive from two words *i.e.*, nutrition and pharmaceutical and the term was coined by Stephen L. DeFelice in 1989 (Wildman et al., 2016). Nutraceuticals refer to dietary supplements that deliver a concentrated form of a bioactive compound from a food delivered in a non-food matrix in a dose that can exceed health enhancement compared to a normal food (Espín et al., 2007). Examples of nutraceuticals are oligosaccharides, organic acids, such as acetic acid and formic acid, synthetic amino acids, such as methionine, lysine; and bioactives, such as probiotics, prebiotics, and postbiotics.

The aggregation of organic acids in abalone aquaculture is limited, and to our knowledge, only Goosen et al. (2011) have shown the positive effects of formic acid and acetic acid on the growth rate of *Haliotis midae*. In that study, abalone grew 15–17% more compared to a control diet that did not include organic acids. An important hint is that growth promotion took place while water temperature was maintained (12–20 °C), otherwise when animals were exposed to high temperature stress of 20.5 °C, growth was not observed (Goosen et al., 2011).

1.1.6.2 Feed optimisation

One of the main problems in aquaculture feed is the waste generated after the feeding (Cho & Bureau, 2001). Uneaten feed residues are considered the biggest cost proportion in aquaculture settings (Cho et al., 1994). Uneaten commercial feeds normally disintegrate quickly, and such waste is collected when tanks are cleaned. Solid waste from aquaculture feed can be reduced through diet formulation (Glencross et al., 2007) and therefore, optimisation can be achieved by improving the ease of collecting solid waste after feeding and reducing the leaching of phosphorus and nitrogen, which have significant eutrophication effects on ecosystems.

One potential area of optimisation in aquaculture feeding is to produce feeds with the right digestibility, palatability, and attractiveness with an increased water stability, which reduces disintegration into small particles and cleaning frequency. For instance, in abalone, feeding usually occurs every 48 hours. Longer periods of feeding can affect the levels of ammonia, nitrate, and nitrite. Excessive nitrogen in the water ponds impacts the growth of algae, microalgae, and bacteria which finally increase the oxygen demand (O_2) and affects the conditions of the abalone farm. A reduction of solid wastes by optimising the feed formulation may allow less nitrogen waste reducing the environmental effects on waste outputs. Another area of optimisation in aquaculture feeding is to increase feed digestibility to reduce faecal density (Glencross et al., 2007). However, the risks of using highly digestible diets are the exclusion of fibres and non-digestible starches that are associated with a healthy digestive function. Fibres, such as non-starch polysaccharides (NSPs) from plants, and non-digestible materials, such as cellulose and hydrolysed lignin are prebiotics that have beneficial effects on gut health. Hydrolysed lignin increased lactic acid bacteria in Atlantic salmon (Colombo et al., 2020) and cellulose supplementation improved the diversity and richness of beneficial bacterial in the gut of yellow catfish *Pelteobagrus fulvidraco* (Zhang et al., 2023). If the generation of highly digestible diets is the mechanism to reduce faecal waste, detailed studies considering gastrointestinal health and solid waste impact should be considered.

1.1.7 Blue revolution, blue aquaculture, blue economy, and aquaculture

Global ocean activities are estimated to be US 1.5 trillion per annum with faster growth than terrestrial activities (Cervigni & Scandizzo, 2017). This growth has caused a concern in different regulating bodies to monitor a sustainable growth to ensure equity for future generations. The ‘blue revolution’ concept appeared to ensure the sustainability of aquaculture activities which contribute to human nutrition. The blue revolution is meant to manage a role of policies and initiatives to promote more sustainable practices of the oceans (Bax et al., 2022). Differently from the ‘green economy’ concept born in 1989 to address land-based resources, the ‘blue economy’ address concerns about ocean-based resources, and the potential danger of poor-sustainable practices (United Nations, 2012). The ‘Blue economy’ concept assesses the cumulative effects of economic sectors on the aquatic environments and diversity (Campbell et al., 2021). Whitin the blue economy framework, any activity that uses marine and coastal environments should be motivated by long term and healthy interactions among communities, stakeholders, and the environment.

The United Nations 2021 agreement (COP21) set the pace for a more equitable and sustainable aquaculture following the United Nations Sustainable Development Goals (UNSDG) 11 (sustainable cities and communities), 12 (consumption and production), 14 (life below water), and 15 (biodiversity) (FAO, 2016). Under these global objectives, promoting a sustainable aquaculture should consider the following dimensions (Figure 1.1): the blue revolution (food production), blue water aquaculture (water for aquaculture use), blue frontiers (contaminations of waters), blue carbon emissions (loss of mangroves due to aquaculture), and blue economy (Ahmed & Thompson, 2019). These activities are oriented to promote the environmental, financial, and community sustainability of the use of water resources globally.

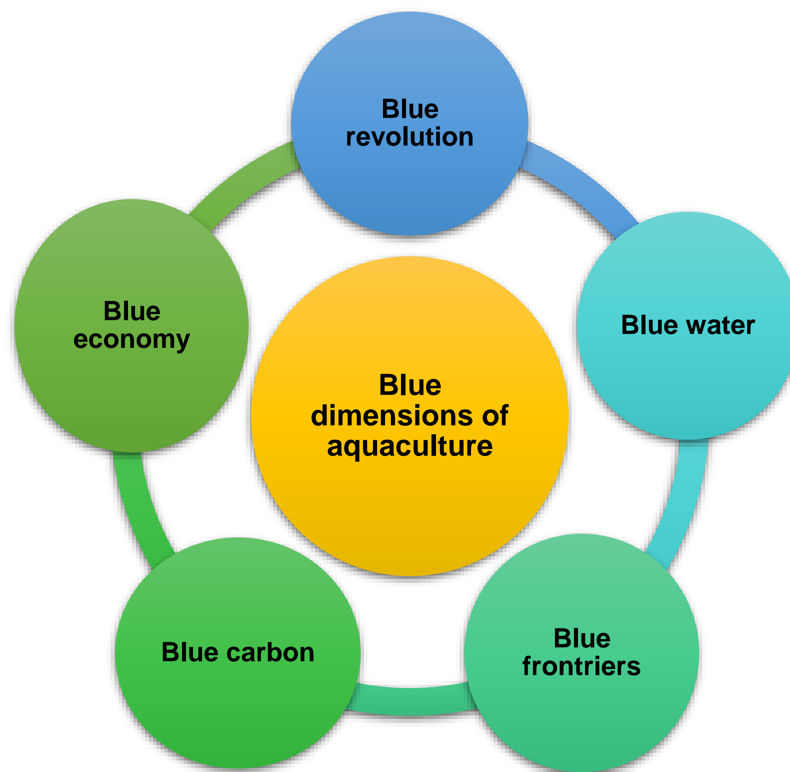

Figure 1.1 A framework of the blue dimensions of global aquaculture from Ahmed and Thompson (2019)

1.1.8 A Māori environmental perspective on feeding technologies for aquaculture

There is a strong impetus in New Zealand to integrate *Mātauranga Māori* (Māori knowledge) with Western sciences (Stevens et al., 2021). In marine practices, this

integration aims to have a more holistic view of production, acknowledging the impacts of our actions on the natural world (Stevens et al., 2021). In Māori worldview, every action should be informed by an ethical view based on *kaitiakitanga* (guardianship) (Cram et al., 2010). In the development of applied technologies, such as feeds for marine species, those ethical aspects should be reviewed and acknowledged. Aquaculture technologies must ensure the priorities of the 21st century in terms of equity, zero waste, and economic justice, going beyond the aim of unlimited growth of both, the operation and animal (New Zealand Parliamentary Counsel Office, 1991). Under this integrative view, the management of the marine estate would need to involve specialists from different areas (e.g., ecologists, fishers, *tōhunga* or Māori knowledge specialists) to ensure that the jurisdictions match the social, ecological, and monetary interests (Reid & Rout, 2020).

Aquaculture growth in New Zealand is oriented to produce more aquatic protein for human consumption and a generation of revenue that is envisaged to triplicate by 2025. Although the Ministry for Primary Industries has outlined the relevance of aquaculture activities in New Zealand socio-political context, little is known about the strategies and ethical framework behind the technologies that offer growth in this sector. First, the expansion of aquaculture will require more available geographical space and therefore, more feed. Second, feed technologies are mainly focus on growth promotion rather than a holistic view of animal nutrition. Third, research is still far away from customising specific diets for New Zealand species. In addition, information about the environmental costs of the introduction of imported feed and ingredients on the New Zealand ecosystem is still unknown.

In that context, any feed ingredient inclusion should be carefully evaluated. The priorities should consider evaluating the nutritional side of ingredients, their potential to affect the environment and the welfare of animals, and reducing the levels of ingredients that can be prioritized for human consumption (fishmeal).

1.2 THESIS AIMS

This thesis aims to evaluate the effect of the inclusion of two alternative ingredients, insect meal and grape marc, on the growth, health, flavour, and nutritional profile of *Haliotis iris*. The inclusion of these sustainable ingredients represents an option to replace fishmeal and to produce more affordable formulated feeds for the abalone industry. The findings of this thesis provide insights into the development of formulated feeds considering their

physical and chemical properties regarding storage, manipulation, and their stability in seawater as well as their effects on all aspects of animal nutrition.

Overall, the thesis includes three main aims:

1. Development of a feed formula, which includes insect meal and grape marc with potential for commercialization for the farming of *H. iris*.
2. Characterisation of the physical and chemical properties of the developed feeds before and after provision to *H. iris*.
3. Evaluation of the developed feeds on the overall health, growth, flavour, shell colour and nutrition profile of *H. iris*.

1.3 THESIS STRUCTURE

To achieve the aims of this thesis, three experiments of different scales were conducted. The results from these studies have been submitted/published as peer-reviewed articles and are presented in subsequent chapters as explained below:

- Introduction and literature review: Chapter 1 & 2
- Nutritional and metabolomic changes of *H. iris* in a farm in Northland (Ruakaka): Chapter 3
- Development of a feed for *H. iris* including insect meal and grape marc: Chapter 4
- Feeding trial of developed feeds for *H. iris* in a farm in Bluff (Invercargill) and their effects on growth performance, nutritional profile, flavour, shell colour, gut health, and metabolome: Chapter 5, 6 & 7
- Overall discussion and conclusions: Chapter 8 & 9

The narration of this thesis starts with the introduction chapter (Chapter 1), which includes an overall view of the molluscan aquaculture globally, and in New Zealand. Also, how new technologies in New Zealand aquaculture industry are aimed to go beyond economic growth. These topics will create the framework for further discussion and conclusions. Chapter 2 provides details about the aspects of abalone nutrition from physiology to food science, highlighting the differences between formulated feeds and seaweeds. Chapter 3 details about the nutritional profile and metabolomic changes of abalone farmed in Northland over one year using a commercial feed. Those fluctuations showed the influence of abalone developmental stages and seasons on the protein proportion and metabolites related to amino acid and fatty acid metabolism. Based on the nutritional profile results from chapter 3, four experimental diets were developed (Chapter

4). This chapter includes the formulation, development, encapsulation, physical, and chemical evaluations before and after seawater immersion, and a pilot study to determine abalone feed intake. The results showed that the diets including insect meal and grape marc promoted growth, were accepted by abalone, and reduced environmental pollution. Chapter 5, 6, and 7 present the results from a 165-days feeding trial using the experimental diets in an abalone farm in Bluff (Southland). The evaluations included the nutritional profile and digestibility of the experimental diets including insect meal and grape marc and their effect on somatic growth, abalone proximate composition, fatty acid, amino acid profile (Chapter 5), flavour and shell colour (Chapter 6), and gut health and metabolome (Chapter 7). The results showed that insect meal and grape marc inclusion did not affect somatic growth nor the gut health of abalone. However, the fatty acid profile and their metabolic products were the most affected providing insights about the role of fatty acids in abalone growth. Shell colour brightness was not affected by the inclusion of insect meal nor grape marc. Flavour compounds were affected suggesting beneficial attributes for grape marc as a natural preservative. Chapter 8 comprises an overall discussion questioning traditional paradigms in aquaculture nutrition and Chapter 9 finalises with overall conclusions.

1.4 CONTRIBUTIONS OF THIS THESIS

- Advancing the understanding of the nutritional requirements for *H.iris* to formulate tailored diets that aligns with the specific needs of the species.
- Development of aquafeed formulations including insect meal and grape marc with improved seawater stability, less nutrient leaching, less nitrogen and phosphorus leaching, and acceptable physical characteristics to resist abrasion during storage time.
- The inclusion of insect meal and grape marc at 10% and 30% respectively is well accepted by *H. iris* in terms of feed intake.
- The inclusion of insect meal and grape marc at 10% and 30% respectively did not produce significant variations in protein and carbohydrate proportion of *H.iris*, but differences were observed in the lipid proportion.
- The inclusion of grape marc at 30% decreased the digestibility of the diets for *H.iris*.
- The inclusion of insect meal at 10% increased the digestibility of the diets for *H.iris*.
- The elevated levels of non-digestible carbohydrates in grape marc reduce the digestibility of the carbohydrate proportion.
- The inclusion of insect meal at 10% did not significantly affect the volatile compounds in the meat of *H.iris*. However, the inclusion of grape marc at 30% affected significantly the compounds associated with fruity flavour.
- The inclusion of insect meal and grape marc at 10% and 30% respectively did not significantly affect the lightness and colour of the shells of *H.iris.*
- The inclusion of insect meal and grape marc at 10% and 30% respectively did not affect the intestinal health of *H.iris* and did not produce inflammation.
- The inclusion of insect meal and grape marc at 10% and 30% respectively increased the relative abundance of gut bacteria possibly associated with the promotion of beneficial species in the gastrointestinal tract of *H.iris*.
- The inclusion of insect meal and grape marc at 10% and 30% respectively did not decrease growth compared to diets without insect meal and grape marc reinforcing that these alternative ingredients can be included in *H.iris* diets.
- Fatty acid synthesis in *H.iris* is associated with higher growth rates.

1.4 ACHIEVEMENTS FROM THIS THESIS

This section includes publications and presentations that directly or indirectly related to this thesis and were conducted during the period 2019–2023.

1.4.1 Peer-reviewed papers

First or main author

1. **Bullon, N**., Seyfoddin, A., & Alfaro, A. C. (2022). The role of aquafeeds in abalone nutrition and health: A comprehensive review. Journal of the World Aquaculture Society. **https://doi.org/10.1111/jwas.12883**

2. **Bullon, N**., Alfaro, A. C., Hamid, N., Masoomi Dezfooli, S., & Seyfoddin, A. (2023). Effect of dietary insect meal and grape marc inclusion on flavour volatile compounds and shell colour of juvenile abalone *Haliotis iris.* Aquaculture Nutrition. **https://doi.org/10.1155/2023/6628232**

3. **Bullon, N**., Seyfoddin, A., Hamid, N., Manivannan, M., & Alfaro, A. C. (2023). Effect of insect meal and grape marc in the nutritional profile, growth and digestibility of juvenile New Zealand farmed abalone. Aquaculture international, 1-30. **https://doi.org/10.1007/s10499-023-01227-z**

4. **Bullon, N**., Seyfoddin, A., Masoomi, S., Young, T., & Alfaro, A. C. (2023). Nutritional and metabolomic changes of juvenile farmed abalone (*Haliotis iris*) in New Zealand. Aquaculture research. **https://doi.org/10.1155/2023/3297576**

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1.4.2 Presentations at conferences and symposia

Oral presentations (first or main author)

1. **Bullon, N**., Seyfoddin, A., Hamid, N.& Alfaro, A. C. (2023). Beyond the fishmeal trap: The potential of insect meal and grape marc to feed the New Zealand farmed abalone. The 11th International Abalone Symposium, Auckland, New Zealand.

2. **Bullon, N.**, Seyfoddin, A., Hamid, N.& Alfaro, A. C. (2022). Promoting a more sustainable aquaculture for New Zealand farmed abalone. AUT Postgraduate School of Science Showcase. Auckland, New Zealand

3. **Bullon, N.**, Seyfoddin, A. & Alfaro, A. C. (2022). Towards a more sustainable feed for New Zealand farmed abalone: Inclusion of insect meal and grape pomace as alternative ingredients. New Zealand Marine Society. Auckland, New Zealand

4. **Bullon, N.**, Seyfoddin, A., Masoomi, S., Moganakumaar, M. Hamid, N., & Alfaro, A. C. (2021). Sustainable aquafeeds to boost abalone nutrition in land-based farming. Physiomar. Auckland, New Zealand

Poster presentations (first or main author)

1. **Bullon, N.**, Seyfoddin, A. & Alfaro, A. C. (2023). A pilot study testing sustainable aquafeed formulations for farmed New Zealand abalone. The 11th International Abalone Symposium, Auckland, New Zealand.

2. **Bullon, N.**, Seyfoddin, A., Hamid, N.& Alfaro, A. C. (2022). Towards a more sustainable feed for New Zealand farmed abalone: Inclusion of insect meal and grape pomace as alternative ingredients. AUT Postgraduate Research Symposium. Auckland, New Zealand

3. **Bullon, N.**, Seyfoddin, A., Masoomi, S., Young, T., & Alfaro, A. C. (2021). Nutritional and metabolomic changes of juvenile farmed abalone (Haliotis iris) in New Zealand. Physiomar. Auckland, New Zealand

Other oral or poster presentations presented by co-authors.

1. Guo J. **Bullon N**. & Alfaro A.C. (2023) Investigation of Bluff's farmed abalone's gut microbiome under various formulated feed pellets. The 11th International Abalone Symposium, Auckland, New Zealand.

1.4.3 Awards

1. August 2022: Postgraduate Teaching Award 2022. Honourable mention for Excellence in Teaching in the School of Science. Auckland University of Technology (AUT)

2. August 2022: Best oral presentation. School of Science Showcase. Auckland University of Technology (AUT). Auckland, New Zealand

3. October 2022: Best poster award. AUT postgraduate Research Symposium. Auckland University of Technology (AUT)

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Chapter

2

The role of aquafeeds in abalone nutrition and health: a comprehensive review

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2.1 ABSTRACT

Overpopulation and the pressure on land-based resources have driven the aquaculture sector to increase its production since the 1980s. To address such demands, new aquafeed technologies have been developed relying on natural and artificial ingredients that are commercially viable. In addition, current global sustainable initiatives require feed technologies to reduce the pressure on limited wild fisheries and minimize negative environmental effects. Although there are numerous studies on abalone nutrition, most tend to focus on animal growth and nutrient utilization. A more holistic research approach to ensure a sustainable future for this industry will require the development of feeds that provide integrated nutrition and health benefits. In this review, we aim to synthesize the most recent scientific literature on the nutritional and health benefits and shortcomings of two main abalone feeding approaches (seaweed and formulated feeds) within aquaculture production practices. We also identify major research gaps and future directions for the development of sustainable abalone feeds.

2.2 INTRODUCTION

Aquaculture is the fastest-growing food production sector in the world, currently providing around 47% of the global seafood demands and is well-positioned to supply the protein requirements of our increasing population (FAO, 2018). The exponential growth of the global aquaculture industry has fostered the development of commercial diets which are cost-effective and optimised for each species. In aquaculture, feed accounts for over 50% of the production costs (Huntington & Hasan, 2009). Fishmeal accounts for almost 73% of aquaculture feeds (Asche & Oglend, 2016; Ringø et al., 2012). However, recent declines in the availability and lack of sustainability in fishmeal has prompted the search for alternative protein sources for aquafeeds.

Abalone are a widespread group of marine gastropods, from the genus *Haliotis*, inhabiting both tropical and temperate waters around the world. Approximately 90 species of abalone are found worldwide, and about 15 species are cultured in Australia, China, Japan, Korea, New Zealand, Philippines, South Africa, and Taiwan (Sales & Janssens, 2004). Although abalone production is relatively small compared to other seafood products, it is one of the most highly prized delicacies globally, especially in Asia (Cook, 2014).

Animal nutritional requirements are based on three main components: endogenous factors (e.g., animal genetics, age, sex, physiology), environmental conditions (e.g., water temperature, oxygen, salinity) and feed properties (e.g., composition, delivery system, digestibility, solubility). Aquafeeds are composed of macronutrients, such as proteins, carbohydrates, and lipids, and micronutrients including vitamins and minerals. In abalone feed formulations, protein is the most expensive component and represents 20 to 40% of the total ingredients. The amount of protein is crucial in abalone feeds since it is the main macronutrient used to build tissues in the animal (Fleming et al., 1996). Carbohydrates make up 50 to 60% of nutritional requirements of abalone, and they are the most preferred source of energy (Lee, Kim, Choi, et al., 2017). Lipids, on the other hand, represent 1 to 5% of the nutritional requirements, and although this percentage is relatively low compared to proteins and carbohydrates, their consumption is essential for the acquisition of polyunsaturated fatty acids (PUFAs) (Mulvaney et al., 2015) which cannot be synthesised *the novo* by the animal (Bautista-Teruel et al., 2011). In addition, lipids supply the phospholipids needed to build cell biomembranes (Bautista-Teruel et al., 2011).

Optimising nutrition involves a comprehensive analysis of not just metabolic provisioning, but also the interplay of other physiological traits (e.g., ingestion, digestion) (Guillaume et al., 2001), biological characteristics (e.g., sex, age, species, genetics, reproduction, health) and environmental conditions (e.g., temperature, water, salinity, oxygen, pH, water quality, tank density). For instance, if the objective is growth-related, nutritional requirements should aim to provide fuel for metabolic expenses and tissue formation. On the other hand, if the priority is to enhance health during summer periods, the feed must primarily strengthen the immune defences to overcome season-related stressors, such as elevated temperatures and high pathogens loads, which predominate in summer months.

Previous studies on optimisation of feed formulations have focused on both, generating nutritionally balanced diets, and reducing feed costs. To this end, strategies have aimed to replace the protein component with more cost-effective ingredients (e.g., seaweed extracts, plant protein) while still achieving good growth performance (Sales et al., 2003). Other approaches have focused on the addition of specific amino acids, immunostimulants, vitamins, and probiotics to the feed mixture. The success of these strategies ultimately requires careful evaluation of potential benefits in feed intake, assimilation, digestion, and metabolic efficiency (Kemp et al., 2015; Yu et al., 2014), health (Tanaka et al., 2003) and flesh composition (Bewick et al., 1997).

The development of optimal abalone diets has taken two main approaches: natural (macroalgae) and formulated feeds. To date, previous studies have evaluated how these types of diets affect abalone growth (Bansemer et al., 2015; Mulvaney et al., 2013), health (Grandiosa et al., 2018; Tanaka et al., 2003), and nutritional quality (Bewick et al., 1997; Preece, 2006) among others. However, the effectiveness of these approaches has been hampered by the lack of a holistic analysis or integrated approach to evaluate the benefits and disadvantages of each type of diet. Such a comprehensive analysis requires a clear review of the current knowledge and identification of knowledge gaps to guide future research. To this end, the present review outlines our current knowledge on 1) abalone feed requirements, including the effect of endogenous and exogenous factors, and 2) the advantages and disadvantages of the application of seaweeds and formulated feeds in abalone aquaculture. Finally, a set of recommendations are provided to guide future research in abalone feed development aiming to enhance this important global production.

2.3 ABALONE NUTRITION

Abalone naturally graze on a range of algal species in intertidal to subtidal high energy coastal habitats. During the early life stages (juveniles), abalone feed on microalgae, usually contained within diverse biofilms on surfaces of rocks and boulders. At some stage in their development, abalone shift to macroalgae, which provide appropriate nutrition to develop somatic and reproductive tissues (Johnston et al., 2005; Shepherd et al., 1992). In the wild, abalone may encounter a range of algal species with varying nutritional compositions, which ultimately affect their growth, reproductive state, and survival. The relationship between animal and food types, as well as the environmental conditions that affect optimal nutrition can be mimicked in controlled aquaculture settings to improve production.

2.3.1 Feeding across life stages

Abalone have four major developmental stages which include larvae, post-larvae, juveniles, and adults (Hahn, 1989). Generally, after 19 hours in the water column, fertilized eggs hatch as trochophore larvae, which subsequently develop into competent veliger larvae after 46–74 hours post-fertilisation, depending on the species (Balkhair et al., 2016; Huang et al., 2012). The lecithotrophic planktonic larvae swim for 40–118 hours (Balkhair et al., 2016; Genade et al., 1988) until they sense biological and chemical cues from suitable habitats to safely settle (Manahan, 1992; Takami, 2003). These biological cues include biofilms (Li et al., 2006), conspecific mucus trails (Gallardo & Buen, 2003), and diatoms (Kawamura et al., 1995) while chemical inductive signals include gamma-aminobutyric acid (GABA). Indeed, GABA has been found to be an exudate component of crustose coralline algae, which is thought to play a major role in inducing settlement and metamorphosis on abalone (Shepherd et al., 1992).

After settlement, benthic post-larvae use a primitive radula with chitinised teeth (Takami & Kawamura, 2003) to feed on crustose coralline algae, benthic diatoms, and the proteins and polysaccharides present in the mucus trails left by adult abalone (Takami & Kawamura, 2003). Diatoms are considered to be one of the most important sources of nutrition for post larvae (Balkhair et al., 2016; Kawamura et al., 1995) with high levels of protein (Courtois de Viçose et al., 2012) and lipids (Dunstan et al., 1996).

The juvenile stage starts approximately 8 weeks after settlement (Hahn, 1989). Juveniles shift from microalgae to macroalgae once the digestive system (*i.e,* more matured

digestive gland, secretion of digestive enzymes) is developed to process seaweeds which are grazed with the radula (Garcia-Esquivel & Felbeck, 2006; Onitsuka et al., 2007; Viana et al., 2007). Macroalgae constitute the main source of nutrition for juveniles due to their high carbohydrate content, which is their main source of energy to grow. Sexual maturity indicates the adult phase (Hahn, 1989). Adult abalone continue to eat seaweeds to supply protein and lipid requirements for gonad development (Bautista-Teruel et al., 2011).

Once abalone start to eat seaweeds, their feeding patterns become nocturnal, which reduce predation pressure (Shepherd & Turner, 1985) and avoid competition with other grazing animals. Abalone graze continuously throughout the night until early morning showing high activity after sunset to a few hours after midnight (Tahil & Juinio-Menez, 1999). Their feeding behaviour is opportunistic when they encounter drift seaweeds (Cornwall et al., 2009; Zeeman et al., 2012), foraging when hydrodynamic forces are favourable (Tahil & Juinio-Menez, 1999).

Adult abalone consume seaweeds at a rate close to 20–35% of their body weight per day (Tahil & Juinio-Menez, 1999), environmental conditions (e.g., water temperature, water movement, habitat characteristics), animal characteristics (e.g., size, species, maturity), and seaweed properties, such as phenolic composition (Shepherd, 1992), drifting or attachment (Cornwall et al., 2009) and digestibility (Tahil & Juinio-Menez, 1999). Colder water temperatures promote a higher consumption of algal biomass in abalone (Barkai & Griffiths, 1986). Water movement enhances the quantity of drifting seaweeds (Zeeman et al., 2012), and abalone may consume certain types of seaweeds in the proportions in which these algae drift nearby (Tutschulte & Connell, 1988; Wood & Buxton, 1996), while still actively selecting from what is abundant in the surrounding habitat (Alcantara & Noro, 2005; Barkai & Griffiths, 1986). Previous studies have shown that abalone may select seaweeds based on their compositional properties that are influenced by the region where seaweeds are located. For example, the Japanese abalone *Haliotis diversicolor* and the New Zealand abalone *Haliotis iris* are more likely to consume brown seaweeds (e.g., *Ecklonia maxima, Ecklonia radiata* and *Macrocystis pyrifera*) due to their better digestibility and less toughness compared to red seaweeds (Alcantara & Noro, 2005; Cornwall et al., 2009), while *Haliotis asinine* from Thailand and Philippines prefer red seaweeds, including *Laurencia*, *Hypnea*, *Gracilaria*, and *Amphiroa* (Tahil & Juinio-Menez, 1999) due to their higher digestibility (Shepherd, 1992) and lack of polyphenols compared to brown seaweeds (Fleming, 1995). In addition, selection of seaweeds for nutritional purposes may be selected according to the needs during different developmental stages (Burtin, 2003). For example, smaller *Haliotis*

midae (< 65 mm) prefer green seaweeds, while bigger abalone prefer brown seaweeds (Barkai & Griffiths, 1986). *Haliotis tuberculata* with mature gonads preferentially consume seaweeds with high omega-3 fatty acids, more soluble carbohydrates, and total protein compared to immature animals (Roussel et al., 2019), and *Haliotis diversicolor* scarcely consume green seaweeds species since they mostly have defensive compounds that deter consumption (Alcantara & Noro, 2005).

2.3.2 Abalone digestive system

The digestive system in abalone is similar in all species (McLean, 1970) and consists of a mouth, which collects the food and passes it to the buccal cavity. After mixing the food with mucus, the bolus enters the oesophagus that extends posterior to a large crop organ, where it is mixed with enzymes and digestive fluids and stored before entering the stomach. The digestive gland or hepatopancreas, which overlies the crop and stomach, moves the food by muscular contractions and ciliary currents. Later, undigested food is transported from the digestive gland to the stomach and intestine, where the rejected material is eliminated through the anus (Hughes, 1986; McLean, 1970; Venter, Loots, Vosloo, et al., 2018).

Ingestion is determined by the development of the radula and its mechanical ability to graze on microalgae or macroalgae. Developmental changes in the radula occur mainly during the larval and juvenile stages. In this period, the number of teeth on the radula increases along with a change in their morphology (from pointed serrations to less pronounced serrations), indicating the shift in diet from biofilms to seaweeds. Lateral teeth are more pointed and longer, which allows them to cut macroalgae when the animal is approximately 2-mm in shell length. In addition, the clearance angle, which is a measure of the function of the radular teeth, evolves from a scoop-shape to a cut-shape, again reducing the ability to consume small particles and increasing the ability to consume macroalgae (Takami & Kawamura, 2003).

Digestion refers to the mechanical and chemical processes that occur in the digestive system, including numerous reactions triggered by proteases, carbohydrases and lipases (Erasmus et al., 1997). The two main digestion regions in abalone are 1) the mouth-intestine region where lipase and aminopeptidase activity are high (Picos-García et al., 2000) and 2) the stomach-digestive gland region where the majority of enzymatic activity occurs (Garcia-Esquivel & Felbeck, 2006) and is characterised by a carbohydrase activity (cellulose and lysozyme). Gut polysaccharide-degrading enzymes produced in the abalone digestive

system include agarase, carrageenase, alginate lyase, carboxymethylcellulase, and laminarinase (Erasmus et al., 1997; Tanaka et al., 2003).

Gut microbiota consist of indigenous and non-indigenous bacteria, and the profile changes according to the life stage (Tanaka et al., 2003), environmental conditions (Gobet et al., 2018), and nutrients (Antonopoulou et al., 2019; Stenberg et al., 2019). At four months of age—when artificial diets are usually started in an aquaculture farm—the gut microbiome is mainly composed of algal polysaccharide-degrading bacteria with mostly facultative anaerobes and non-motile fermenters (NMF), such as *Vibrio halioticoli* (Tanaka et al., 2003). Although seasons and dietary intake affect the gut microbiome, abalone possess a core group of bacteria that remains stable (Gobet et al., 2018). For example, the core bacterial community in the digestive gland of adult *Haliotis tuberculata* fed on macroalgae has been found to be consistently dominated by the Phyla Fusobacteria (e.g., *Psychrilyobacter*), Tenericutes (e.g. *Mycoplasma*), and Gammaproteobacteria (e.g., *Vibrio*) (Gobet et al., 2018). This core bacterial composition varies from species to species, found to be different in the gut of 1-year of *Haliotis discus hannai*, where Alphaproteobacteria, Firmicutes, Mollicutes, Mycoplasm and Bacillales were predominant (Tanaka et al., 2004).

2.3.3 Abalone metabolism

Metabolism is defined as the chemical reactions involved in the synthesis of biological macromolecules and the generation of energy for vital functions through the processes of catabolism, anabolism, and amphibolism. Catabolism involves the oxidation of molecules to generate adenosine triphosphate (ATP), the main substrate of energy. Anabolism corresponds to the synthesis from precursors where ATP is used for endergonic reactions and amphibolism, which includes catabolic or anabolic processes (Garrett & Grisham, 2010).

In abalone, the energy sources (carbohydrates, proteins, and lipids) tend to be attributed to specific activities. Indeed, carbohydrates are mainly used for locomotion and respiration (Fleming et al., 1996), while proteins are prioritised for tissue building and maintenance, and lipids are utilised for cell membrane formation and gonad maturation (Dunstan et al., 1996; Uki et al., 1986). When carbohydrate intake is restricted or abalone are under stress, they use protein as an energy source (Lee et al., 2019). Carbohydrates are broken down into pyruvate, which is subsequently used as a substrate for the tricarboxylic acid cycle (TCA) or opine, lactate, and oxaloacetate derivatives. Coenzyme A is the base for the generation of ATP, NAD, FAD, and CO_2 via the mitochondrial oxidative

phosphorylation system (OXPHOS) (Van Rensburg & Coyne, 2009). Proteins are broken down into amino acids, which then undergo a series of transformations to produce citric acid intermediates. Furthermore, triacylglycerols are broken down into fatty acids, which are oxidised in the mitochondria into acetyl CoA.

Abalone are facultative anaerobes and can make use of anaerobic metabolism (lactate and opine synthesis) for functions, such as crawling locomotion and survival during stress conditions. In normal conditions, their metabolism is controlled by anabolic activity involving the synthesis of molecules for growth and recovery (Garrett & Grisham, 2010). During stressful conditions, catabolic metabolism is activated ensuring available energy to restore equilibrium (Venter, Loots, Mienie, et al., 2018).

The environment, diet, and animal characteristics have a great influence on abalone metabolism, thus modifying feed intake. For example, abalone food intake in warm waters is higher than in cold waters due to the high energetic expenses involved in respiration (Lopez & Tyler, 2006) resulting in more algal consumption in warmer seasons. In addition, seaweeds have a lower caloric content (Allen et al., 2006) producing an increased feed consumption compared to formulated diets (Bansemer et al., 2015; Bautista-Teruel & Millamena, 1999; Coote et al., 2000; Mai et al., 1995; Sales et al., 2003). The body weight of abalone also has been shown to affect feed intake. Juvenile *Haliotis fulgens* consume 1.43–1.65% of their body weight (BW) per day (Gómez-Montes et al., 2003) compared to 1% in juvenile *Haliotis midae* (Britz, 1996). *Haliotis fulgens* of 1–2 cm in shell length require about 1% BW per day compared to the 0.2–0.3% required by larger animals (Farías et al., 2003).

2.3.4 Abalone nutritional profile

Abalone have been considered a functional human food that improves health beyond basic nutrition. The range and quality of available nutrients from abalone products depend on whether the animal comes from the wild or farm. Abalone in aquaculture facilities, where formulated feeds are used, are exposed to higher levels of dietary protein and lipids compared to those in the wild which feed on seaweeds. Thus, the protein proportion in the flesh of wild abalone is 14–18% (Chiou et al., 2001; Hatae et al., 1995) compared to 40–56% in farmed abalone (Mai et al., 1995; Tung & Alfaro, 2011). Indeed, farmed juvenile *Haliotis laevigata* fed on higher amounts of dietary protein were shown to have 65% greater flesh protein than juveniles fed on macroalgae (Stone et al., 2013).

The protein profile of abalone is determined by the type of amino acids available in the body. It has been documented that both farmed and wild abalone meat contains all essential amino acids: Arg – arginine, His – histidine, Ile – isoleucine, Leu – leucine, Lys – lysine, Met – methionine, Phe – phenylalanine, Thr – threonine, Trp – tryptophan, and Val – valine, and nonessential amino acids: Ala – alanine, Asp – aspartic acid, Cys – cystine, Glu – glutamic acid, Gly – glycine, Pro – proline, Ser – serine, and Tyr – tyrosine (Fleming et al., 1996; Latuihamallo et al., 2015). Amino acids, such as Arg, Met, Thr, Leu, and Phe are considered crucial for abalone growth and therefore, should be included in the feed (Mai et al., 1994; Roussel et al., 2019). From these amino acids, leucine is the most abundant in abalone meat (Shi et al., 2020).

The carbohydrate proportion in abalone meat is less than the protein and includes many types of sugars. In general, the carbohydrate content varies between 0.5–7% (Shi et al., 2020) and fluctuates depending on the season, being higher in the summer and lower in the autumn (Hatae et al., 1995) and if abalone is farmed or wild. Sulphate and neutral polysaccharides are the most significant sugars in abalone flesh due to their immunomodulatory (Zhu et al., 2009), antithrombotic, and antifatigue effects (Liu et al., 2020), which have nutraceutical applications. Other carbohydrates include L-rhamnose, D-xylose, D-mannose, D-glucose, D-galactose, glucuronic acid, and fucose.

The lipid fraction in abalone is minimal and depends on the feed given (Chiou et al., 2001). Lipid levels vary from 0.2–1% in wild abalone (Hatae et al., 1995) compared to 5–7% in farmed ones (Stone et al., 2013; Thongrod et al., 2003). Abalone are a good source of omega-3 polyunsaturated fatty acids (PUFAs) (Su et al., 2004), which have been found to be higher compared to beef, pork, and chicken (Mulvaney et al., 2015). Among these acids, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and α-linolenic acid (ALA)are at least three times higher in wild abalone than farmed abalone (Su et al., 2004). Sterols are another class of lipids available in abalone with an essential role in their growth and metabolism (Nelson, Leighton, et al., 2002). Cholesterol is the main component accounting for 87–96% followed by desmosterol which accounts for 1–7.6% and phytosterols are present in almost undetectable amounts (Dunstan et al., 1996; Zhang et al., 2009).

2.4 TYPES OF FEEDS AND THEIR EFFECTS ON NUTRITION AND GROWTH

The most used indicators to measure aquaculture efficiency and productivity are animal growth, performance, and survival (Kankainen et al., 2012). External conditions, such

as water quality, climatic conditions, feed quantity and quality, and tank design, as well as animal characteristics, such as sex, immune system, genotype and age, have an impact on stock performance, ultimately affecting the profitability of production. Among these conditions, the feed has an extensive impact on animal health and growth (Guillaume et al., 2001). The type, quantity, and quality of the nutrients influence many aspects of the animal's performance, such as growth, immunity, nutritional profile, meat taste, and meat quality. For this reason, aquaculture nutritional studies have been highly significant over the last decade.

General growth indicators include physical measurements of body size and shape and meat to shell ratio. Nutrient utilisation parameters include feed conversion ratio (FCR), nutrient deposition (ND), protein efficiency ratio (PER) and energy efficiency ratio (EER). These parameters are calculated based on the sum and difference of the feed given, consumed, and excreted. In summary, growth performance indicates objectively how the feed is translated into weight, length, and width, and nutrient utilisation parameters identify how the animal can effectively transform the feed into muscle tissue.

2.4.1 Nutritional Composition of Seaweed

In general, seaweeds contain between 5–27% protein, 1–5% lipids and 32–65% total carbohydrates (Bansemer, Qin, Harris, Howarth, et al., 2016; Viera et al., 2011; Viera et al., 2005). The crude protein varies according to the algal species (Lourenço et al., 2002), habitat conditions and season (Gaillard et al., 2018). For instance, crude protein in red algae (20–31%) is higher than brown algae (6–19%) (Dawczynski et al., 2007; Ruperez & Saura-Calixto, 2001), and higher amounts of crude protein are often found in seaweeds during spring compared to autumn (Gaillard et al., 2018). Generally, seaweeds appear to have all essential amino acids and high amounts of non-essential amino acids, such as glutamic acid (Glu) and aspartic acid (Asp) (Lourenço et al., 2002). However, red seaweeds tend to have higher concentrations of total amino acids than green and brown seaweeds (Gaillard et al., 2018). Other differences among the major seaweed groups include the fact that green algae generally have lower percentages of both aspartic and glutamic acid compared to red and brown algae (Lourenço et al., 2002).

Compared to terrestrial plants, seaweeds have a relatively low content of total lipids. Total lipids in red algae vary from 1–2.8%, brown algae from 1–4.5% (Dawczynski et al., 2007) and green algae from 2–3% (Nelson, Phleger, et al., 2002; Rodrigues et al., 2015). Seaweeds contain different lipid classes, such as fatty acids, sterols and triacylglycerols. Three types of FAMEs (fatty acid methyl esters) are present in seaweeds: saturated fatty

acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). Among these fatty acids, PUFAs represent the highest proportion (31–74%) with more n-3 species (10–51%) than n-6 (Dawczynski et al., 2007; Nelson, Phleger, et al., 2002). In general, linoleic acid (C18:2, n-6, LA), α-linoleic acid (C18:3, n-3, ALA), stearidonic acid (C18:4, n-3, SDA), and arachidonic acid (C20:4, n-6, AA) are the most representative fatty acids, while eicosapentaenoic (C20:5, n-3, EPA) is the most abundant PUFA (10–42%) in seaweeds (Dawczynski et al., 2007). High proportions of saturated fatty acid, such as palmitic acid (C:16) and monounsaturated fatty acid, such as oleic acid (C18:1, n-9), are found in red, green, and brown algae (Nelson, Phleger, et al., 2002; Rodrigues et al., 2015). Seaweeds contain low n-6:n-3 ratios of 0.1–1.1 (Dawczynski et al., 2007; Floreto et al., 1996) compared to the well-known high levels (0.2–0.3) found in salmon (Berge et al., 2009). Macroalgae are considered to be the most important contributors of long chain fatty acids (n-3) (Arterburn et al., 2007), which are only synthesized *de novo* in photosynthetic organisms.

Sterols are another type of lipids present in seaweeds and are involved in cell structure and function (Mouritsen et al., 2017). Some sterols, such as fucosterols are dominant in brown algae (>70% of total sterols), while ergosterols are common in green algae and cholesterols and desmosterols are predominant in red algae (34–88%) (Lopes et al., 2011).

Carbohydrates are the main macronutrient in seaweeds accounting for 60–70% of the composition and are available in two types: storage and structural polysaccharides. Non-starch polysaccharides account for 30–50% of total carbohydrate (Dawczynski et al., 2007) placing seaweeds as a very important source of dietary fibre compared to terrestrial plants. Generally, the storage sugars are laminarian (brown algae), floridian starch (red algae) and starch (green algae) whereas the cell wall polysaccharides comprise cellulose; alginic acid, and fucan in brown algae; agar, carrageenan, and xylan in red algae; and mannan and xylan in green algae (Stiger-Pouvreau et al., 2016). Sulphated polysaccharides are other types of carbohydrates available in seaweed, which have bioactive relevance as anti-coagulant, anti-proliferative, anti-tumoral, anti-inflammatory and anti-viral (Chojnacka et al., 2012).

Vitamins (Norziah & Ching, 2000) and polyphenols are also in the nutritional profile of seaweeds. Carotenoids, such as α- and β-carotene, lutein, zeaxanthin, and fucoxanthin are present in the three groups of macroalgae (Burtin, 2003) while phlorotannins are the most abundant phenolic compounds in brown and red seaweeds (De Quirós et al., 2010). Vitamin B-12 levels are higher in seaweeds than terrestrial plants (Takenaka et al., 2001),

whereas vitamin C is high especially in brown seaweeds compared to other seaweed groups (Matanjun et al., 2009).

Seaweeds can absorb inorganic substances from the marine environment and store them as mineral content. This value is higher in seaweeds than edible terrestrial plants and animal sources, accounting for 30.1–39.3% in brown algae and 20.6–21.1% in red algae (Rupérez, 2002). Additionally, previous reports highlight seaweeds as one of the most important vegetable sources of calcium (Ca) (Norziah & Ching, 2000) (Rajapakse & Kim, 2011), sodium (Na), potassium (K), magnesium (Mg), phosphorous (P) and trace elements, such as iron (Fe) and iodine (I), zinc (Zn) and cooper (Cu) (Robledo & Freile-Pelegrin, 1997; Vieira et al., 2020).

2.4.2 Seaweeds as a food source for abalone farming

In many countries, such as China, Korea, South Africa, and Chile, macroalgae are a common food source in abalone farming (Flores-Aguilar et al., 2007; Robertson-Andersson et al., 2007). In these countries, seaweeds are extensively harvested or cultivated offshore to feed abalone. When seaweeds are harvested from the wild for aquaculture production, the final operation relies on seasonal availability, which can hinder abalone production. In other countries, legislation protects seaweed harvests against intense depletion. In these countries, formulated feeds are the only option to promote growth in abalone aquaculture.

The selection of seaweeds depends on what grows and/or can be cultivated locally. The species of macroalgae mostly cultivated for abalone aquaculture are *Laminaria, Undaria* and *Porphyra* in Japan, China, and Korea (Hwang et al., 2009), *Macrocystis* in Chile (Macchiavello & Bulboa, 2014), and *Ulva* and *Ecklonia* in South Africa (Robertson-Andersson et al., 2007; Troell et al., 2006). These species can be used as a single source, or in conjunction with pelletised feed.

Feeding farmed abalone on an exclusive live seaweed diet has both advantages and disadvantages. Diets based on one type of live seaweed can negatively affect weight gain and nutrient utilisation of *Haliotis* species. For example, *Haliotis midae* fed exclusively on macroalgal diets gained less weight and showed less protein in their tissues compared to animals fed formulated diets (Britz, 1996). Similarly, *Haliotis rufescens* fed live kelp showed lower growth than animals fed formulated feeds containing 25–38% protein (Garcia-Esquivel & Felbeck, 2009). *Haliotis discus Reeve* fed *Undaria pinnatifida* grew 10% less than the ones fed on formulated feed, including *Undaria pinnatifida* meal (Ansary et al., 2019). The

low growth shown in such studies suggests that an exclusive seaweed food type is not the best choice for abalone. Indeed, a single algal species may provide a limited or poor nutritional profile, and relatively low protein content compared to balanced commercial feeds (Bansemer, Qin, Harris, Duong, Hoang, et al., 2016).

Alternatively, a mixture of live seaweeds may provide a better amino acid range and overall nutritional profile for abalone. For example, Kemp et al. (2015) demonstrated that a combination of two live seaweeds (*Macrocystis pyrifera* and *Lessonia berterona*) improved the growth performance of *Haliotis rufescens* compared to a fed regime exclusively based on formulated pellets. In that study, animals fed on those two live seaweeds increased their weight gain ratio by 20% and length by 40% when compared to formulated pellets. In addition, Bansemer, Qin, Harris, Duong, Hoang, et al. (2016) showed that a mixture of seaweeds (*Gracilaria* sp. and *Ulva* sp.) resulted in enhanced growth and nutrient utilisation (e.g., tissue protein deposition and protein efficiency ratios) compared to commercial feeds in *Haliotis laevigata*.

In the last years, many attempts have been made to increase the nutritional profile of seaweeds, particularly in terms of protein, through enrichment techniques. These techniques aim to increase protein, carbohydrate, and lipid levels in seaweeds by growing them in high nitrogen content media (Viera et al., 2015). For example, Bansemer, Qin, Harris, Duong, Hoang, et al. (2016) found that using fishpond effluents to cultivate seaweeds resulted in increased protein levels by >33%. Another study by Viera et al. (2015) showed an increase in *Halioitis coccinea Reeve* growth rate by 10% and weight gain by 30–169% when animals were fed enriched algae compared to animals fed on formulated feeds. In that study, an enriched-live mixture of *Gracilaria cornea* and *Ulva rigida* promoted 50% more growth and led to a weight gain of more than doubled when compared to formulated feeds. Furthermore, the above-mentioned studies concluded that enriched seaweeds improve not only growth performance, but also nutrient utilisation.

2.4.3 Composition of formulated feeds

Generally, formulated feeds are made up of a mixture of traditional and alternative sources of proteins, carbohydrates, and lipids to supply the on-farm energy demand. Traditional ingredients include fishmeal and casein as protein sources, starch or cornflour as carbohydrates, and fish oil and cod liver oil as lipid sources. Alternative sources have been incorporated into aquafeeds to reduce the use of traditional feedstuffs which are prioritised for human consumption. These alternatives include seaweed extracts, seaweed

meals, terrestrial plant meals, agriculture by-products, insect meals and oils, and microalgae extracts. In addition, supplements, such as immunostimulants can be added into the formulation to boost growth by strengthening the immune status of aquatic animals. Formulated feeds have demonstrated to be effective in generating good growth rates and nutrient utilisation parameters. The reason for their effectiveness is the high load of macronutrients, especially protein, and the synergy of the variety of ingredients in the final formulation. The high diversity of nutrients positively affects nutrient delivery and the digestibility of the whole formula which in turns impact abalone growth (Agbidye et al., 2009)

Seaweeds are incorporated in aquafeeds as supplements, whether dry or wet, to increase the animal feed response. This incorporation ensures good feed intake without compromising growth, survival, and nutrient utilisation. For example, Allen et al. (2006) mixed dried *Gracilaria* sp. and *Macrocystis* sp. with commercial feed, which resulted in a significant increase of weight and shell growth of 110.6 ± 3.2 μmd^{-1} with *Gracilaria* ssp. compared to 86.9 ± 4.0 $\mu m\ d^{-1}$ in animals fed AB-Feed™. Also, the study of Nel, Pletschke, Jones, et al. (2017) reported that the inclusion of a 0.44 to 5.54% dry kelp, *Ecklonia maxima*, promoted faster growth and higher biomass in *Haliotis midae* compared to animals fed on non-supplemented feed. Ansary et al. (2019) demonstrated that abalone fed on pelletised diets containing dry seaweeds showed an improved survival rate of 92.2-96.1% compared to 86.1% in the ones fed on exclusive live seaweeds with a survival of 86.1%. Seaweed inclusion in aquafeeds not only increases feed intake, but also improves nutritional markers, such as protein efficiency ratio and digestibility. For instance, abalone fed on aquafeeds supplemented with *Macrocystis* sp. (Allen et al., 2006), *Ecklonia maxima* (Nel, Pletschke, Jones, et al., 2017) or *Ulva* sp. (Bates et al., 2017) increased their meat yield percentages compared to animals fed on exclusive live seaweeds (Kemp et al., 2015).

Abalone diets without seaweeds also provide good growth rates and nutritional markers. Bansemer, Qin, Harris, Duong, Currie, et al. (2016) developed a seaweed free diet that despite its lower intake, produced higher protein deposition levels and protein efficiency compared to aquafeeds with dried macroalgae. The formula included three different sources of protein (non-vegetable and vegetable) that might improve nutrient deposition due to a synergistic effect between different amino acid profiles. In fact, aquafeeds composed of a mixture of fish and vegetable protein sources produce better growth than feeds based on singular sources of protein (Bautista-Teruel et al., 2003). For instance, the inclusion of a mixture of corn gluten meal, silkworm pupae meal, and soy meal along with crustacean meal

has shown to improve growth performance of abalone by providing a good amino acid profile of lysine, arginine, methionine, threonine, and histidine (Cho, 2010).

Agriculture by-products have been included in aquafeeds with promising results in abalone. These ingredients include abalone silage (Viana et al., 1996), fermented fish by-products (Jung et al., 2016), feather meal (Campos et al., 2017), insect meal (Henry et al., 2018), and grape marc (Currie et al., 2019). The improved growth rate as a result of the inclusion of these ingredients is dependent on the percentage used. For instance, Guzmán and Viana (1998) showed that abalone viscera silage can entirely replace fishmeal producing comparable feed consumption rate and growth in *Haliotis fulgens*. Similarly, Jung et al. (2016) concluded that fermented tuna by-products can be included at a maximum of 21% in the whole formula without causing retarded growth and reduced weight in *Haliotis discus*. Other atypical sources, such as silkworm pupae meal, can only be included up to 16% to produce higher growth rates in *Haliotis discus* compared to other fishmeal-based feeds (Cho, 2010).

In addition to the protein, carbohydrate and lipid proportions included in the feed, other ingredients are added to improve growth performance by stimulating the immune system. These substances are called immunostimulants and have a direct effect on the immune response of the animal, leading to good growth and improved survival. Examples of immunostimulants are vitamins, minerals, nucleotides, marine- polysaccharides, prebiotics, and probiotics. Most of these compounds have been successfully included in the feed of many aquatic species, but limited research has been performed in abalone. One of the best-known immunostimulants is vitamin C. Nevertheless, the inclusion of this vitamin in *Haliotis discus hannai* has not shown significant differences in growth and survival compared to animals not receiving vitamin supplements (Mai, 1998). To our knowledge, the effect of other vitamins on abalone growth has not been studied. Other types of immunostimulants are polysaccharides. The inclusion of these polymers, such as alginate and chitosan have been tested in common carp and shrimp with successful results (Gopalakannan & Arul, 2006; Wang & Chen, 2005). However, studies on abalone species are limited. The study by Cheng and Yu (2013) is probably the only one which reported the beneficial effect of the inclusion of alginate in abalone diets. However, the results in that study were aimed to evaluate the immune capacity rather than the growth performance. The most recent immunostimulants used in aquaculture are probiotics. These bioactives have shown positive effects on abalone health and growth. For instance, Grandiosa et al. (2018) showed that *Haliotis iris* fed on a diet supplemented with multi-strain probiotic improved growth by 32.3%

and weight gain by 109.6% compared to 31.9% and 72.8% in animals fed on non-supplemented feeds. Similarly, Zhao et al. (2018) concluded that *Haliotis diversicolor* fed on a supplemented commercial feed with probiotics showed 70.51% growth rate compared to 35.73 % in animals fed on non-supplemented diet.

Different from probiotics, prebiotics have been included in aquafeeds due to its gastrointestinal benefits and easy management. Ingredients, such as grape marc could provide many benefits to aquatic species due to its high levels of non-digestible carbohydrates. For example, *Haliotis laevigata* fed on a diet with a 5–20% inclusion of grape marc showed 10–12% higher biomass and 5–6% better growth rate compared to animals fed on non-grape meal feed (Currie et al., 2019). In that study, animals consumed 9% less grape marc-based feed, yet the protein deposition and feed conversion ratio were superior. The results from that study indicate that good nutrient utilisation parameters are not necessarily linked to feed intake.

2.5 FORMULATED FEED

Most on-land abalone farms tend to use some kind of formulated feed, which in some cases also may contain a certain amount of dried seaweed or seaweed derivatives. Formulated feeds may also contain other sources of nutrients, such as fishmeal, shrimp meal or plant-based protein along with other trace and essential ingredients.

Aquafeed development is often started by designing practical diets with a single source of protein, carbohydrates, and lipids, along with minerals and vitamins premixed to enhance growth. To shorten the growth period, abalone aquafeeds may include high amounts of protein (20–50%), lipids (1–5%) and carbohydrates (30–60%) (Fleming et al., 1996). Macronutrients are usually included in high quantities in aquafeeds, which make it possible for abalone to consume the carbohydrates and lipids for energy production. This leaves all the existing protein to be used for building muscle via the ‘protein-spare effect’ (Lee et al., 2019). The right percentage of protein in formulated feeds is the amount that increases the current growth rate of 2–3 cm per year (achieved with natural seaweed), reducing the on-farm rearing period to less than 5 years (Hahn, 1989).

Protein is the most studied ingredient in aquafeed due to its high price and main role in abalone growth improvement. For a protein source to be adequate for aquafeeds, the amino acid profile of the source of protein must resemble that of the animal (Bautista-Teruel & Millamena, 1999). Additionally, the protein source must have a good digestibility index to

promote feed consumption. Sources, such as fishmeal, casein, and defatted soybean meal are commonly used due to their excellent amino acid profiles. Other protein sources, such as cottonseed, peanut meal, and canola meal, show a high digestibility index of almost 96%, which also make them suitable for aquafeeds. However, these sources of protein are not popular. From all these types of protein, fishmeal is the preferred ingredient due to its positive effect on animal growth, palatability, and nutritional value (Britz, 1996). Despite the excellent nutritional properties of fishmeal, it is considered an unsustainable source since it comes from wild fish. Nevertheless, almost 63% of the fishmeal produced worldwide is used for aquaculture purposes (Nugroho & Nur, 2018), making it scarce and expensive.

There is a wide range of carbohydrate sources that can be used in aquaculture feeds with a maximum inclusion of 50%. The most used are dextrin, glucose, rice starch, corn starch, cellulose, maltose, sucrose, cornflour, and wheat flour, which have been demonstrated to produce good growth (Lee, Kim, Kim, et al., 2017). The selection of the right carbohydrate type is based on the composition, interaction with other co-ingredients, digestibility, and price. For example, carbohydrate sources with higher degrees of polymerization are preferred for *Haliotis discus* due to the high polysaccharidase activity common for this seaweed grazers (Garcia-Esquivel & Felbeck, 2006; Thongrod et al., 2003). Carbohydrates are included for their energy supply and binding properties that ultimately influence water stability, which facilitates nutrient delivery and feed absorption.

Lipids, as with other macronutrients, are essential due to their role in reparation and generation of new tissues in aquatic animals (Lee et al., 2019). The most used lipid sources in abalone aquafeeds are single sources or combination of fish oil, soybean oil and cod liver oil. Other sources, such as microalgal lipids (Sarker et al., 2016) and insect oil (Belghit et al., 2018) have also shown good results in other aquatic species. From these sources, fish oil is the most popular ingredient in aquafeeds due to its polyunsaturated fatty acid profile, which includes eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The lipid requirements of herbivores, such as abalone, are relatively low due to a limited lipase activity found in their digestive system (Garcia-Esquivel & Felbeck, 2006). Thus, diets high in lipid content lead to low feed intake in herbivores (Thongrod et al., 2003). The maximum level of lipid inclusion depends on the lipid source. However, it is generally considered that 5% is the maximum inclusion for abalone aquafeeds (Uki et al., 1985). Higher levels of lipids deteriorate the growth of certain species, such as *Haliotis discus* (Lee et al., 2019) and *Haliotis asinina* (Bautista-Teruel et al., 2011). As Durazo-Beltrán et al. (2003) reported, *Haliotis fulgens* grew best when diets contained 1.5% lipid, and *Haliotis asinine* grew best

at a maximum of 6.1% (Bautista-Teruel et al., 2011). Abalone feeds should contain approximately 1% of omega-3 fatty acids (Uki et al., 1986) and 0.23% cholesterol to promote optimal growth (Zhang et al., 2009).

2.6 ABALONE HEALTH

Blood cells or haemocytes are the main cell types involved in cell-mediated immune responses. They possess similar functions to macrophages in vertebrates (Loker, 2010). These cells are capable of chemotaxis, antigen recognition, attachment followed by agglutination, phagocytosis, and elimination of invaders by respiratory burst or exocytosis of antimicrobial factors (Loker, 2010). Other haemocyte responses include the generation of immune mediators called chemotaxins, such as Interleukin-8, Lipopolysaccharide (LPS) and formyl-methionyl-leucyl-phenylalanine (fMLP) (Hooper et al., 2007). The humoral immune response includes lectins, lysosomal enzymes, and antimicrobial peptides.

Most of the immune responses in abalone are centred on the haemocyte. These blood cells are involved in phagocytosis, an important process which eliminates microorganisms or foreign particles (Bayne, 1990). During phagocytosis, several kinds of reactive oxygen intermediates (ROIs) are produced. These species include superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), singlet oxygen and hydroxy radical ($OH_{\cdot}$) (Klebanoff, 1982). The release of superoxide anion is known as respiratory burst, which plays a microbicidal role. Haemocytes release enzymes which also have a role in the humoral immune response. Examples of these enzymes are acid phosphatase (ACP), alkaline phosphatase (ALP), superoxide dismutase (SOD), lysozyme and phenoloxidase (Hooper et al., 2007). These enzymes are used as markers of immune response. Generally, the most common immune measurements in abalone include total haemocyte count (THC), phagocytic rate, intracellular superoxide anion, phenoloxidase activity, respiratory burst, lysosomal membrane stability (NRR time) and antibacterial activity.

Abalone immune responses are dependent on the type of stressor and the stress duration. Prolonged presence of stressors can lead to immunosuppression, which in turns can cause early mortality. Studies have shown that stressed abalone may present a transient drop in haemocyte count (haemacytopenia) (Yang & Min, 2019), increased haemocyte apoptosis and necrosis (Yang & Min, 2019), increased phagocytic activity (Cheng, Juang, et al., 2004; Cheng & Yu, 2013; Hooper et al., 2014), reduced phenoloxidase activity (Cheng, Hsiao, et al., 2004; Hooper et al., 2014), and respiratory burst.

In addition to these cell-mediated changes, there is an upsurge of antioxidant enzyme levels, such as glutathione peroxidase and superoxide dismutase under stress conditions (Vosloo et al., 2013). The high antioxidant enzyme activity serves to prepare the animal from potential oxidative stress. Superoxide dismutase (SOD) converts free radicals to H_2O_2, where-after glutathione peroxidase (GPX) and catalase (CAT) reduce H_2O_2 to H_2O and O_2 (Matés & Sánchez-Jiménez, 1999).

2.7 EFFECT OF FEED ON HEALTH

The immune markers used to evaluate the impact of environmental stressors in aquatic animals are currently used to determine the effect of aquafeeds in different animal life stages during different exposure periods. The type of nutrition affects the immune status of aquatic species as a result of the vast number of chemical feed-host interactions. Particularly, nutrition has a high impact on gastrointestinal processes, such as digestion and ingestion. Diets can provide an important proportion of the daily fibre, which stimulates the growth of gut bacteria (prebiotic) (Nayak, 2010), affect digestive endogenous/ exogenous enzymatic activity, and cell-mediated and humoral immunity responses (Erasmus et al., 1997).

2.7.1 Effects of seaweed diets on health

Seaweed diets have been shown to produce beneficial effects on animal health. These benefits are linked to the activity of the many bioactive compounds present in seaweed, such as sterols, polyphenols, fatty acids, polysaccharides, and sulphated polysaccharides. These bioactive molecules are responsible for the anti-microbial (García-Bueno et al., 2014), antiviral (V. T. Dang et al., 2011; Kitikiew et al., 2013), and anti-inflammatory effects (Chojnacka et al., 2012) in living animals (Kemp et al., 2015).

From these molecules, polysaccharides are highly valued due to their tested immunomodulatory effects. Seaweed polysaccharides are considered a potential prebiotic in aquatic animals (Mohan et al., 2019). These polymers improve intestinal health by stimulating the secretion of endogenous digestive enzymes, such as amylases (Erasmus et al., 1997), proteases (García-Carreño et al., 2003), lipases, and exogenous enzymes produced by the host microbiota. The most common non-soluble polymers in seaweeds are carragenate-oligosaccharides (COS), alginate-oligosaccharides (ALGOS), fucoidans (FUCOS), galactofucans, and laminarin (Gomez-Zavaglia et al., 2019).

Many studies have reported the positive immunomodulatory effects of extracted polysaccharides in aquatic species, such as stellate sturgeon, sea bream and European sea bass (Akrami et al., 2013; Guerreiro, Enes, et al., 2015; Guerreiro, Oliva-Teles, et al., 2015). However, only one study evaluated the prebiotic effect of dietary live seaweeds on farmed abalone. The study of Dang et al. (2011) reported that *Haliotis laevigata* fed on a combination of macroalgae (*Spyridia filamentosa* and *Ulva lactuca*) increased THC and improved the haemolymph anti-bacterial activity against *Vibrio anguillarum* and *Herpesvirus* compared to animals fed on commercial diets. Nevertheless, this combination did not generate significant changes in phagocytosis, superoxide anion, phenoloxidase levels, and haemolymph anti-viral activity. In the same study, an *Ulva lactuca* diet produced abalone extracts with higher anti-viral activity of 64% compared to 47% in abalone fed on commercial pellets. Some studies have evaluated the effect of enriched seaweeds on the immune responses of aquatic animals, such as rainbow trout (Araújo et al., 2016) and Nile tilapia (Valente et al., 2016). However, the literature on gastropod immune activity is still limited. The only study available reported that the cell-mediated immune response in abalone fed on enriched *Ulva* sp. had a stronger antioxidant potential (FRAP – Ferric ion reducing antioxidant power) and higher superoxide dismutase (SOD) activity compared to animals fed on commercial diets (Stone et al., 2014)

2.7.2 Effect of formulated feeds on health

There is a wide range of ingredients included in aquafeeds that can assist in promoting health and boosting growth simultaneously. Seaweed meals, seaweed extracts, microalgae extracts, probiotics, postbiotics, symbiotics, prebiotics, nucleotides, vitamins, minerals, and antioxidants are some examples of the ingredients that can both replace traditional ingredients and supplement aquafeeds for specific purposes in farms.

Seaweed meals have been included in aquafeeds in the past as attractants. However, their inclusion has recently shown positive effects on the general welfare of abalone. One of the effects of seaweed meal inclusion is on the abalone bacterial gut environment as reported by Nel, Pletschke and Britz (2017). In their study, the inclusion of kelp up to 3.5% in formulated diets for *Haliotis midae* promoted a more balanced gut bacterial composition than in animals fed non-supplemented feeds. The researchers found that the main components of the abalone gut microbiota were Proteobacteria and Tenericutes, which can be modified by the presence of *Mycoplasma* sp. when animals are exposed to a kelp-supplemented diet. The authors claimed that kelp diets promoted a more balanced and regulated gut bacterial environment. Another effect of seaweed meal inclusion

is the effect on digestive enzyme activities. The inclusion of 5% of *Ulva* sp. meal in feeds for *Haliotis laevigata* produced higher trypsin activity levels, an indicator of improved digestion and growth, compared to animals fed non-supplemented feeds (Bansemer, Qin, Harris, Schaefer, et al., 2016).

The inclusion of probiotics and prebiotics has been widely used in aquaculture with outstanding results during the last decade. Probiotics have been included in abalone aquafeeds not only to promote growth, but also to boost immunity and antioxidant capacity. The mechanisms of action of probiotics can be classified as (i) modifying the host-associated or ambient microbial community, (ii) improving the use of feed or enhancing its nutritional value, and (iii) improving the quality of the intestinal environment. As reported by Zhao et al. (2018), abalone fed on a probiotic-enriched diet showed a more diverse gut microbial environment which led to higher survival (Jiang et al., 2013), improved total haemocyte count, higher haemocyte viability, higher reactive species of oxygen (Grandiosa et al., 2018), lysozyme activity (Gao et al., 2018) and non-apoptotic cell levels compared fed commercial feeds without probiotics.

Prebiotics have been included in aquaculture research since the last three decades (Hanley et al., 1995), and they are defined as "*non-digestible food ingredients that stimulate the growth and activity of bacterial species in the host's colon improving the host's intestinal health*" (Gibson & Roberfroid, 1995). Prebiotics are carbohydrates which can be monosaccharides, oligosaccharides, or polysaccharides according to their molecular size (Ringø et al., 2010). By altering the gastrointestinal microbial activity and/or composition (Dimitroglou et al., 2009), prebiotics regulate the innate immune response of the host. This regulation may positively affect the animal's growth (Li & Gatlin, 2004; Staykov et al., 2007) and ability to fight pathogen infections (Wu et al., 2016). One of the advantages of prebiotics is their easy management and lower cost compared to probiotics, especially in large-scale applications. Common prebiotics used in fish aquaculture to date include inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mananoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligo-saccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO), and Grobiotic™ (Ringø et al., 2010).

Prebiotics have been tested in rainbow trout (Staykov et al., 2007), hybrid striped bass (Li & Gatlin, 2004), common carp carp (Hoseinifar et al., 2017), and shrimp (Rungrassamee et al., 2014). However, only a couple of studies have included them in abalone feeds. Meng et al. (2019) reported that *Haliotis discus hannai* fed on a diet

containing MOS, extracted from the cell wall of *Saccharomyces cerevisiae*, showed 20 times more total antioxidant capacity and three times more total superoxide (SOD) activity than animals fed non-supplemented feeds. In that study, the inclusion of MOS caused 30% less mortality when animals were exposed to *Vibrio parahaemolyticus*. Other prebiotics included in aquafeeds are β-glucans, which can be extracted from yeast, fungi, plant, and bacterial cell walls. β-glucans have not been included as a supplement in abalone feeds, but have had success in other species, such as pompano fish *Trachinotus ovatus* Linnaeus (Do Huu et al., 2016) and Nile tilapia *Oreochromis niloticus* Linn (Amphan et al., 2019). In these two species, β-glucan inclusion increased survival and resistance to bacterial infections. Although β- glucans have not been included in feeds for abalone, Wu et al. (2016) demonstrated a positive effect of the injection of β-1,3; 1,6-glucan on *Haliotis diversicolor supertexta* immune response. The study showed an increased superoxide anion and phenoloxidase response, phagocytic index, phagocytic rate, and reactive oxygen intermediates. Sodium alginate is another polysaccharide with prebiotic effects (Van Doan et al., 2016) that has been tested in some abalone species. Alginic acid, its precursor, is a polysaccharide with proven immunomodulatory activity derived from brown algae and certain bacteria. In a bacterial challenge were *Haliotis diversicolor* was exposed to *Vibrio parahaemolyticus*, Cheng and Yu (2013) reported that a 0,1–0,3% sodium alginate dietary supplement improved immune responses. The study found that animals with sodium alginate supplementation had higher phenoloxidase, superoxide dismutase, phagocytic activity, and respiratory bursts than animals fed non-supplemented feeds.

Other types of prebiotics which have demonstrated an immunostimulant effect on aquatic species are chitin and the deacetylated product chitosan. Chitin is a natural polymer abundantly found in the exoskeletons of crustaceans and insects and fungal cell walls. To our knowledge, there are no studies of dietary chitin and chitosan in abalone. However, the effects of dietary chitin and chitosan have been tested in other species, such as rainbow trout, O*ncorhynchus mykiss* (Siwicki et al., 1994) and gilthead seabream, *Sparus aurata* L (Esteban et al., 2001). These studies indicate a positive regulation between chitin/chitosan and non-specific immune responses. Although chitin or chitosan have not been included in aquafeeds for abalone, their origin sources, such as shrimp meal or insect meal, have been used. The study of Cho (2010) obtained good growth rates with the replacement of fishmeal by shrimp meal and insect meal in aquafeeds for *Haliotis discus hannai Ino.* Unfortunately, that study only evaluated growth performance.

Certain prebiotics have been successfully commercialised for their use in aquaculture. An example is Grobiotic™, a prebiotic mixture of partially autolyzed brewer's yeast, dairy ingredient components, and dried fermentation products. Although Grobiotic™ has not been included in abalone feeds, it has been included in other aquatic species, such as striped bass, *Morone chrysops* x *M.saxatilis* (Li & Gatlin, 2004), Nile tilapia, *Oreochromis niloticus* (Zheng et al., 2011) and largemouth bass, *Micropterus salmoides* (Yu et al., 2019). The study performed by Li and Gatlin (2004) showed that the inclusion of 1 and 2% of Grobiotic™ in feeds for striped bass led to higher feed efficiency and growth after 7 weeks of feeding. Additionally, animals fed on diets containing Grobiotic™ showed enhanced fish survival rates of 73–90% compared to 53% in animals fed diets without Grobiotic™ when animals were challenged with *Streptococcus iniae*. Also, Yu et al. (2019) reported that the inclusion of Grobiotic™ contributed to the abundance of *Bacillus* bacteria, which promotes disease resistance in largemouth bass. Furthermore, the study by Zheng et al. (2011) reported that the inclusion of 1.2% of Grobiotic™ improved growth rates and survival of Nile tilapia infected with *Aeromonas hydrophila*.

2.8 CONCLUSION AND FUTURE DIRECTIONS

According to the World Bank, global food demand from aquatic systems will reach 150.000 million tons in 2030, 27% more demand compared to 2010 (FAO, 2018). The increasing demand for aquaculture products is promoting the development of new feed technologies with the primary goal of attaining more growth in shorter time periods. However, with increasing consumption, food quality awareness, and sustainability, cost-effective productions are no longer the only considerations for the aquaculture industry. The studies highlighted in this review indicate that the recent focus of abalone feed research has been directed towards alternative nutritional sources and ingredients, as well as innovative techniques and applications to improve the efficiency and effectiveness of feeds leading to enhanced stock nutrition and health. However, the body of published literature also suggests that while a high number of studies have focused on the effects of feed composition on abalone growth and overall nutrition, few studies have looked at the effects of feeds on abalone health. With the increasing stocking densities and deteriorating environmental conditions associated with the continued growth of the aquaculture sector, it is expected that health parameters will have a more prominent role to play in feed formulation in the coming years.

There is no doubt that one of the main drivers of using alternative ingredients in aquafeeds is the reduction of fishmeal inclusion without compromising growth performance

and nutritional quality of abalone. Therefore, future studies should evaluate key essential metabolites in abalone, such as amino acids and fatty acids that can be impacted by different feeding approaches and ingredient replacements. In a similar fashion, future studies should include chemical profiling of the feed and its impact on abalone metabolites of special interest, such as free amino acids, volatile compounds, and organic acids which are indicators of flavour.

The use of molecular tools in nutritional studies can help address the specific effects of alternative ingredient inclusions in aquafeeds for abalone. Analytical platforms, such as GC-MS, LC-MS can be used to establish possible links between different feeding schemes and their effects on gut bacterial diversity, microbiome and metabolomic or enzymatic profile. In addition, histo-morphological studies can also be included in future research to provide insights of gastrointestinal response, such as inflammation due to the exclusive use of natural or artificial feeds. These techniques can be used to improve our understanding of the relationship between a healthy abalone production and feed requirements within commercial settings.

It is imperative that future nutritional studies in abalone are placed into a holistic framework that includes environmental sustainability of alternative ingredients along with the financial profitability of the entire production. Current methodologies to determine environmental impacts, such as life cycle assessment (LCA) have been used to assess the entire production of other aquatic species. However, such tools have not been used to evaluate abalone aquafeeds to date.

Another trend that has been elucidated from this review is that many of these studies have not been extrapolated from the laboratory to the farm, which may represent a gap in the transfer of research outcomes to the industry. Thus, it is recommended that future studies clearly outline a path for research uptake from the start, and that the abalone industry engages more closely with research experts and innovators in this field.

The application of aquafeeds in farm environments introduces other variables, such as the feasibility of feed production at an industrial scale, and ease of storage and handling which are critical for the sustainability of commercial operations. In summary, it is suggested that future research and development should focus on:

- Simultaneous evaluation of the effect of aquafeeds on animal health, growth, and nutritional quality.

- Development of long-term nutritional studies that evaluate the effect of aquafeeds across different seasons and life stages.
- Development of nutritional studies which include a solid economic assessment of the feed production costs for the farm and costs related to environmental impacts.
- Development of targeted nutritional studies, such as evaluating the effect of specific ingredients on the production of bioactive compounds in abalone, which may have specific human application.
- Inclusion of histo-morphological studies to evaluate the impact of alternative ingredients and different feeds on the digestive health of abalone.
- Inclusion of molecular tools, such as metabolomic and microbiome analysis to evaluate the effect of different feeds in abalone nutritional quality and digestive health.
- Development of nutritional studies in abalone integrated in multi-trophic aquaculture settings.

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Chapter

Nutritional and metabolomic changes of juvenile farmed abalone (*Haliotis iris*) in New Zealand

3

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3.1 ABSTRACT

Seasonal variations play a crucial role in the physiology, immune responses, and nutritional profile of aquatic animals. Unpredictable water temperature fluctuations, especially those caused by climate change, may negatively affect feed consumption and growth of cultured organisms, such as abalone. In addition, metabolic and nutritional changes across different seasons may have significant effects on aquaculture production. This study aimed to investigate biochemical and metabolic alterations in healthy abalone (*Haliotis iris*) during one year of grow-out in a land-based farm in New Zealand. Proximate analyses were used to identify nutritional variations in whole animal tissues, and a gas chromatography-mass spectrometry (GC-MS)-based metabolomics approach was used to identify metabolic changes in adductor muscle of abalone during different seasons in the one-year sampling period. Results showed that protein content was higher in warmer months compared to colder months, whereas lipid, ash, and carbohydrate contents remained generally constant throughout the year. Metabolic profile fluctuations indicated higher amounts of glutamic acid, glutathione, methionine, lysine, serine, tyrosine, and glycine in January and March (warmer months) compared to October and July (colder months), indicating possible amino acid breakdown and collagen degradation due to warmer temperatures. While the proximate analyses findings revealed no signs of nutritional deficiencies in abalone among seasons, the metabolic profiles suggested possible thermal stress during summer months. This study provides a foundation for further nutritional studies to optimize seasonal diets for farmed *H. iris*, and highlights the need to monitor thermal stress effects, especially during summer and/or heatwave events.

3.2 INTRODUCTION

The abalone industry forms part of a dynamic primary production sector that has been continuously growing due to global demands for seafood. The high nutritional quality and meat flavour of abalone make this seafood especially sought after in Asia, where it is considered a delicacy. Global abalone production has dramatically increased from 20,370 Mt in the 1970's to 73,206 Mt in 2010 and to 174,162 Mt in 2016/17 (Cook, 2016, 2019). From the total abalone production in 2015, abalone aquaculture contributed 95%, while fisheries contributed only 5% (Cook, 2016). This proportion shift from fisheries to aquaculture reflects a growing demand for reliable and sustainable products, which necessitate responsible farming practices. To this end, a significant number of innovations have been developed to enhance abalone culturing systems, allowing farms to improve production, especially in those countries where abalone farming is just starting to develop. However, one of the most significant bottlenecks of land-based abalone aquaculture is the high cost of the feed, which has been documented to be up to the 50% of the production cost (Fleming et al., 1996). Thus, there has been a growing interest in feed innovation and technology to optimise abalone nutrition according to the requirements of the species and the availability and supply of formulated and natural diets. Another challenge for the abalone aquaculture industry is the length of time required to rear individuals, which is normally 4–5 years to attain market size (Sales & Janssens, 2004). To overcome this limitation, new feed technologies have been developed to optimise nutrition and growth. These technologies use different approaches, such as feeding a combination of formulated and natural diets (Allen et al., 2006), increasing protein content in artificial feeds (Britz & Hecht, 1997), and adding specific amino acids (Venter et al., 2019) or fatty acids (Dunstan et al., 2000) to enhance the nutritional profile.

In New Zealand, the abalone industry has grown to 700 t per year in 2019 (Seafood New Zealand, 2019). This production is based on one species, the New Zealand black-footed abalone (*H. iris*), which is mainly exported to China, Japan, Hong Kong, Australia, Taiwan, Cambodia, Singapore, and Malaysia (Seafood New Zealand, 2020), generating export revenues of almost NZD 20 million in 2021 (Seafood New Zealand, 2021). While abalone land-based operations have appeared and disappeared over the years, one major farm (Moana New Zealand Ltd.) has persisted and contributes most of the production of

cocktail-sized (60–94mm) farmed abalone worth NZD 2.4 million in 2021, as well as wild caught abalone sales of NZD 22.6 million (Moana New Zealand, 2021).

Currently, farmed *H. iris* are fed artificial diets from early juveniles to adults. However, the artificial diets are not species- or season-specific and are not optimised to supply the exact nutritional requirements for different seasons. To date, only few studies have investigated the changes in nutritional profile of abalone meat as a function of the type of feed and seasonal changes. Some studies have focused on the development of formulated feeds using alternative ingredients (e.g., insect- and plant-based proteins) to enhance growth and nutritional composition. For example, Bautista-Teruel and Millamena (1999) reported that the growth of *Haliotis asinina* was faster on artificial feeds compared to natural feeds due to the high levels of protein and more balanced amino acid profile. Other studies have also shown that growth is enhanced when the amino acid profile in the feed formulation resembles the amino acid profile of animal's tissues (De Silva & Anderson, 1994). Mai et al. (1994) determined that the presence and amount of some essential amino acids, such as arginine, methionine, and threonine, in diets directly affected the nutritional profile of *Haliotis tuberculata* and *Haliotis discus hannai*. In terms of lipid composition, Bautista-Teruel et al. (2011) reported that the lipid content of *Haliotis asinina* increased when animals received higher amounts of dietary lipids. Specifically, the supplementation of linoleic acid (C18:2n-6), alpha linoleic acid (C18:3n-3), and n-3 highly unsaturated fatty acids (HUFA) produced animals with higher contents of these fatty acids in the animals, which also grew faster, probably due to the contribution of these fatty acids on biomembrane composition (Sargent et al., 1999). Britz and Hecht (1997) found that the protein composition in the soft bodies of *Haliotis midae* was directly affected by the level of protein and lipids in their diets, resulting in better growth when 44% protein and 6% lipid contents were given. However, a diet containing 10% or more in lipids impaired growth (Britz & Hecht, 1997). These studies highlight the need for a deep understating of the nutritional profile of abalone species when wanting to provide the optimal feed to maximise productivity.

The environmental conditions for cultivation are also important factors to consider when optimising nutritional value and growth of abalone. For example, oxygen levels, temperature, and water chemistry may affect animals in different ways during different seasons and may also alter the composition and quality of the feed. These effects are reflected in the animal's nutritional composition, immunity (Dang et al., 2012), and growth (García-Esquivel et al., 2007). For example, abnormally high ocean water temperatures,

which have been reported in the last years due to climate change, have been associated with increased mortality in different *Haliotis* species (Cheng et al., 2004; Nguyen & Alfaro, 2020; Travers et al., 2009; Travers et al., 2008). Warmer seasons have also been found to slightly increase protein levels and decrease moisture in *Haliotis discus* (Hatae et al., 1995), whereas glycogen, an indicator of palatability in abalone meat, has been found to be higher in summer than in winter in *H. discus* (Watanabe et al., 1992). Indeed, abalone with low levels of glycogen in their soft tissues have been characterised as watery and lacking taste. In terms of amino acid profiles, the total amount of free amino acids also varies from season to season. In *H. discus,* free amino acids, such as glutamine, arginine, glycine, glutamic acid, alanine, and serine tend to be higher in animals right after summer compared to other months. Coincidentally, glutamic acid and glycine are taste-active components that are associated with *umami* taste, a reason for harvesting abalone in summer months in Japanese traditions (Hatae et al., 1995). Other nutrients, such as polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), tend to be found in higher concentrations in *Haliotis rubra* and *Haliotis laevigata* harvested in winter and spring than in summer. Conversely, saturated fatty acids are significantly higher in summer than other seasons (Su et al., 2006).

From a sustainability point of view, information on the nutritional profile of commercial aquatic species over different seasons is relevant to the development of a more sustainable aquaculture industry. This knowledge is particularly important during the juvenile phase, when animals experience rapid growth and start to build resources for reproduction. Thus, farmers aiming to improve and optimise production practices may adjust the inclusion of certain ingredients or bioactives in artificial feeds accordingly. Furthermore, the effect of specific feed ingredients on the performance of abalone may be of interest when testing alternative sources of proteins replacing fishmeal for more sustainable aquafeeds.

Previous nutritional studies have evaluated the benefits of macronutrients, such as lipids, carbohydrates, and proteins as well as amino acids, fatty acids, vitamins, and minerals in molluscan species, such as mussels (Fernández-Reiriz et al., 1996; Okumuş & Stirling, 1998; Orban et al., 2002) and clams (Beninger & Lucas, 1984). For abalone, nutritional profiles have been well documented for *Haliotis diversicolor* (Chiou et al., 2001), *H. discus* (Hatae et al., 1995), *Haliotis laevigata* x *Haliotis rubra* (Mateos et al., 2010), *H. rubra* and *H. laevigata* (Su et al., 2006), but limited information is available on the nutritional profile of *H. iris*.

The nutritional status of a species can be investigated with a range of analytical techniques, such as proximate analysis and metabolomics. Proximate analysis provides a general overview on macronutrient levels within broad types of compounds (e.g., lipids, proteins, carbohydrates), whereas metabolic profiling provides a snapshot of endogenous metabolites, such as amino acids, fatty acids, organic acids, and other biomarkers involved in biological processes (Nguyen & Alfaro, 2020). Previous studies have successfully demonstrated the effectiveness of using metabolic profiling techniques to investigate the nutritional state of abalone stocks within different cultured conditions, such as diet quality (Shen et al., 2018; Venter et al., 2019), water temperature (Nguyen & Alfaro, 2020), and handling (Nguyen et al., 2021). Such studies provide valuable information about the capacity to optimise nutritional state and profitability in aquaculture settings using these techniques. Thus, the aim of this study is to evaluate the nutritional status and metabolic profiles in juvenile New Zealand black-footed abalone (*H. iris*) within farm conditions across seasons. To this end, we applied traditional proximate analyses and novel metabolomics techniques to identify animal growth and meat quality parameters and their variations over different seasons.

3.3 MATERIALS AND METHODS

3.3.1 Experimental Setup

This study was conducted within a commercial abalone (*H. iris*) farm at Moana New Zealand Limited, Ruakaka, Northland, New Zealand. Healthy juvenile abalone (1-year old) were randomly selected from the farm stock for this experiment. The animals ranged in size from 21 to 38 mm in shell length and had wet weights of 1.2 to 5.9 g. About 500 animals were placed within each of three experimental cylindrical tanks (85 cm radius x 85 cm height x 7 cm of water coverage), which was consistent with the culturing densities at the farm. Densities of tanks were maintained throughout the study, by replacing dead or sampled abalone with the ones from a stock tank designated at the beginning of the trial. A total of 50 animals from each tank were tagged with a special tag designed in the farm (vinyl tags with a spring). Tags were adhered and embedded into the shell with the aid of forceps. The tagging process was performed over one day and the water temperature was 15.7 °C.

The tanks were all linked to the farm's recirculating water system with 100% water exchange every 4 hours with filtered seawater (60-micron filter). The water flow through the

containers was at a rate of 10 L/min, which equates to a total water exchange of 12 times per hour. The tanks were drained and cleaned once a month. Briefly, the stopper at the bottom of the tank was pulled off from the tank, and with the tipping action of the water supply faeces, debris and uneaten food residues were flushed out. Animals remained in the tank while cleaning was performed. Water temperature was measured according to the farm procedures with an electronic device that monitored water temperature every ten minutes per day for the whole farm. Water pH and dissolved oxygen was measured in the experimental tanks with a multiparameter device every day and the values were maintained within 7.9–8.1 and dissolved oxygen of > 99%, respectively. Mortalities were recorded daily, by inspection of animals in the tank.

At the beginning of the twelve months of sampling (October 2019) abalone started being fed with a commercial diet (Marifeed® S34) according to their body weight of 1.11–1.25% per day. Feeding rate was adjusted according to the higher temperature, especially during December, January, February being increased by 10% of the initial proportion of feed. Abalone were fed once every other day in the morning.

3.3.2 Sampling of Animals

Every four weeks (Nov–19, Dec–19, Jan–20, Feb–20, Mar–20, Jun–20, Jul–20 and Oct–20) tagged abalone were measured. April, May, August, and September 2020 measurements did not occur due to COVID lockdowns. For growth measurements, removal of tagged abalone from tanks was performed with the aid of a blunt knife by carefully lifting the foot off the surface of the tank. Then, the animals were dried with paper towels, and their maximum shell lengths and widths (mm) and total animal wet weights (g) were recorded. Lengths were measured with a vernier calliper (Mitutoyo 0–125mm, Warwickshire, UK) to the nearest 0.1 mm, and weights were measured with a digital balance to the nearest 0.1 g.

At each sampling point, 20 animals per tank were collected for proximate analysis and seven animals in total for metabolomic analyses (Figure 3.1). For proximate analysis, the whole soft body was collected and placed in dry ice (-80 °C) until further processing. For metabolomic analyses, the adductor muscle was dissected, placed into 2 mL cryovial (Biostor™), quenched in liquid nitrogen for 10 min and then stored in dry ice for

transportation (2h drive) to the -80 °C freezer located at the Auckland University of Technology (Auckland, New Zealand) where samples were stored until further analysis.

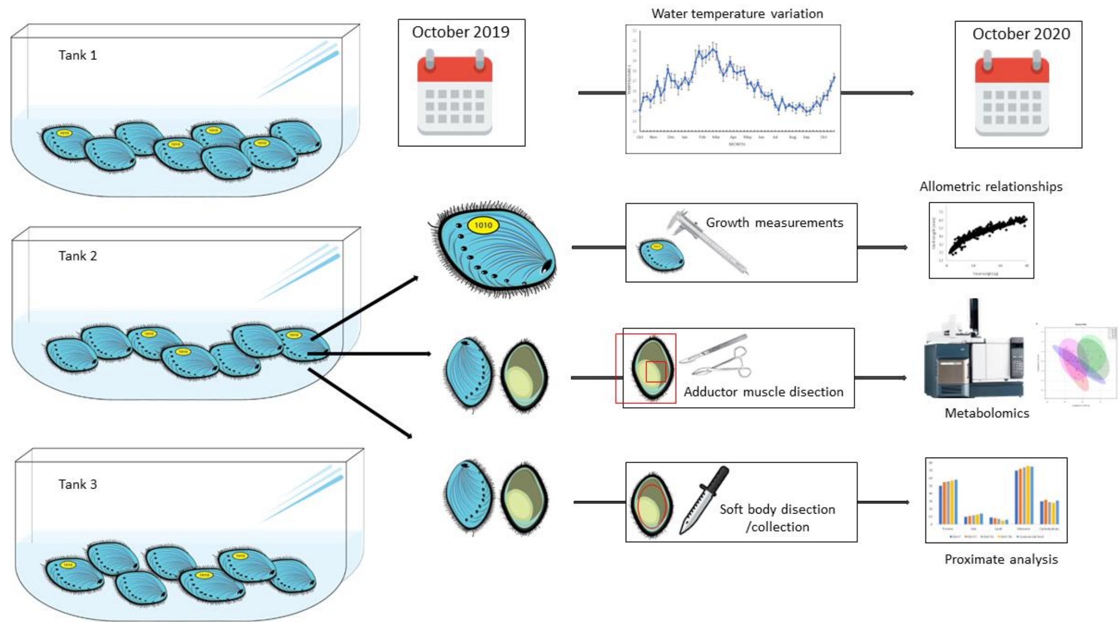

Figure 3.1 Summary of the experimental workflow

3.3.3 Growth parameters

Tagged animals were used to provide information about growth with the following equations:

$$Weight\ gain = \frac{final\ weight - initial\ weight}{initial\ weight}\ x\ 100$$

$$Shell\ gain = \frac{final\ shell\ length - initial\ shell\ length}{initial\ shell\ weight}\ x\ 100$$

Specific growth rates were calculated to determine the effectiveness of animal growth using the following equation:

$$SGR\ (specific\ growth\ rate)\ \left(\frac{\%}{day}\right) = 100\ x \frac{Ln\ average\ final\ weight - Ln\ average\ initial\ weight}{number\ of\ days}$$

Daily growth rate was calculated to describe the longitude of growth as:

$$DGR\ (daily\ growth\ rate)\left(\frac{\mu m}{day}\right) = 1000\ x\ \frac{Final\ shell\ length - initial\ shell\ length}{number\ of\ days}$$

Muscle yield (%):

$$MY\ (\%) = (muscle\ weight\ \div\ total\ body\ weight)\ x\ 100\%$$

Condition factor (CF) is an index to evaluate the relationship between the weight of the abalone per unit shell length (Dlaza et al., 2008). It was calculated following Britz (1996)

$$CF = \ [5.575\ x\ (final\ weight\ \div shell\ length\ \hat{}2.99)]$$

3.3.4 Proximate composition analyses

Proximate analyses were conducted on the commercial feed and animal soft tissues to obtain crude protein, crude lipid, ash, and moisture contents following AOAC (1985). For moisture determination, three replicates of frozen tissue from each tank were freeze-dried (Christ alpha series freeze dryer, Osterode am Harz, Germany) for 48 h. Then, the dried tissues were ground up to powder using a grinder (IKA A11 model analytical mill, Germany) with the addition of liquid nitrogen to avoid denaturation/oxidation of metabolites. The Kjeldahl method was used for crude protein determination and the Bligh and Dyer method (1959) for crude lipid quantification, adapted for small samples. For crude protein extractions, 0.2 ± 0.02 g of dried abalone samples was used with a factor of 6.25 for nitrogen conversion. Briefly, the samples were digested in Velp tubes with 7 g of catalyst (9:1 w/w mixture of potassium sulphate and cupric sulphate) and 10 mL of concentrated sulfuric acid. The tubes with samples and blanks (no sample material) were placed in a digestor (Velp Scientifica Ltd. Usmate, Italy). First, all samples were boiled at 420 °C for 1 h and once cooled, diluted with 40 mL distilled water. The samples were distilled in a Kjeldahl system with NaOH 35% and boric acid as indicator. The distilled solution was collected in a 250 mL conical flask where the indicator was placed, and the distilled ammonia was received until the solution was 150 mL. The change of colour from red to green indicated that the collection of ammonia was successful. Titration was performed with standardized 0.1M HCl solution. The percentage of crude protein was calculated with the formula:

$$Crude\ protein\ \left(\frac{g}{kg}\right) = 6.25\ x\ 100\%\ x\ \frac{(Vs - Vb)x\ Conc.HCl\ standard\ (0.1M)\ x\ NaOH\ molar\ (14.01)}{m\ (sample)g}$$

V_b = amount of standard 0.1 M HCl solution used in the blank titration, V_s = amount of standard 0.1 M HCl solution used in the sample, S = sample weight (g) and 6.25 = conversion factor to determine protein percentage in meat.

A lipid extraction method was modified for small samples (Bligh & Dyer, 1959). Dried abalone soft tissue (0.1 g) samples were hydrated in 0.8 mL distilled Millipore water and 3 mL mixture of methanol (2 mL) and chloroform (1 mL) in 15 mL screw-top glass tubes. A vortex was used for 2 min to enhance the extraction process. After this time, 1 mL chloroform was added and vortexed for 30 s. One mL Millipore distilled water was added after this time and mixed again for 30 s. The final solution was centrifuged for 5 min at 3000 rpm. The lower organic solvent layer was collected into a pre-weighted 8 mL glass tube. A nitrogen stream was used until all solvent was vaporized. Finally, the net weight of the lipids in the sample was recorded.

The ash content was obtained by complete combustion in a furnace at 550 °C for 6 h. Carbohydrate content in dried samples were calculated as follows:

$$\% \, carbohydrate \, (\, in \, dry \, tissue) = 100\% - (protein \, \% + ash \, \% + lipid \, \% \,)$$

3.3.5 Metabolomic analysis

Seven abalone at each sampling point were analysed. Five sampling points (Oct–19, Jan–20, Mar–20, Jul–20, and Oct–20) were examined to reflect endpoints for summer (Jan, Mar), winter (Jul) and spring (Oct) seasons. Samples of abalone adductor muscle were freeze dried for 48 h (Christ alpha series freeze dryer, Osterode am Harz, Germany) and ground up using a mortar and pestle into fine powder before analysis. Metabolite extractions were derivatized via methyl chloroformate (MCF) alkylation, following Nguyen et al. (2018). Briefly, 7 mg of powdered tissues were slowly thawed on ice and mixed with 20 μL of d_4-alanine (10 mM) as an internal standard. Extractions were performed using 400 μL of cold (-20 °C) 50% MeOH:H_2O solution. The mixture was vortexed vigorously for 2 min using a tissue homogenizer, frozen in dry ice and then thawed again. Extracts were cold (-6 °C) centrifuged at 2500 rpm for 5 min at 4 °C (Hermle laboratories, Model Z216MK, Germany) and the supernatants from the extractions were collected in 1.5 mL Eppendorf plastic vials placed on dry ice. Similarly, the second extraction was performed with 400 μL of cold (-20 °C) 80% MeOH:H_2O. The supernatant was collected and mixed with the previous supernatant from the first extraction on ice.

Derivatized samples were transferred into 2 mL amber GC-MS glass vials fitted with 300 µL inserts with bottom springs (Sigma- Aldrich, St. Louis, MO, USA) and then analysed on an Agilent 7890B gas chromatograph (GC) coupled to an Agilent MSD5977A mass spectrometer detector (Agilent Technologies, CA, USA), with an electron ionisation (EI) source operated at 70 eV. The system was equipped with a ZB-1701GC capillary column (30 m x 250 µm internal diameter x 0.15 µm film thickness with a 5 m guard column) (Phenomenex, Torrance, CA, USA). The instrument parameters were set according to Smart et al. (2010). The detailed protocol is accessible through Nguyen et al. (2021).

Different types of quality controls (QC) were used to guarantee reproducibility of GC-MS measurements, including d_4-alanine, blank samples, and pooled biological QC samples from all samples after extraction. Blank samples contained only 20 µL of 10 mM d_4-alanine. Blank samples and pooled QC samples were extracted and derivatized with the other samples. For QC purposes, chloroform solvent and non-derivatized n-alkanes (C10–C40) were injected at the beginning of the analysis. This was followed by pooled QC samples and blank. The samples were injected in a random fashion after QCs. Injections of pooled QCs were repeated after every 5 samples. On the final day of the analysis, all pooled QC samples were run again to compare with the previous days.

3.4 STATISTICAL ANALYSES

For size measurements, simple linear regression was used. For proximate composition, data followed a normal distribution (Kolmogorov-Smirnov test, $p < 0.05$) and were statistically treated by one-way ANOVA and Tukey's test was applied for multiple comparison of means at a $p < 0.05$ on the statistical package XLSTAT (Addinsoft, Version 2022.3.1). For ANOVA purposes, month of sampling was considered a fixed factor and tank a random factor.

For metabolomic profiling, statistical analyses were performed using the integrated web-based platform MetaboAnalyst 5.0 (metaboanalyst.ca). Data were normalized by auto-scaling (mean-centered and divided by the standard deviation of each variable). A one-way ANOVA ($p <0.05$) was used to assess effects of months on abalone metabolite profiles. Chemometric analysis via partial least squats- discriminant analysis (PLS-DA) was performed to facilitate visualization of the major trends. A heatmap of detected metabolites in adductor muscle was generated to visualize differences.

3.5 RESULTS

3.5.1 Water temperatures

The water temperatures showed weekly and seasonal variations, with a minimum of 10.6 °C in Oct–19 and a maximum of 21.9 °C in Jan–20 (Figure 3.2). Temperatures fluctuated from 10.6–19 °C in spring 2019 (Oct, Nov, Dec), 15.7–21.4 °C in summer 2020 (Jan, Feb, Mar), 14.1–19.7 °C in autumn 2020 (Apr, May, Jun), 12.9–16.2 °C in winter 2020 (Jul, Aug, Sep), and 13.3–18.1 °C in spring 2020 (Oct 20).

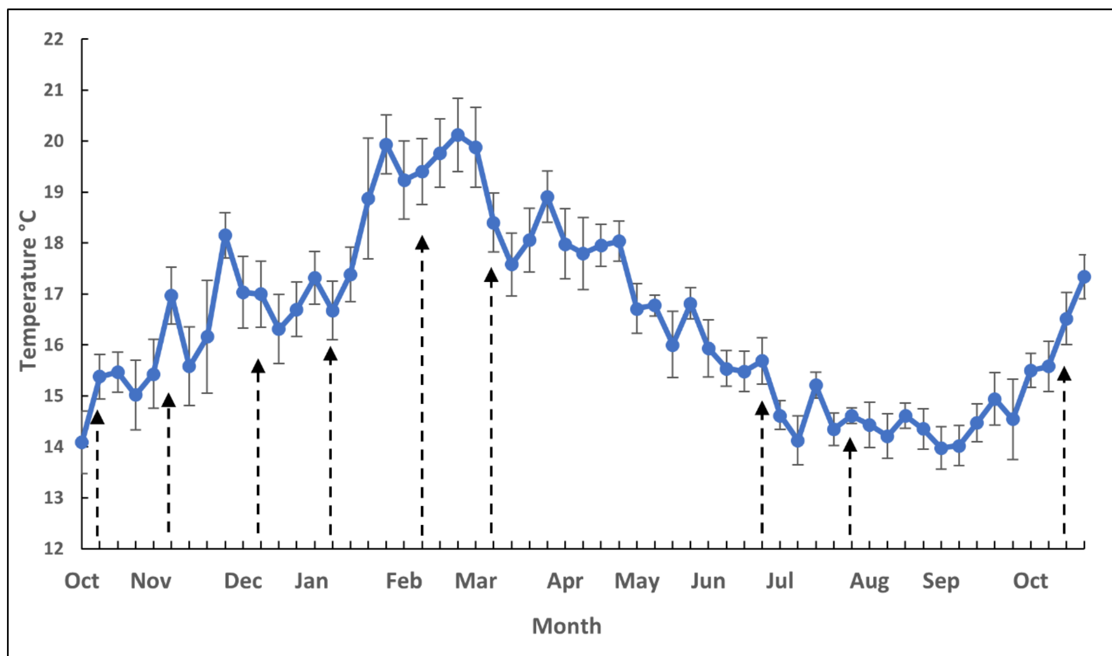

Figure 3.2 Average weekly water temperatures in the system from October 2019 to October 2020. Error bars denote minimum and maximum weekly temperatures. Dotted arrows indicate sampling events.

Table 3.1 Monthly water temperatures during the study period

Month	Min °C	Max °C	Mean ± SD
Oct–19	10.6	16.3	15 ± 0.7
Nov–19	14	19	16.6 ± 1.3
Dec–19	15.2	19	16.9 ± 0.8
Jan–20	15.7	21.9	17.9 ± 1.3
Feb–20	17.4	21.6	19.6 ± 0.8
Mar–20	16	21.4	18.6 ± 1.1
Apr–20	16	19.7	18.1 ± 0.8
May–20	14.6	18.7	16.8 ± 0.8
Jun–20	14.1	17.2	15.7 ± 0.5
Jul–20	12.9	15.8	14.6 ± 0.5
Aug–20	12.9	15.2	14.4 ± 0.4
Sep–20	13	16.2	14.3 ± 0.6
Oct–20	13.3	18.1	15.9 ± 0.9

3.5.2 Survival

Abalone survival was relatively high during the one-year grow-out period, with an overall average of 86% from the beginning to the end of the study (Figure 3.3).

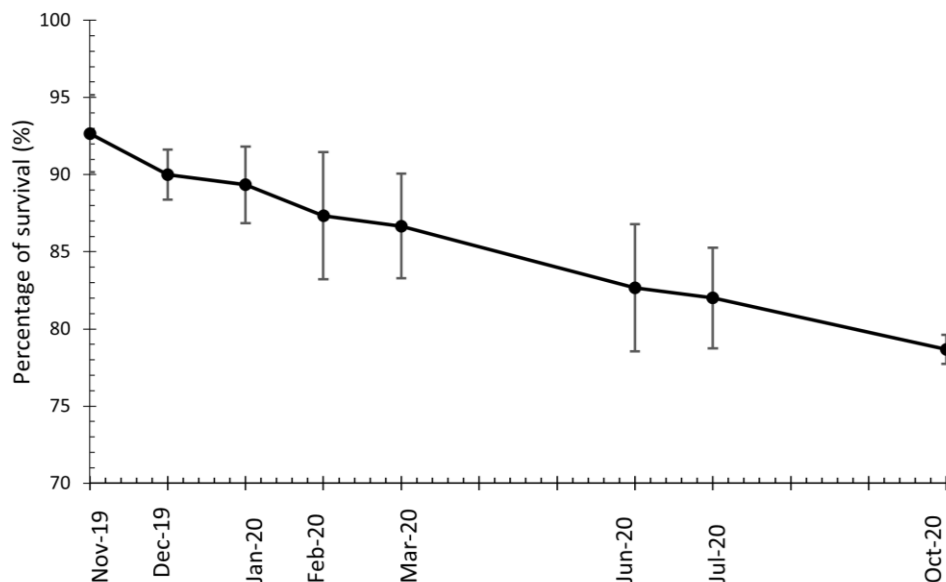

Figure 3.3 Survival percentages for farmed abalone during one year of grow-out. Data represents means and bars standard deviation (n = 500 animals per tank).

3.5.3 Growth parameters

The size relationships showed expected results based on normal growth behaviour of molluscs (Figure 3.4). Most of the relationships clearly showed that while animals mature, the variability of sizes and weight increased.

Relationships such as shell length – total weight and shell width – total weight showed a low r^2 indicating a wide range of weights in animals with the same shell length or width. In addition, in the shell weight – shell length relationship ($r^2 = 0.9216$), shell weight was more variable as the shell increased in size. The relationship shell weight – total weight ($r^2 = 0.9835$) indicated a reliable regression model. Similarly, the relationship soft body weight – total weight ($r^2 = 0.9967$) showed that soft body weight is a good predictor variable that explains 99.7% the total weight. Animals showed an overall SGR% of 0.82 ± 0.08, an overall shell length gain (%) of 93.33 ± 28.51 and a total weight gain (%) of 665.48 ± 234.13 (Table 3.2). The highest SGR% per day was in Nov–19 (0.72 ± 0.27 %) and Dec–19 (0.74 ± 0.25 %) compared to the lowest value in Jul–20 (0.42 ± 0.23 %). The highest weight gain occurred in Nov–19 and the lowest in Jul–20. In addition, animals showed an overall shell length gain of 70.44 ± 14.00 µm/day. The highest shell length gain was observed in Jan–20 with 8.87 ± 3.94 % followed by Nov–19 with 7.48% ± 3.86 and the lowest gain was in Jul–20 with 3.29 ± 1.69 %. Daily growth measurements revealed that animals grew at the highest rate of 101.68 ± 44.42 µm/day during Jan–20 and at a lowest rate of 54.52 ± 27.34 µm/day during Jul–20.

The soft body: shell ratio (SB/S) ranged from 3.72 ± 1.27 in Oct–19 to 2.27 ± 0.17 in Oct–20, the condition factor (CF) ranged from 0.64 ± 0.04 in Oct–19 to 0.67 ± 0.06 in Oct–20, and the muscle yield ranged from 77.15 ± 4.04 in Oct–19 to 69.35 ± 1.64 in Oct–20 (Table 3.3). The SB/S and CF showed that animals had heavier soft bodies in the first months (Oct–19, Nov–19, Dec–19, Jan–20) compared to the last months (Feb–20, Mar–20, Jun–20, Jul–20, and Oct–20).

Table 3.2 Growth measurements of *H. iris* during one year of grow-out.

Growth measurements	Nov-19	Dec-19	Jan-20	Feb-20	Mar-20	Jul-20	Total (Oct-19-Oct-20)
Initial weight (g)	2.70 ± 0.73	3.47 ± 0.96	4.19 ± 1.16	5.01 ± 1.40	6.14 ± 1.80	12.25 ± 3.62	2.70 ± 0.73
Final weight (g)	3.47 ± 0.96	4.19 ± 1.16	5.01 ± 1.40	6.14 ± 1.80	7.14 ± 2.13	13.88 ± 3.95	19.93 ± 5.63
Weight gain per day (%)	2.22 ± 1.07	2.84 ± 1.28	2.78 ± 1.99	3.40 ± 1.75	3.45 ± 2.07	5.43 ± 3.22	658.94 ± 243.54
Weight gain (%)	29.40 ± 12.26	23.36 ± 8.45	17.97 ± 9.96	23.71 ± 10.28	15.79 ± 8.82	12.72 ± 7.52	665.48 ± 234.13
Initial length (mm)	28.35 ± 2.78	30.35 ± 2.76	32.15 ± 3.07	34.88 ± 3.63	37.07 ± 3.92	47.16 ± 4.97	28.35 ± 2.78
Final length (mm)	30.35 ± 2.76	32.15 ± 3.07	34.88 ± 3.63	37.07 ± 3.92	39.45 ± 4.11	48.02 ± 4.76	55.00 ± 5.78
Shell length gain per day (µm)	59.33 ± 29.39	87.20 ± 14.87	101.68 ± 44.42	61.35 ± 33.99	89.90 ± 41.17	54.52 ± 27.34	70.44 ± 14.00
Shell length gain (%)	7.48 ± 3.86	6.32 ± 3.32	8.87 ± 3.94	6.20 ± 3.58	6.89 ± 3.42	3.29 ± 1.69	93.33 ± 28.51
SGR (%) day	0.72 ± 0.27	0.74 ± 0.25	0.58 ± 0.30	0.60 ± 0.24	0.51 ± 0.27	0.42 ± 0.23	0.82 ± 0.08

Data represents means and standard deviations (n = 150). Abbreviations: SGR = Specific Growth Rate (% body weight per day).

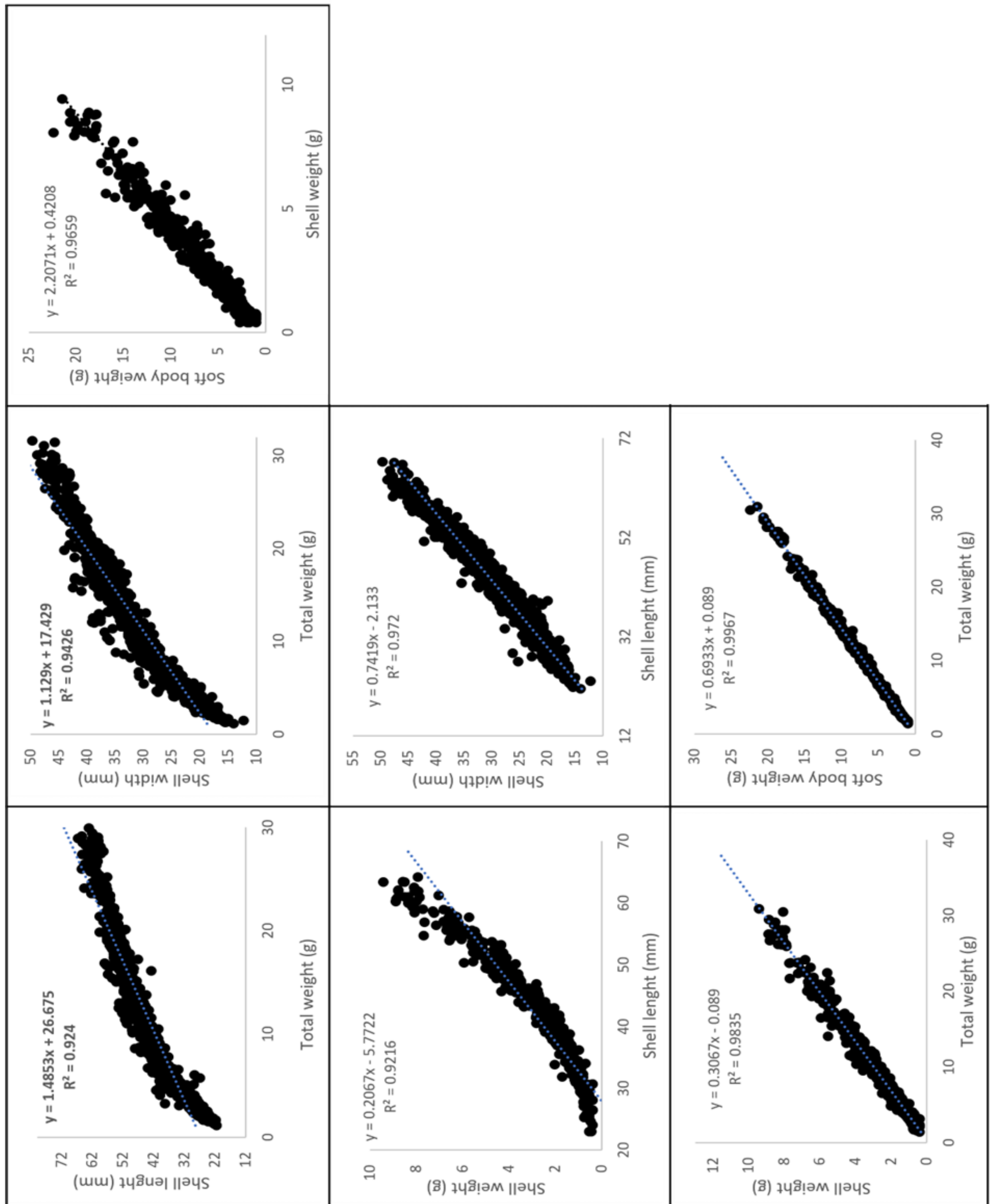

Figure 3.4 Growth relationships for farmed abalone from October 2019 to October 2020.

Table 3.3 Shell weight, shell length, shell width, soft body weight, soft body: shell ratio, and condition factor of *H. iris* collected in different months October 2019-October 2020.

Growth parameters	Oct-19 (Baseline)	Nov-19	Dec-19	Jan-20	Feb-20	Mar-20	Jun-20	Jul-20	Oct-20
Wet shell weight (mg)	0.61 ± 0.16	0.93 ± 0.25	1.17 ± 0.31	1.58 ± 0.40	2.23 ± 0.44	2.67 ± 0.62	4.85 ± 1.02	5.39 ± 1.05	7.84 ± 0.86
Wet soft body weight (mg)	2.16 ± 0.66	2.60 ± 0.79	3.06 ± 0.81	4.06 ± 1.09	5.02 ± 1.19	6.15 ± 1.61	11.03 ± 2.56	12.34 ± 2.53	17.80 ± 2.21
Soft body: shell ratio SBS/S	3.72 ± 1.27	2.80 ± 0.52	2.62 ± 0.32	2.58 ± 0.27	2.25 ± 0.26	2.30 ± 0.23	2.27 ± 0.27	2.30 ± 0.26	2.27 ± 0.17
Muscle yield (%)	77.15 ± 4.04	73.14 ± 4.14	72.21 ± 2.27	71.87 ± 2.10	68.99 ± 2.55	69.53 ± 2.14	69.24 ± 2.62	69.47 ± 2.49	69.35 ± 1.64
Condition factor	0.64 ± 0.04	0.63 ± 0.07	0.63 ± 0.06	0.6 ± 0.05	0.61 ± 0.07	0.63 ± 0.04	0.66 ± 0.04	0.67 ± 0.05	0.67 ± 0.06

Data represents means and standard deviation (n= 150).

3.5.4 Proximate composition analyses

The proximate composition of the commercial feed used during the study was 34.7% protein, 5.2% lipid, and 42.6% carbohydrate.

Differences among carbohydrate, protein, lipid, and ash content in wet and dry weight of abalone meat are presented in Table 3.4. Both are presented, but only wet weight-based results are discussed. The protein levels (wet weight) in the soft tissues of abalone were significantly higher in Feb–20 (14.79 ± 0.48) and Mar–20 (14.55 ± 1.03) compared to colder months Oct–19 (12.38 ± 0.07) and Nov–19 (12.37 ± 0.76). There were no significant differences among the lipid levels (wet weight) from different months and they ranged between 1.22–1.32%. The ash content was significantly higher in Oct–19 (2.49 ± 0.01) compared to colder months Jun–20 (2.07 ± 0.11) and Jul–20 (2.24 ± 0.28). The moisture content varied from 76.57–81.03% with significantly higher values in warmer months Oct – 19 (79.21 ± 0.17), Nov–19 (81.03 ± 1.11), and Oct–20 (79.65 ± 0.90) compared to other months. The carbohydrate content varied from 2.99–5.45%. Significantly lower values were observed in Nov–19 (2.99 ± 0.34) and Oct–20 (3.27 ± 0.56) compared to other months.

Table 3.4. Proximate composition (whole body) in *H. iris* based on dry weight and wet weight (%). Data represents means and standard deviation of three technical replicates. Means with the same superscript (a, b, c) in each column are not significantly different from Tukey post hoc tests ($p < 0.05$).

Proximate composition		Oct-19	Nov-19	Dec-19	Jan-20	Feb-20	Mar-20	Jun-20	Jul-20	Oct-20
Carbohydrate (%)	dry weight	22.3 ± 0.03^{abc}	15.76 ± 1.69^{d}	22.15 ± 2.36^{abc}	20.65 ± 2.76^{abc}	19.56 ± 1.43^{cd}	20.01 ± 2.19^{bcd}	24.37 ± 6.36^{ab}	24.61 ± 2.70^{a}	16.06 ± 2.42^{d}
	wet weight	4.64 ± 0.05^{a}	2.99 ± 0.34^{b}	5.21 ± 0.81^{a}	4.64 ± 0.74^{a}	4.49 ± 0.41^{a}	4.54 ± 0.42^{a}	5.44 ± 1.59^{a}	5.45 ± 0.64^{a}	3.27 ± 0.56^{b}
Protein (%)	dry weight	59.57 ± 0.17^{cd}	65.19 ± 1.14^{a}	62.78 ± 1.92^{abcd}	62.53 ± 2.86^{abcd}	64.45 ± 1.52^{ab}	63.82 ± 2.05^{abc}	60.85 ± 6.36^{bcd}	59.48 ± 2.29^{d}	66.65 ± 2.01^{a}
	wet weight	12.38 ± 0.07^{cd}	12.37 ± 0.76^{d}	14.69 ± 0.86^{ab}	13.98 ± 0.52^{abc}	14.79 ± 0.48^{a}	14.55 ± 1.03^{ab}	13.51 ± 1.37^{bcd}	13.21 ± 1.19^{cd}	13.56 ± 0.71^{bcd}
Lipid (%)	dry weight	6.13 ± 0.16^{ab}	6.85 ± 1.19^{a}	5.53 ± 0.37^{b}	5.89 ± 0.65^{b}	5.52 ± 0.49^{b}	5.67 ± 0.49^{b}	5.49 ± 0.88^{b}	5.86 ± 0.78^{b}	5.30 ± 0.78^{b}
	wet weight	1.28 ± 0.05	1.30 ± 0.18	1.29 ± 0.14	1.32 ± 0.17	1.29 ± 0.14	1.29 ± 0.15	1.22 ± 0.2	1.3 ± 0.21	1.08 ± 0.16
Ash (%)	In dry weight	12 ± 0.33^{a}	12.20 ± 0.29^{a}	9.54 ± 0.65^{d}	10.92 ± 0.54^{b}	10.47 ± 0.47^{bc}	10.50 ± 0.30^{bc}	9.29 ± 0.34^{d}	10.05 ± 0.70^{c}	11.99 ± 0.52^{a}
	In wet weight	2.49 ± 0.01^{a}	2.32 ± 0.18^{ab}	2.24 ± 0.13^{bc}	2.44 ± 0.07^{ab}	2.4 ± 0.07^{ab}	2.39 ± 0.13^{ab}	2.07 ± 0.11^{c}	2.24 ± 0.28^{bc}	2.44 ± 0.13^{ab}
Moisture (wet weight)	-	79.21 ±0.17^{ab}	81.03 ± 1.11^{a}	76.57 ± 1.66^{b}	77.62 ± 0.89^{b}	77.05 ± 0.70^{b}	77.22 ± 1.17^{b}	77.76 ± 0.93^{b}	77.79 ± 1.68^{b}	79.65 ± 0.90^{a}
Protein: Lipid ratio	-	9.72^{b} ±0.30	9.63^{b} ± 1.02	11.42^{ab} ± 0.87	10.79^{ab} ± 1.73	11.87^{ab} ± 1.09	11.34^{ab} ± 0.98	11.34^{ab} ± 2.03	10.33^{b} ± 1.45	12.87^{a} ± 1.70

3.5.5 Metabolomics

Metabolite identification yielded 100 annotated compounds from spectra of abalone muscle tissues. Most of these compounds consisted of amino acids, organic acids, and fatty acids. The multivariate data analysis via PLS-DA score plot revealed some clear separations between sampling times through the year (Figure 3.5A). Overall, samples in October (year 2019 and 2020) were clearly distinct from other months (Jan–20, Mar–20, Jul–20). However, there was no good separation between October samples in 2019 and 2020. Similarly, there were some overlaps in distribution of samples in Jan–20, Mar–20 and Jul–20. The first two components contributed 49.7% of the total variation. The PLS-DA model cross validation *via* LOOCV showed accuracy of 0.6, R^2 of 0.83 and Q^2 of 0.61, indicating a good prediction model. Furthermore, PLS-DA analysis also identified 27 metabolites with VIP scores greater than 1 which are important classifiers (Figure 3.5B).

The univariate data analysis *via* one-way ANOVA identified 31 metabolites that were significantly different between sampling times. A heatmap of these metabolites was generated to visualize the detail differences (Figure 3.6), which divided the metabolites into 3 main clusters (A, B & C). Metabolites in cluster A are fatty acids while most of amino acids are in cluster C. Cluster B includes some amino acids, fatty acids, and organic acids. As the PLS-DA score plot, the heatmap also grouped sampling times into two main groups: The first group with October samplings (2019 and 2020) and the remaining sampling times (Jan–20, Mar–20, Jul–20) in the second group. The first sampling group (Octobers) shared a similar abundance of metabolites in cluster C, but had some differences with cluster A and B, respectively. Similarly, most metabolites in cluster C of the second sampling group (Jan–20, Mar–20, Jul–20) were similar between three sampling times while metabolites in cluster A and B of March 2020 sampling were higher than those of January and July 2020 samplings. The January and July 2020 sampling shared similar levels of metabolites identified by one-way ANOVA. On the other hand, the most obvious difference among sampling times was observed between Oct–20 and Jul–20, where most metabolites were higher in Jul–20 than in Oct–20, with exception of cysteine and aspartic acid.

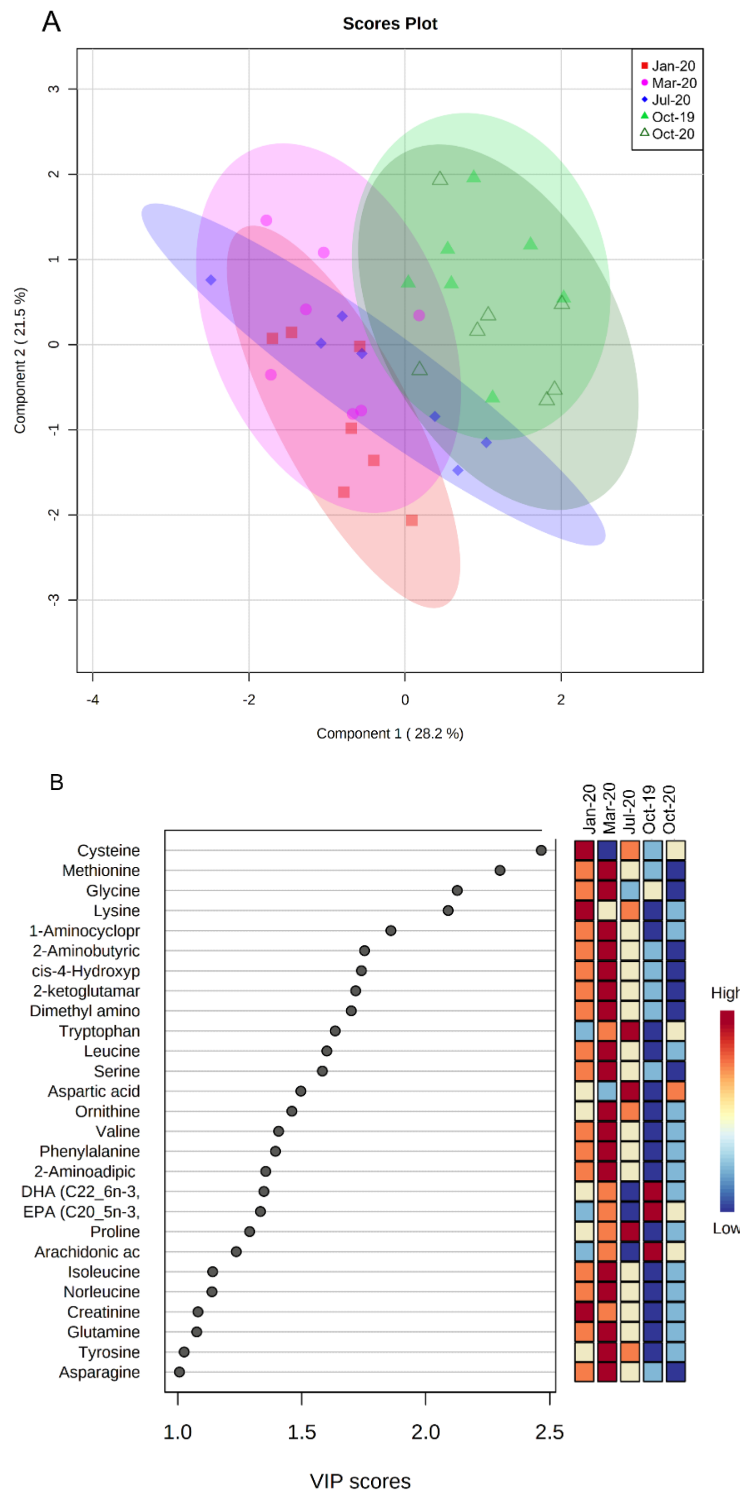

Figure 3.5 PLS-DA analysis of abalone metabolite profiles at different sampling times. A) PLS-DA score plot. B) List of 27 metabolites with PLS-DA VIP scores greater than 1.

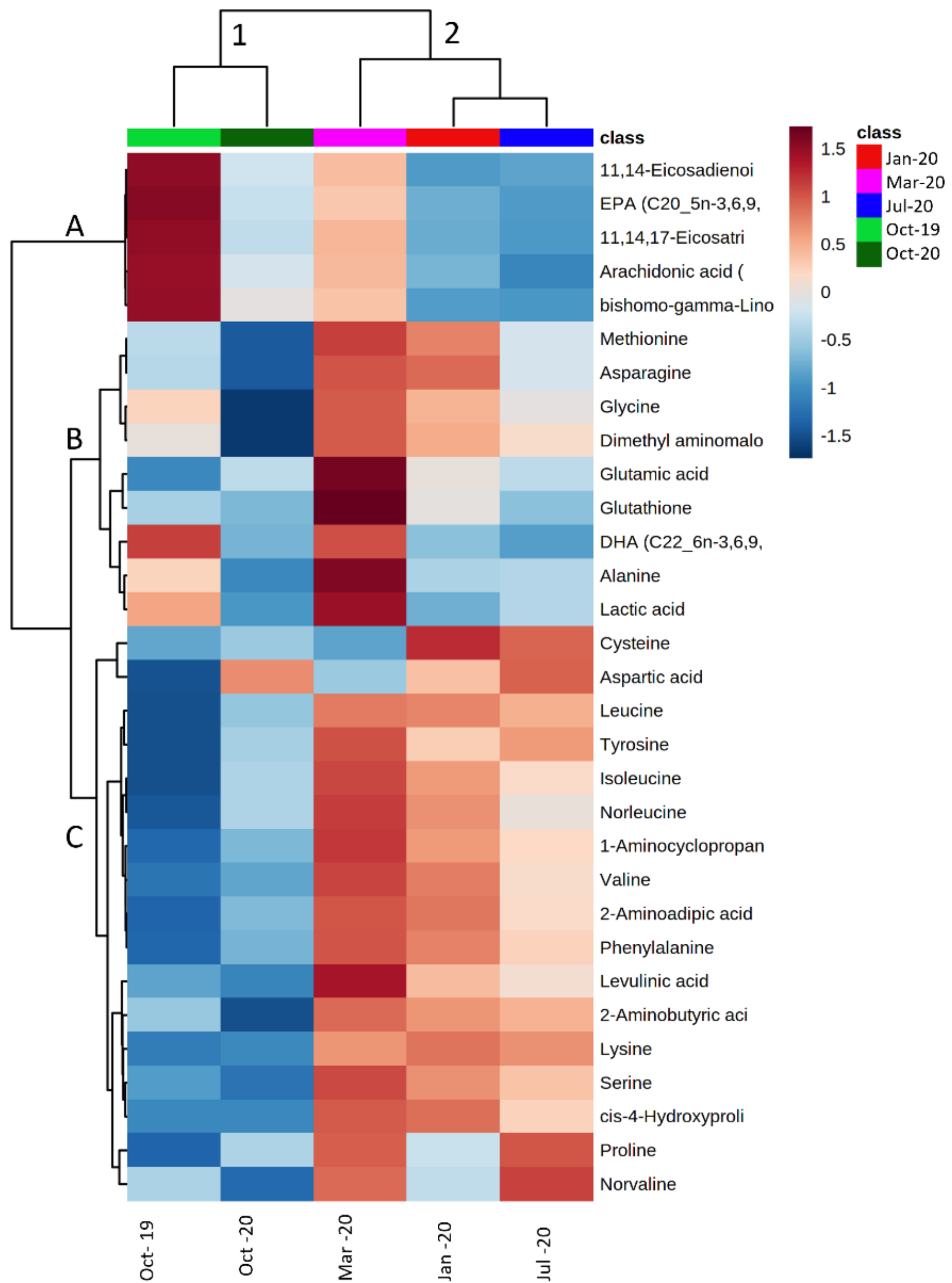

Figure 3.6 Heatmap of 31 metabolites of abalone muscle tissue different among five sampling times (one-way ANOVA, $p < 0.05$).

3.6 DISCUSSION

Growth variations revealed similar trends for *H. iris* as other *Haliotis* species with better growth in warmer water temperatures compared to colder temperatures. Our study reported an overall SGR of 0.82% per day in 12-month trial with an SGR of 0.42% in winter and 0.74% in the beginning of summer. These values indicate that *H. iris* in the North Island of New Zealand grow faster in summer compared to winter, following the trend documented for other abalone species (Troynikov et al., 1998). However, previous studies differ which season produces the most rapid growth in abalone as the variation might be related mainly to microscopic food supply coming from ocean waters (Shepherd & Hearn, 1983). The SGR values in this study are higher than the ones reported by Allen et al. (2006) in the same species with an overall SGR of 0.45–0.51% and the SGR of 0.24% and 0.52% in winter and summer respectively. This discrepancy in SGR between the two studies is expected as growth is a result of a multiplicity of factors including different feeds, species, environment, and intrinsic characteristics of the animals.

Apart from determining the growth performance of *H. iris* in a monthly basis, another objective of this study was to identify the proximate composition variation of juvenile *H. iris* during one year of grow-out. In general, many marine species show proximate composition variation relative to the season. These variations are influenced by factors such as availability of food, temperature of the water, differences in diet and physical-chemical changes brought about by the maturity of the animal (Ramesh & Ravichandran, 2010). The results of this study indicate that proximate composition of *H. iris* varies along the year with protein levels within the expected range for the species higher in summer than winter, and ash, carbohydrate, and moisture levels without much variation among months. The protein values found in this study mirror those of previous studies that have examined the effect of different feeds in proximate composition. Similar to our results, Tung and Alfaro (2011) and Allen et al. (2006) determined *H. iris* protein levels between 10.6–12.9% and 18.6–19.3%, respectively. Allen et al. (2006) used commercial feed AbFeed pellets (South Africa) and Tung and Alfaro (2011) used commercial feed Adam and Amos abalone foods (Australia). Hatae et al. (1995) reported protein levels between 14.2–18.4% in *Haliotis discus* (wild abalone).

In wild abalone, protein has been reported within 14.5–18.4 %, lipids within 0.26–0.93%, ash within 1.11–1.92% and moisture within 72.4–82.1%, all of them in a dry basis (Hatae et al., 1995). As protein levels are directly related to muscle building, their fluctuations affect the final product weight and monetary value, therefore its understanding becomes relevant to the abalone industry (Fleming et al., 1996). Significant differences in protein levels have only been reported in wild abalone (Hatae et al., 1995) and farmed abalone (Green et al., 2011; Tung & Alfaro, 2012) when exposed to different water temperatures. The protein level differences presented here might be explained by better feed intake during warmer seasons compared to winter that leads to increase protein consumption and protein animal composition. This is possible as temperature is one of the limiting factors in feed intake (Stone et al., 2013). For example, it has been observed that when temperature exceeds 26 °C, *Haliotis laevigata* feed intake is substantially reduced and abalone severely stressed (Stone et al., 2014). In addition, protein levels in the soft body of abalone are ascribed to be directly related to the dietary protein content when temperature remains the same (Stone et al., 2013). Since only one commercial feed was used during the whole trial, this may suggest that the differences are attributable to water temperature, reproductive status, and age of abalone. Further studies to evaluate the influence of these factors on the nutritional composition of abalone are recommended.

While protein was the most significant nutrient varying during the year; moisture, ash, lipid, and carbohydrate showed differences without much variation among months. Seasonal variation of moisture might indicate that *H. iris* possess higher values of moisture right before summer compared to other months. This trend has also been reported for other aquatic species, such as Atlantic Mackerel (Ramesh & Ravichandran, 2010) and blue mussel (Slaby et al., 1978). Moisture is an important attribute for juiciness and determines marketability of abalone, therefore the harvest of abalone before and during summer would be correlated to better sensory attributes. Significant moisture variations have been reported in abalone exposed to 20 and 25 °C in a laboratory setup showing higher levels in the latter (García-Esquivel et al., 2007) compared to lower temperatures, however, there are no studies outside the laboratory.

The ash content varied from 2.1–2.5% (wet weight) and was significantly lower in Dec–19, Jun–20 and Jul–20 compared to other months. These results differ from previous studies documenting ash values of 1.4% (Shi et al., 2020), 1.1–1.9% (Hatae et al., 1995), and 1.8–2.8% (Chiou et al., 2001). A possible explanation of the different mineral content

along the year might be the salinity fluctuations of coastal waters, which usually fluctuate between 34–36 g/Kg (Heath, 1985). Mineral content, most importantly sodium and potassium, increase when water salinity increases (Boamah et al., 2020).

The lipid content variations were not significant across months (wet weight). These results are similar to those reported by Hatae et al. (1995). It has been documented that lipid levels significantly change in abalone meat when lipid or carbohydrates are modified in the diet (Lee et al., 2019; Thongrod et al., 2003) or when temperatures are significantly low (Hernández et al., 2013), but no reports have documented significant seasonal lipid variation (Hatae et al., 1995).

Metabolomic profile

Another objective of our study was to identify significant metabolic variations during the year of grow-out. To our knowledge, previous studies have reported abalone metabolite profiles facing different stressors, such as heat and hypoxia (Huo et al., 2019), heat stress (Xu et al., 2020), and harvesting and transportation (Nguyen et al., 2020), but not seasonal variation in *H. iris*. In this study, metabolomic profiling showed that only 31 metabolites were significantly different at five different sampling times associated with different seasons (Oct–19, Jan–20, Mar–20, Jul–20, and Oct–20). From these metabolites, proteinogenic and non-proteinogenic amino acids, fatty acids and organic acids were identified.

Free amino acids (FAA) in *H. iris* have been mostly reported using non-GC-MS techniques such as Liquid Chromatography -Mass Spectrometry (LC-MS). So far, this is the first study evaluating the metabolomic profile of *H. iris* in different seasons. Our study shows that many amino acids such as methionine, asparagine, glycine, cysteine, aspartic acid, leucine, tyrosine, isoleucine, norleucine, valine, phenylalanine, lysine, serine, and proline were higher in warmer months Jan–20 and Mar–20 compared to other months, while glutamic acid, glutathione, and alanine were higher in Mar–20 and lower in the rest of the months. Cysteine, glutamic acid, glutathione, glycine, lysine, methionine, and serine belong to the glutathione metabolism pathway, which is important for the antioxidant and immune activities within cells. In addition, these amino acids are related to flavour and taste, and they have been documented to increase when abalone is exposed to mild stressors, such as transportation (Alfaro et al., 2021). Possibly, higher water temperatures might stress abalone's metabolism favouring the increased levels of flavour-related free amino acids which are an indicator of the best time for harvesting. From all metabolites contributing to

flavour, glutamic acid is considered the strongest whereas glycine provides a fresh sweetness flavour. Both metabolites contribute to the *umami* flavour and sourness (Cochet et al., 2015), and their levels have been documented to increase during summer in *H. discus* (Hatae et al., 1995), *Haliotis diversicolor* (Chiou et al., 2001) and other mollusc species such as oysters (Gao et al., 2021).

This study documents the higher levels of valine, serine, and proline in summer compared to winter, which is similar to *H. discus* (Hatae et al., 1995). Although more data introducing other variables is needed to establish a clear link between the season and the levels of free amino acids, warmer months produced higher fluctuations of amino acids related to flavour, tenderness, and taste. Decreased levels of amino acids during colder months might reflect amino acid breakdown and their usage for energy supply (Venter et al., 2019) due to high energy demands in winter (Alfaro et al., 2021). Valine is an essential amino acid, and its accumulation is associated with altered protein turnover and thermal stress (Tripp-Valdez et al., 2019). Proline is a non-essential amino acid that accumulates in response to temperature variations and osmotic stress. In contrast, its reduced levels are seen when protein catabolism takes place for energy production (Venter et al., 2019). In addition, proline, hydroxyproline and glycine are the main degradation products of collagen and their increment during summer months Jan–20 and Mar–20 might indicate more tenderness of the meat. Based on a previous work by Hatae et al. (1995) on *H. discus*, collagen degradation products were higher in summer compared to winter, leading to a more tender carcass.

Other amino acids such as leucine, isoleucine, aspartic acid, tyrosine, and phenylalanine were found to increase in warmer compared to colder months. These compounds, which are mostly linked to osmoregulation, have been found to be elevated in thermal stress and hypoxia (Lu et al., 2016; Nguyen et al., 2021), transportation (Alfaro et al., 2021), and muscle damage (Bautista-Teruel et al., 2003), but to our knowledge no elevation has been reported in different seasons. From these amino acids, aspartic acid has been suggested to be an important biomarker of stress and health status in *H. iris* (Alfaro et al., 2021). However, its levels have not been found elevated in summer in our study suggesting that thermal stress did not occur during this period. To elaborate further in this premise, further research should be done to investigate the impact of seasons on abalone nutritional composition in studies longer than 1 year. Significant elevated free amino acids such as arginine, glycine and a lesser quantity of methionine, glutamic acid, and alanine

account normally for 81–94% of the total free amino acids in abalone muscle (Chiou et al., 2001). Their main role is osmoregulation; therefore, their levels fluctuate according to season (Hatae et al., 1995), developmental stage (Litaay et al., 2001), feed (Bautista-Teruel & Millamena, 1999; Mai et al., 1994), and culture setup (Bewick et al., 1997; Latuihamallo et al., 2015).

In our study, some key fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) showed high levels in October 2019, but low levels in October 2020. The decreasing levels of these metabolites along the months is unclear and might not be directly related to the season. These changes can also be associated to many other factors such as developmental age of the animal (Lee et al., 2009), farm conditions (aquaculture setup), and muscle disposition preparing for maturity which were not controlled in this study. In terms of seasonality, previous studies have reported different results; for example, Su et al. (2006) concluded that abalone had higher levels of n-3 polyunsaturated fatty acids (PUFAs) in winter compared to summer. However, a previous study performed by the same research group concluded no significant differences in these metabolites in Australian abalone during different seasons. The available evidence on fatty acid composition in abalone do not appear to be uniform as their levels might be linked not only to temperature fluctuations, but also to diet composition (Bautista-Teruel et al., 2011), culture system (Latuihamallo et al., 2015), and life stage. Our findings agree with other studies concluding EPA, ARA and DHA are the most relevant PUFAs in this species. In fact, abalone is a rich source of n-3 PUFAs, such as EPA, DHA and docosapentaenoic acid (DPA) (Su et al., 2006) and n-6 PUFAs, such as linoleic acid (LA) and arachidonic acid (ARA) (Su et al., 2001).

EPA and ARA, relevant n-3 PUFAs for maintaining the membrane fluidity under lower temperatures in aquatic animals (Toledo-Agüero & Viana, 2009) were reduced during winter (Jul–20) and after winter (Oct–20). The decreased levels of EPA and ARA in our study might be related to the higher expenditure of these metabolites for abalone metabolism and its reduction on muscle concentration. As poikilotherm, abalone does not possess a temperature regulation system and therefore must rely on membrane protection for temperature adaptation (Hernández et al., 2013). ARA is the major polyunsaturated fatty acid found in *H. discus* (Chen et al., 2021; Su et al., 2001) and *Haliotis rubra x Haliotis laevigata* (Mateos et al., 2010) and it has been found to be higher close to winter and summer in the latter species (Su et al., 2006). These findings match with our results showing

that ARA levels in *H. iris* are higher after summer (Mar–20) and winter (Oct–19) compared to the following months. Apart from temperature regulation, ARA plays a vital role in muscle metabolism opening up ATP-sensitive potassium channels to hyperpolarize and activate muscle contraction in abalone. The decreasing levels of EPA and ARA along the year might also suggest a better capability of bio membranes to cope with temperature fluctuations as animals mature. In addition, DHA levels decrease as animals age. DHA is commonly found in the lowest proportion in abalone (Chen et al., 2021) and this might be attributed to an adaptation to a low lipid diet such as seaweed, characteristic of aquatic herbivores (Dunstan et al., 1996). DHA has found to be in low levels as abalone has low conversion rate from docosapentaenoic acid (DPA) to DHA (Uki et al., 1986).

3.7 CONCLUSIONS

In conclusion, this study provides evidence of significant variations of *H. iris* in terms of growth performance, nutritional profile and specific metabolites during summer and winter months. Protein composition was the most relevant macronutrient affected during the year of grow-out, whereas carbohydrate, ash, and moisture showed less variation but significant. Lipid level variation was not significant across months. Significant metabolites, such as some amino acids related to flavour and tenderness, fatty acids related to membrane temperature regulation and organic acids showed strong seasonal changes with higher levels mostly in summer compared to winter. Our findings suggest that abalone pass through metabolic and nutritional changes along one year of grow-out and those variations are not exclusively associated to seasonal changes, as they are also affected by other factors that were not considered in this study such as temperature, feed availability and developmental stage. The data obtained from this study provides a baseline information for further studies that envisage to evaluate longer effects of seasons on the nutritional status of *H. iris*. In addition, the results of this study provide the nutritional profile of *H. iris* that can be used to design formulated feeds specifically for this species in juvenile stage.

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Chapter

Sustainable aquafeed formulations containing insect larval meal and grape marc for the New Zealand farmed abalone

4

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4.1 ABSTRACT

The aquaculture industry has been criticised for the excessive use of fishmeal (FM) in feeds due to the utilisation of wild fish in the formulation and the exacerbation of overfishing marine resources. Land-based abalone aquaculture mainly uses commercial feeds (CFs) to promote faster growth, which include FM as a primary protein component. Alternative ingredients, such as insect meal (IM) and grape marc (GM) are potential candidates for aquafeeds due to their suitable nutritional profile and sustainable production. This paper reports on a novel diet for the New Zealand farmed abalone (*Haliotis iris*), which replaces FM with IM by 10% and includes a waste by-product (GM) by 30% as a potential prebiotic source. The study was performed in two stages: (a) physico-chemical determination of diets delivered in an alginate matrix (four experimental diets with graded levels of IM and GM) and their stability in seawater compared to CF and (b) evaluation of growth performance, gastrointestinal enzyme activity and feed intake in juveniles *H. iris* over a period of 14 days. There were significant differences between experimental diets and CF in terms of sinking rate, particle weight, and microscopic observations. Water stability of the experimental diets was increased by 50% in 24 h and 48 h compared to CF, producing less solid waste, and potentially reducing cleaning efforts in the farm. The inclusion of IM and GM did not compromise overall animal growth or their feed conversion ratio (FCR), however, further evaluation need to be explored in future research. The enzyme activity of amylase was substantially increased with the inclusion of dietary GM. The findings revealed that the developed encapsulated feeds are a more stable food delivery method for *H. iris* compared to the CF. Furthermore, both IM and GM can be included in feed formulations as a more sustainable strategy without compromising weight and shell gains in abalone farming.

4.2 INTRODUCTION

Aquaculture is the fastest growing industry in the world in terms of protein supply, providing almost 50% to the total aquatic animal production (FAO, 2022). In New Zealand, aquaculture is still a growing industry, exporting to 81 countries with annual sales of around NZD $650 million (Ministry for Primary Industries, 2022). Although the New Zealand aquaculture industry is expected to grow five times by 2035, its current advancement has been hampered by the lack of knowledge of optimal nutritional profiles and effective feeding technologies to promote growth and reduce environmental impacts simultaneously (Rivera et al., 2017). One of the main contributors to the reduced sustainability of current aquafeeds is the use of fishmeal (FM). The high price of FM is the result of its global scarcity and unsustainable future. Great efforts to replace FM in formulations with plant meals and concentrates have been investigated. However, the use of plant protein is still problematic due to the large cultivation area and water needed. Therefore, alternative ingredients, especially those derived from wastes from other productions are seen as potential candidates in aquaculture feeds. Alternative ingredients for replacement, such as insect meals (IMs) and waste-by-products (e.g., grape marc (GM) have been included in some aquafeeds to a certain extent but are often accompanied by a reduced feed quality due to poor digestibility of ingredients or lack of attractiveness to the species cultivated.

IMs have a good nutritional profile of protein within 42–63% comprising all essential amino acids, and lipids up to 36% with high levels of unsaturated fatty acids from 19–70% (Makkar et al., 2014) and other important nutrients, such as chitin that make them a good replacement for FM (Marono et al., 2015; Nogales-Mérida et al., 2018). Insect production is more sustainable than fish production because it uses less land area (Van Huis, 2015), produces less greenhouse gas (Nugroho & Nur, 2018) and less ammonia emissions (Van Huis, 2015), has an efficient feed conversion (Ferrer et al., 2019), and can be grown on organic waste-products from other industries.

GM, a by-product of the winery industry derived from *Vitis vinifera*, consists of the skins and seeds left after pressing. New Zealand has an abundant supply of this material, but its disposal has become problematic. GM possesses several nutritional qualities, including low levels of digestible carbohydrates, polyphenols (anthocyanins, catechins, flavonols, and phenolic acids), large amounts of resveratrol found in red grape skins and pulp, high levels of polyunsaturated fatty acids (over 60%), and more than 2% being omega-3 fatty acids (Habeanu et al., 2015). These nutritional attributes make it promising for use in aquaculture nutrition. However, the physical characteristics of GM need to be enhanced to improve water stability and nutrition retention. GM is primarily composed of

polymers including 18.5% cellulose, 8.2% hemicellulose, and 56.4% lignin (Perez-Ameneiro et al., 2014), giving it excellent adsorption capabilities (Perez-Ameneiro et al., 2014). Despite these qualities, the incorporation of GM in aquafeeds is very limited. To our knowledge, only GM derivatives have been used in feeds for *Haliotis laevigata* with great success (Currie et al., 2019). The use of steam distilled GM (Acti- Meal®) has resulted in improved growth rates and feed conversion ratios (FCRs) for *H. laevigata* when compared to commercial feeds and feed without GM supplementation (Currie et al., 2019). However, the direct use of GM in aquaculture feeding practices has not been previously explored.

Two of the most important concerns that come along with the development of feeds for aquaculture are the possible effects of alternative ingredients on the stability of the diet during seawater immersion and the potential pollution of the water column due to excessive phosphorus, carbon, and nitrogen (Cho & Bureau, 2001). Out of the solid wastes produced in aquaculture, uneaten feed residues are considered the biggest cost proportion in aquaculture settings (Cho et al., 1994). Uneaten commercial feeds (CFs) normally disintegrate quickly, especially during abalone feeding activity, and such waste is collected when tanks are cleaned. Improvement of apparent digestibility of diet formulations, reduction of excessive leaching of nutrients (e.g., phosphorus, nitrogen, and carbon), and promoting faecal consistency constitute the first steps to improve diet formulations and feeding strategies to reduce solid waste excretion and improve farm water quality. Many types of IM can be included in aquafeeds, resulting in differences in terms of floatability, pellet duration, water absorption, and expansion ratio (Irungu et al., 2018; Weththasinghe et al., 2021). The level of IM inclusion affects the fat content of the final feed, decreasing the pellet expansion and therefore affecting floatability (Irungu et al., 2018). Increased moisture in feeds increases pellet floatability due to increased starch gelatinization, maintaining air bubbles inside the pellets (Irungu et al., 2018).

Around 75% of the nitrogen (N) and phosphorus (P) from the feeds are usually unutilized and remain as waste in the culturing tanks (Enduta et al., 2011). Phosphorus is an essential element required by all fish for normal growth and may be included in excess to promote growth in aquatic animals. This cultivation practice can cause excessive discharge of phosphorus into the environment, thus, increasing eutrophication with associated ecosystem impacts. Although some effluent P (such as faecal and uneaten P from feed) can be collected, soluble P is non-recoverable and is inevitably discharged into the environment without any existing regulation. Nitrogen waste is mostly associated with dissolved nitrogen in the form of amino acids released from the feeds and animal excretion of ammonia and faeces.

The inclusion of new ingredients into aquafeeds requires evaluation of feed physical characteristics, such as floatability, matrix erosion, texture profile analysis, and nutritional composition, and chemical characteristics, such as carbon, nitrogen, and phosphorus components, which are retained or released once the feed is placed in the water. These evaluations may provide insight on the physico-chemical and nutritional potential of alternative ingredients, such as IM and GM when included in abalone feed formulations.

The aim of this study was to develop innovative encapsulated feeds, formulated with alternative ingredients (IM and GM) for abalone aquaculture. The study provides a comprehensive characterisation of physical and chemical properties of the various experimental diets, as well as an evaluation of animal feed intake and growth during 14 days. It is envisioned that this work will provide the foundation for future aquafeed formulations with a wider range of supplements optimised for different species and farm requirements.

4.3 MATERIALS AND METHODS

4.3.1 Experimental animals and tank systems

Juvenile abalone (*Haliotis iris*) (n = 45) with an initial mean weight of 5.7 ± 0.8 g, mean shell length of 34.9 ± 1.6 mm and shell width of 23.5 ± 1.1 mm were provided by The New Zealand Abalone company (Bluff, Invercargill, New Zealand) and transported to the facilities of the Auckland University of Technology (Auckland, New Zealand) overnight in December 2022 (Figure 4.1). Upon arrival, the animals were immediately placed in seawater with an ambient temperature of 14°C. Abalone were acclimated for 2 days and then kept for 14 days for the feeding trial. During this time, water parameters, such as pH, nitrate, nitrite, ammonia, salinity, and temperature were measured before and after water exchanges every day. An electronic thermometer was used for temperature measurement and was calibrated according to manufacturer instructions. A liquid saltwater master kit (Api Fishcare Inc.) was used for pH, nitrite, nitrate, and ammonia measurements and was calibrated against standard solutions provided by the manufacturer. Salinity was measured with a salinity refractometer and calibrated with a standard solution provided by the manufacturer.

Three tanks containing 5 L of seawater were allocated per treatment. Each tank contained three abalone, which provided nine abalone per dietary treatment. Tanks were aerated and maintained in a photoperiod of 24 h of darkness unless feeding and cleaning activities were performed. This conditioning was executed to attenuate direct sunlight. Four experimental diets (labelled F: fishmeal; FI: fishmeal + insect meal; FG: fishmeal +

grape marc, and FIG: fishmeal + insect meal + grape marc) and a CF were used in the experiment. The full description of ingredients for each experimental diet is described in Table 4.1. The commercial feed used was Marifeed S34 ® (Hermanus, South Africa). Food was provided daily in excess (0.5 g per tank) at 15:00 h after cleaning. Tanks were cleaned daily, and uneaten feed was collected in a 1-mm mesh. Uneaten feed was dried for 20 h at 60 °C in a convection oven and then stored until further analysis.

Apart from the feed provided, a cloth bag (200-μm nylon mesh filter) containing 1.2 g of dried experimental diets or CF was placed on the surface of the experimental tanks with abalone. Those feeds were used to measure how the uneaten feed was affected in terms of phosphorus, carbon and nitrogen levels over time, and the impact of the seawater as it was modified by the abalone's physiological activities (e.g., respiration, faeces production). After 24, 48, and 96 h, the bags with feed were removed from the tanks, the residue was removed and dried for 20 h at 60 °C in a convection oven and then stored until further analysis could be performed (see sections 4.3.6.2 and 4.3.6.3).

At the end of the 14-days feeding trial, all animals were measured and dissected, and their tissues collected for digestive enzyme analysis (see section 4.3.1.2).

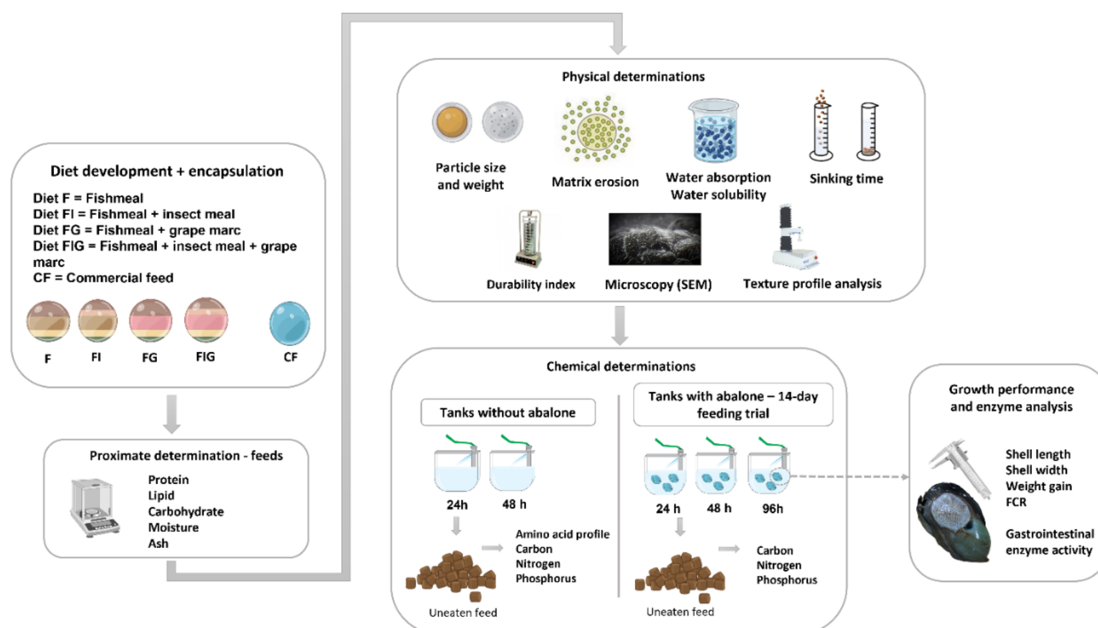

Figure 4.1 Summary of the experimental design and the experimental workflow.

4.3.1.1 Growth performance

Growth measurements were recorded at the start and at the end of the 14 day-feeding trial. Removal of abalone from tanks was performed with the aid of a blunt knife by carefully lifting the foot off the surface of the tank. Then, abalone were dried with paper towels, and their maximum shell lengths and widths (mm) and total abalone wet weights

(g) were recorded. Lengths were measured with a vernier calliper (Mitutoyo 0–125mm, Warwickshire, UK) to the nearest 0.1 mm, and weights were measured with a digital balance to the nearest 0.1 g. The following measurements were calculated:

Difference of weight (D_W)

$$Dw = Tfw - Tiw$$

Where T_{fw} is total weight at the end of the trial and T_{iw} is total weight at the beginning of the trial.

Difference of shell length (D_{SL})

$$Dsl = SLf - SLi$$

Where SL_f is the shell length (mm) at the end of the trial and SL_i is the shell length (mm) at the beginning of the trial.

Difference of shell width (D_{SW})

$$Dsw = SWf - SWi$$

Where SW_f is the shell width (mm) at the end of the trial and SW_i is the shell width (mm) at the beginning of the trial.

Feed conversion ratio (FCR)

$$FCR = \frac{Feed\ consumed}{abalone\ weight\ gain}$$

Where FCR and the feed consumed is the difference of the feed given and the uneaten feed.

4.3.1.2 Enzyme analysis

Abalone shells were removed before dissection. The gastrointestinal region (combined tissue as no clear distinction was possible) was separated from the adductor muscle. The samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C prior to the analysis of digestive enzyme activity.

Before analysis, the samples were thawed, and 0.7–0.8 g of tissue was weighed and homogenised in 5 mL of MiliQ water. A homogeniser (IKA Ultraturrax T25, Germany) was used at 17000 rpm for 60 s. Samples were then centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was removed completely and saved for the different determinations of enzymes which were performed at different pH.

The supernatants were analysed for trypsin, α-amylase and lipase activity using spectrophotometric techniques and commercial enzyme test kits. Trypsin was determined at 405 nm at 3 and 60 min (Catalogue AB102531; Abcam, Australia) in a kinetic mode. Amylase was determined at 405 nm at 0 and 3 min due to the high activity of the samples (Catalogue AB102523; Abcam, Australia) in a kinetic mode. Lipase was determined at 412 nm at 0 and 30 min (Catalogue AB102525; Abcam, Australia) in a kinetic mode. Total protein was determined using a bicinchoninic acid (BCA) protein assay kit with bovine serum albumin solution as the standard (Catalogue 23225; Thermo Fisher Scientific, USA). Specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one µmol of substrate per minute per mg of protein (*i.e.*, U mg protein $^{-1}$).

4.3.2 Diet preparation

Four experimental diets were formulated to contain graded levels of FM, corn meal (CM), IM, and GM (Table 4.1). The CF Marifeed® (Hermanus, South Africa) was provided by The New Zealand Abalone Company and used as the control feed.

Experimental diets were prepared by initially mixing pre-weighed, ground ingredients in a commercial blender (Nutri Bullet 600 household mixer). All ingredients were mixed except starch. Starch syrup at 10% was prepared and sequentially added to the dried mixture to form a dough of event consistency. The dough was placed on a flat tray to be dried at 65 °C for 16 h. Then, the dried dough was ground using a commercial blender (Nutri Bullet 600 household mixer) for 30 s and the powder was sifted two times using a kitchen sieve (approx. 40 mesh number)

Table 4.1 Percentage (dry weight) composition of the experimental diets (g/100g)

	Diet			
Ingredients (g/100g diet)	F	FI	FG	FIG
Fishmeal[1]	35	25	35	25
Insect meal[2]	-	10	-	10
Corn meal	30	30	-	-
Grape marc[3]	-	-	30	30
Seaweed (dry) *Macrocystis pyrifera*[4]	4	4	4	4
Starch (Native maize flour)[5]	10	10	10	10

[1] Fishmeal supplied by Sandford, NZ.
[2] Insect meal supplied by Mahurangi Technical Institute (MTI).
[3] Grape marc supplied by Bragato Research Institute, NZ.
[4] Seaweed (*Macrocystis pyrifera*) supplied by Southern Clams.
[5] Starch supplied by New Zealand Starch.

4.3.3 Encapsulation of experimental diets

Encapsulation was performed according to Masoomi et al. (2022) with small modifications using a calcium chloride and alginate solution. Alginate solution 1% w/v was mixed with the feed powder. Encapsulation took place after placing the mixture feed powder-alginate in a calcium chloride solution (0.1 M). Formed beads were washed with 100–200 mL distilled water and then arranged on an oven tray covered lined with aluminium foil. These beads were left to air dry inside a commercial oven (Piron PF8906, Italy) maintaining a temperature of 65 °C for 16 h. After that, dried beads were carefully sealed in a vacuum-sealed bag to prevent potential contamination. Proximate analyses were conducted on all diets in triplicate.

4.3.4 Proximate analyses of feeds

Proximate compositional analyses, including crude protein, crude lipid, ash, and moisture content were carried out on both the experimental diets and the CF following AOAC guidelines (1985). The moisture content was determined in three replicates using a convection oven at 135 °C for 3 h. To prevent denaturation or oxidation of metabolites, the experimental diets and the CF were ground up into powder using a grinder (IKA A11 model analytical mill, Germany) with the addition of liquid nitrogen.

Protein analyses

The nitrogen levels of the experimental diets and the CF were assessed using a CE-440 Elemental Analyser (Exeter, Chelmsford, Massachusetts, USA). The process involved combustion and reduction temperatures were 980 and 700 °C, respectively, with pure oxygen as the combustion gas and pure helium as the carrier gas. The purge and combustion times were 15 and 20 s, respectively. The protein content was calculated by multiplying the nitrogen value by the conversion factor 6.25 (AOAC NO. 960.52).

Lipid analyses

For lipid determination in diets, the extraction of crude fat was carried out following the method described by Bligh and Dyer (1959) for small samples. In summary, 0.1 g of the dried and ground experimental diets or CF were mixed with 1 mL chloroform and 2 mL methanol and 800 μL distilled water. The mixture was then vortexed for 2 min, after which 1 mL chloroform was added. The solution was vortexed again for 30 s and 1 mL distilled water was added to separate layers. The mixture was vortexed again for 30 s and centrifuged at 3000 rpm for 5 min. The lower organic solvent layer was collected

into a pre-weighed 8 mL glass tube and evaporated using a nitrogen stream. Finally, the net weight of the lipids in the sample was recorded.

Ash analyses

A sample of 0.5 g from each dried ground experimental diets and CF were placed in a furnace for combustion at 550 °C for 6 h. Ashes were weighed, and the difference expressed as ash percentage (%) in the total sample.

Carbohydrate analyses

Carbohydrate contents were determined using the Anthrone method (Yemm & Willis, 1954). In brief, 30 mg of ground samples were digested with 2.5 N HCl for 3 h. Following this, the resulting homogenate was centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was collected and diluted 50 times for a better determination. Each tube containing 1 mL sample (diluted if needed for better determination) had 4 mL of Anthrone reagent added to it. These tubes were sealed and incubated at 100 °C for 15 min. After cooling, the developed colour was measured against a glucose standard and a blank at 620 nm in a UV–visible spectrophotometer using a quartz cuvette. The results were expressed in milligram of glucose/g sample.

Fibre analysis

Total dietary fibre was determined in experimental diets and CF using Megazyme kit (Catalogue K-TDFR-100A/K-TDFR-200A 04/1, Megazyme, Ireland). Briefly, 1 g of ground samples were hydrolysed subsequently with α-amylase, protease, amyloglucosidase, and further precipitated with ethanol to produce a residue that was used for protein and ash determination.

4.3.5 Physical characterisation of feeds

4.3.5.1 Particle size and weight of feeds

Experimental beads for diets F, FI, FG, FIG, and CF were measured (n = 50) in the longest axis using a calliper Mitutoyo (0.01mm). Weight was measured using a digital balance (Sartorius CPA2P).

4.3.5.2 Sinking rate of feeds in seawater

The sinking rate of particles (n = 25) was measured by placing individual beads or pellets in a measuring cylinder filled with seawater. The height of the seawater column

was 16 cm. The time that a particle travelled the column height from top to bottom was recorded as sinking time and divided by 16 cm.

4.3.5.3 Water absorption index (WAI) and water solubility index (WSI) of feeds

WAI measures the volume occupied by the granules after swelling in excess of water. WSI indicates the percentage of polysaccharides released from the granule after the addition of excess water. WAIs for the experimental diets and CF were determined by the method outlined by Anderson (1969). The ground diets (0.85 g) were suspended in deionised water (10 mL) in a tared 50 mL centrifuge tube. The suspension was stirred constantly for 3 minutes (Vortex genie 2, Scientific industries, USA) and centrifuged at 5000 rpm for 10 min. The supernatant was decanted into a tared aluminium cup and dried at 120 °C for 2 h. The cup and its contents were cooled in a desiccator and re-weighed on a sensitive weighing scale, and the difference in weight (W_{SS}) was obtained. The mass of the gel remaining in the centrifuge tube (W_g) was also obtained. The WAI and WSI were calculated using the following equations:

$$WAI = \left(\frac{Wg}{Wds}\right)$$

Where W_g is the weight of gel (g), and W_{ds} is the weight of dry sample (g).

$$\mathrm{WSI}\ (\%) = \left(\frac{\mathrm{Wss}}{\mathrm{Wds}}\right) \mathrm{x}\ 100$$

Where W_{ss} is the weight of dry solids of supernatant (g), and W_{ds} is the weight of dry sample (g).

4.3.5.4 Matrix erosion of feeds

This experiment was conducted in tanks in the farm (Bluff, Invercargill, New Zealand) without abalone. The purpose was to evaluate the percentage of solids disintegrated from the capsule or pellet after 24 and 48 h of immersion in seawater. Three mesh cloth bags (200 µm nylon mesh filter) containing 2.5 g of experimental diets or CF were placed at the bottom of a tank containing 90 L of filtered seawater (100 µm nylon mesh filter). A flow-through water system was maintained at a rate of 1.5 L/min, which equates to a total water exchange of 40 times per day. Water temperature was maintained between 16.1–19 °C and dissolved oxygen 92.7–99.6%. After 24 and 48 h the bags were removed and dried at 50 °C for 16 h and stored for further analysis.

The dried weights of particles were measured and the percentage of matrix erosion in the particles was calculated as follows:

$$Matrix\ erosion\ \left(\%\frac{w}{w}\right) = \left(\frac{W0 - W}{W0}\right) X\ 100$$

Where W0 is the initial dry weight of the beads and W is the mass of dry beads after incubation in seawater.

4.3.5.5 Microscopy of feeds

The morphology and microstructure of the experimental diets and CF were examined using a scanning electron microscope (SEM) (Hitachi SU-70, Japan). For SEM imaging, samples were dehydrated at 70 °C (experimental diets) and randomly selected. The dried diets were placed on double sided adhesive carbon tapes on aluminium stubs and then coated with a thin layer of platinum under vacuum for 60 s by an ion sputter coater (Hitachi E-1045, Japan). The elemental analysis was performed using an energy dispersive spectrometer (EDS). Samples were observed under SEM at 15 kV and EDS spectra were obtained to screen the elemental composition of the beads using Noran System 7 (NSS) microanalysis system software (Thermo Scientific, USA).

4.3.5.6 Texture profile analysis of feeds

Texture analysis was performed using a Stable Micro Systems Texture Analyser equipped with a Film Support Rig (HDP/FSR) on a Heavy-Duty Platform (HDP/90) with a 5 mm stainless steel probe (P/55) and a 5 kg load cell. The texture analyser was set to measure force in the compression mode with a pre-test speed of 2.0 mm/s, a test speed of 0.5 mm/s, and a post-test speed of 2.0 mm/s ,strain 1%, trigger force 1 g and a time of 5 s for diets in time 0 h and 16 h, and a pre-test speed of 2.0 mm/s, a test speed of 0.5 mm/s, and a post-test speed of 2.0 mm/s, strain 50%, trigger force 1 g and a time of 5 s for diets in time 24 and 48 h. Target mode was set to a distance of 5 mm. Data acquisition rate was set at 500 pps. Hardness, gumminess, and chewiness data were collected for statistical analyses. Hardness is described as the force required to compress a material by a given amount, gumminess as the energy required to break down a semi-solid ready for swallowing, and chewiness as the energy required to chew a solid food into a state ready for swallowing (all of them with unit N – newtons) (Delgado & Bañón, 2015).

4.3.5.7 Durability index

The durability index is a measure of the strength of pellets/capsules to resist mechanical handling during transportation, storage, and subsequent use. The durability index was determined as outlined by Irungu et al. (2018) with modifications. About 15 ± 0.1 g of each dried sample was sifted on a series of seven sieves placed in this order: 2,

1, 0.6, 0.425, 0.25, 0.15, and 0.075 mm. The samples were placed at the highest pore sieve 2 mm (Wi) and the series of sieves placed in a flask mounted on a Lab-Line shaker, which was shaken for 20 min. The beads were re-weighed (Wr) in each sieve and the pellet durability index (PDI) calculated using the following equation:

$$PDI\ (\%) = \left(\frac{Wr}{Wi}\right) \times 100$$

4.3.6 Chemical characterisation of feeds

These determinations were conducted in two different settings. The first setting included the evaluation of the nutrient leaching (amino acids, phosphorus, carbon, and nitrogen levels) from the uneaten feeds in tanks without abalone at 0, 24, and 48 h. These data were useful to understand the nutrient leaching/adsorption in uneaten feed when abalone excretion waste was not present in the tanks. The second setting included the evaluation of nutrient leaching/adsorption (phosphorus, carbon, and nitrogen levels) from the uneaten feed in tanks with abalone at 0, 24, 48, and 96 h as per section 4.3.1. These data were used to evaluate if abalone excretion waste has a role to play in the nutrient leaching/adsorption of the uneaten feeds. In both cases, the uneaten feed was collected, dried for 20 h at 60 °C in a convection oven and then stored until further analysis.

4.3.6.1 Amino acid level of feeds

The procedure for extracting total amino acids followed the methodology outlined by Paramás et al. (2006). Three replicates of 50 mg dried samples of diets were hydrolysed with 1.5 mL 12M concentrate hydrochloric acid in a heating block at 110 °C for 22 h. The solution was filtered through filter paper and adjusted to pH 4–6 with NaOH 1M. Once neutralised, samples were centrifuged at 3000 rpm for 5 mins and supernatants were kept frozen until analysis. After extraction, samples were derivatised using the AccQ-Tag (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate method for liquid chromatography- mass spectrometry (LC-MS) as detailed by Bullon et al (2023). The amino acid standard used contained 37 amino acids (A9906 amino acid standard Sigma 485845-1G) and d-4 alanine was used as an internal standard. LC-MS system using an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA) was used for determination with a column Phenomenex Kinetex evo C18 (2.1 x 150mm, 1.7µm). The mobile phase was composed of water containing 0.1%(v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The initial gradient condition was 99:1 (A:B). The total run time was 30 min.

4.3.6.2 Phosphorus level in feeds

Microwave plasma atomic emission spectroscopy (MP-AES) was used to determine phosphorus in experimental diets and CF at different times of seawater immersion. Dried samples (0.5 g) were digested with 10 mL of 65% HNO_3 in a microwave at 195 °C over a period of 20 min and at 180 °C for 20 min. Samples were filtered using a plastic funnel with filter paper (541 Whatman filter paper) and 50 mL 2% nitric acid and diluted 25 times to be measured against a calibration curve with diammonium hydrogen orthophosphate $(NH_4)_2HPO_4$.

4.3.6.3 Carbon and nitrogen level of feeds

Total carbon and nitrogen concentrations were measured in experimental diets and CF at different times of seawater immersion. Ground samples were weighed at 2–4 mg in a tin capsule. Nitrogen and carbon detections were determined by a CE-440 Elemental Analyser (Exeter, Chelmsford, Massachusetts, USA). The combustion and reduction temperatures were 980 and 700 °C, respectively, with pure oxygen as the combustion gas and pure helium as the carrier gas. The purge and combustion times were 15 and 20 s, respectively. Samples were measured in three replicates and acetonitrile was used as a standard.

4.4 STATISTICAL ANALYSES

One-way analysis of variance was carried out to detect the effect of the diet formulation on the physical characteristics, proximate composition, growth measurements and abalone gastrointestinal enzyme activity when a normal distribution was found (Kolmogorov – Smirnov test, $p < 0.05$) followed by pairwise comparisons with Tukey's post hoc test. Homogeneity of variances was analysed using Levene's test when samples followed a normal distribution ($p < 0.05$). Two-way analysis of variance was carried out to detect the influence of the type of feed and time of immersion in seawater on the amino acid profile, phosphorus, carbon, and nitrogen levels. Significant differences between readings of the different samples were evaluated using the statistical software XLSTAT 2022.3.1 (Addinsoft, New York, USA) with Tukey's post hoc comparison tests, where statistical significances were found.

4.5 RESULTS

4.5.1 Physical characterisation of the feeds

The particle weight of experimental diets ranged depending on the ingredients (Table 4.2). Diet F (4.5 ± 1.2 mg) and FI (4.1 ± 1.3 mg), which did not contain GM, were significantly lower in weight compared to diet FG (10.3 ± 4.2 mg) and FIG (7.9 ± 3.1 mg). Regarding particle size, diet F (2.1 ± 0.5 mm) and FI (2.4 ± 0.7 mm) were significantly smaller in size compared to diet FG (4.4 ± 1.7 mm), FIG (3.9 ± 1.2 mm) and commercial feed (3.9 ± 0.3 mm). In terms of sinking rate, dried experimental beads performed better than fresh beads and frozen beads better than dried beads. In the frozen form, diets containing GM had significantly higher sinking rates compared to diets without GM. The matrix erosion results showed that all experimental diets had significantly higher seawater stability compared to the CF in 24 and 48 h. The diets containing GM had significantly higher WAIs compared to diets not containing GM. The WSI indicated that diet FG had lower water solubility compared to other diets, diet FI and the CF had the highest solubility with 9.3 ± 0.1 % and 9.9 ± 0.2%, respectively.

Table 4.2 Physical characterisation of four experimental diets and a commercial feed (CF)

		Diet				
Physical characteristics		F	FI	FG	FIG	CF
Particle weight (mg)	-	4.5 ± 1.2^{d}	4.1 ± 1.3^{d}	10.3 ± 4.2^{b}	7.9 ± 3.1^{c}	16.6 ± 2.3^{a}
Particle size (mm)	-	2.1 ± 0.5^{b}	2.4 ± 0.7^{b}	4.4 ± 1.7^{a}	3.9 ± 1.2^{a}	3.9 ± 0.3^{a}
Sinking rate (s)	Fresh beads	7.4 ± 2.0^{bc}	6.3 ± 0.7^{c}	8.3 ± 1.5^{b}	11.1 ± 4.8^{a}	NA
	Frozen beads	3.6 ± 0.6^{b}	3.5 ± 0.5^{b}	4.8 ± 0.8^{a}	5.3 ± 0.8^{a}	NA
	Dried beads	4.0 ± 0.5^{c}	4.3 ± 0.6^{c}	9.1 ± 4.6^{b}	12.0 ± 6.3^{a}	2.6 ± 0.4^{c}
Matrix erosion (%)	24 h	4.6 ± 1.6^{b}	0.1 ± 0.01^{c}	0.5 ± 0.8^{c}	0.7 ± 0.4^{c}	13.9 ± 0.9^{a}
	48 h	13.3 ± 1.1^{ab}	7.2 ± 2.3^{c}	8.5 ± 0.7^{bc}	7.3 ± 1.6^{c}	17.5 ± 3.1^{a}
WAI[1] (g)	-	3.3 ± 0.2^{c}	3.3 ± 0.1^{c}	4.4 ± 0.2^{a}	4.0 ± 0.05^{b}	3.9 ± 0.04^{b}
WSI[2] (%)	-	8.0 ± 0.4^{b}	9.3 ± 0.1^{a}	5.1 ± 0.1^{c}	7.6 ± 0.3^{b}	9.9 ± 0.2^{a}

Data represent means and standard deviation. For each parameter, significant differences are shown by different superscripts (Tukey post hoc tests, $p < 0.05$).
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).
Particle weight and size (n=50), sinking rate (n=25), matrix erosion (n=3), WAI and WSI (n=3).
Sinking rate test performed in seawater temperature 22 °C.
[1]WAI (g) Water absorption index.
[2]WSI (%) Water solubility index.
NA = not applicable.

Microscopy of the feeds

The scanning electron microscopic images of the experimental diets showed that the alginate beads were formed by three-dimensional porous structures consisting of a high number of connected irregular sheets with some grain-shaped bulges on the surfaces. There was no presence of scaffolds, typical in alginate hydrogels. The surface of the four experimental diets was even, without many breakages in different drying temperature (35, 50, and 70 °C). This consistence in bead surfaces exposed to different temperatures suggest that the drying temperature did not affect the porous structures of the beads. The morphology of the experimental diets was well-compacted without observable significant changes in the porous structures which indicate the absence of effect of GM or IM inclusion in the morphology of the dried beads. However, the shape of the beads was different between diets with grape marc and without grape marc. Diet F (Figure 4.2) and FI (Figure 4.3) were generally rounded compared to diet FG (Figure 4.4) and FIG (Figure 4.5), which were elongated. Comparably, the CF showed a more porous structure the surface suggesting more water absorbance (Figure 4.6).

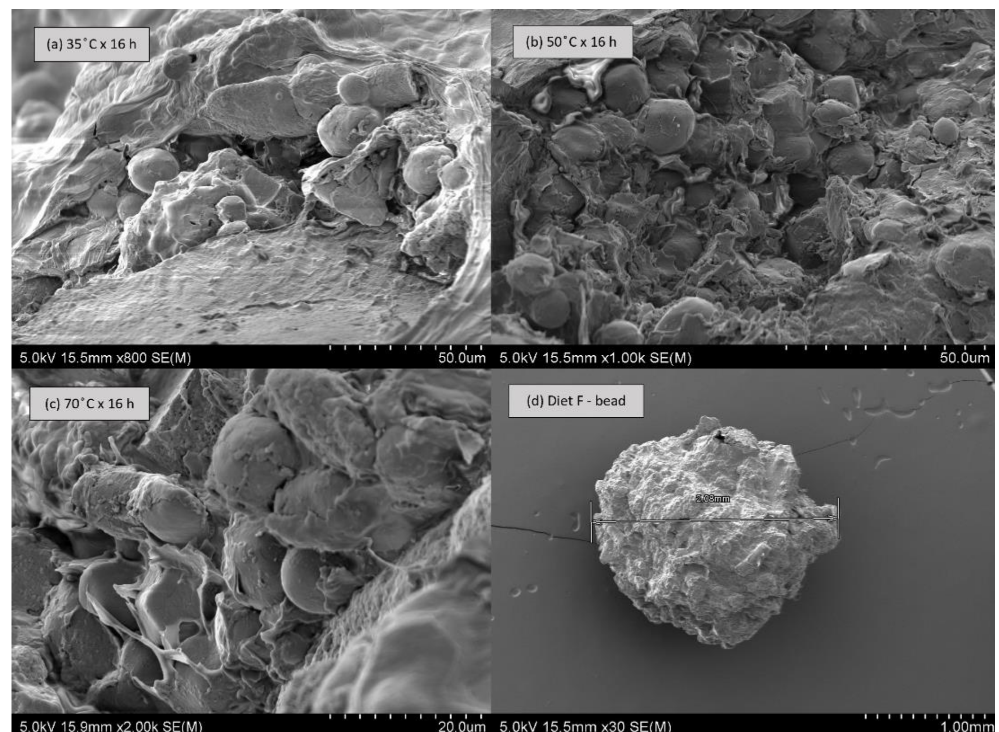

Figure 4.2 The SEM photographs of diet F. (a) diet F dried at 35 °C for 16 h (b) diet F dried at 50 °C for 16 h and (c) diet F dried at 70 °C for 16 h. (d) Entire photo of alginate bead from diet F. Abbreviation: diet F (fishmeal based).

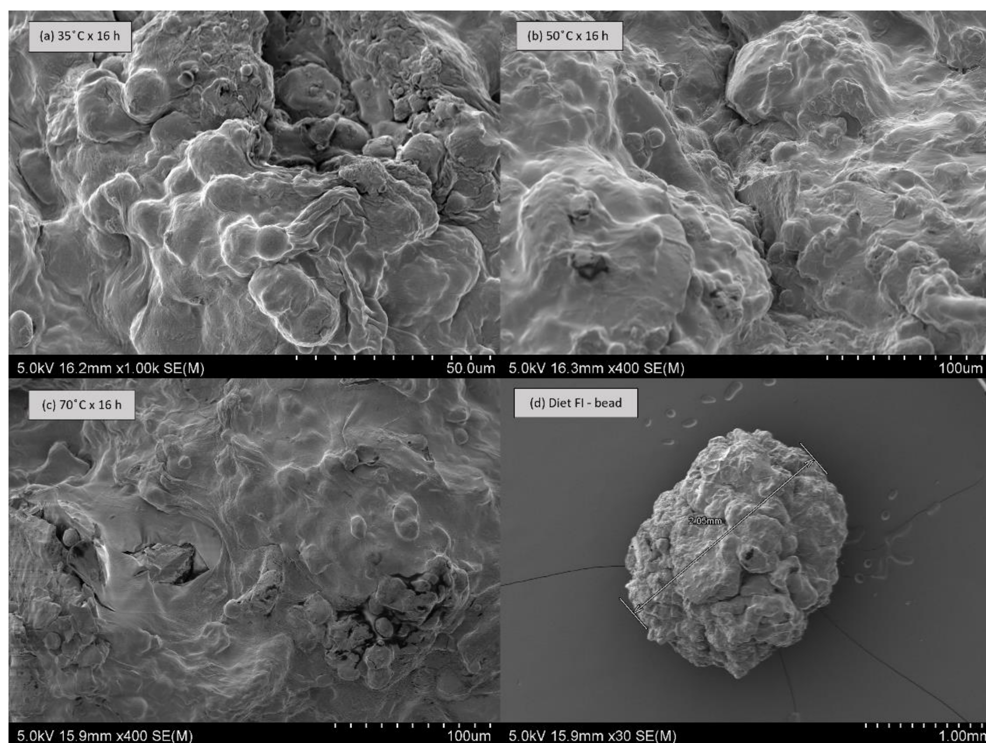

Figure 4.3 The SEM photographs of diet FI. (a) diet FI dried at 35 °C for 16 h (b) diet FI dried at 50 °C for 16 h and (c) diet FI dried at 70 °C for 16 h. (d) Entire photo of alginate bead from diet FI. Abbreviation: diet FI (fishmeal + insect meal).

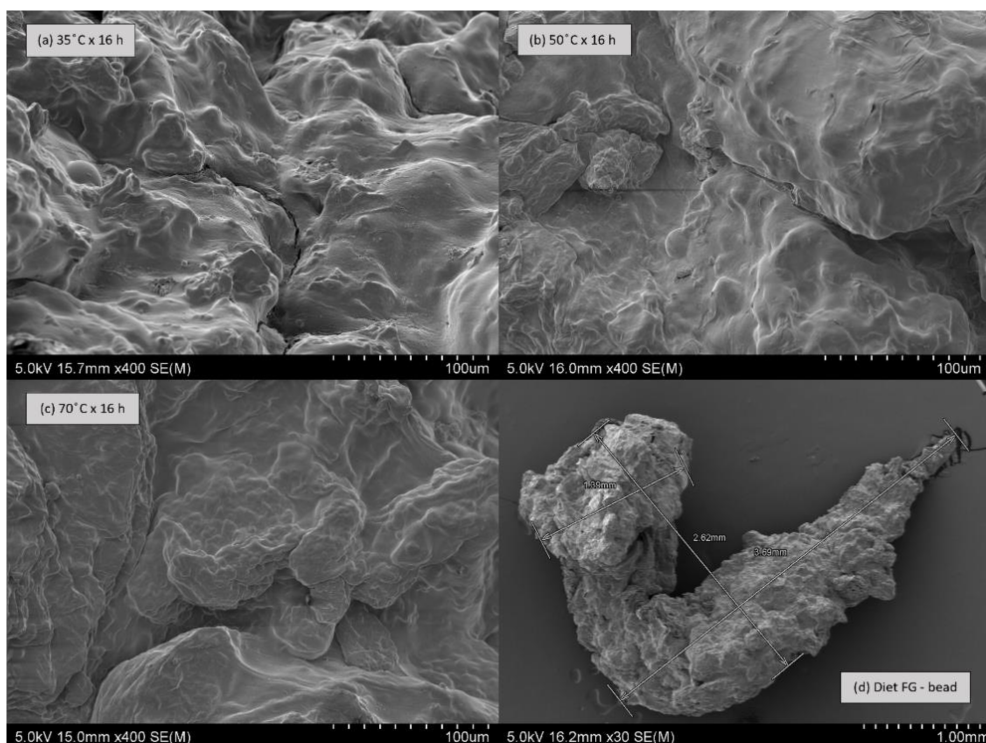

Figure 4.4 The SEM photographs of diet FG. (a) diet FG dried at 35 °C for 16 h (b) diet FG dried at 50 °C for 16 h and (c) diet FG dried at 70 °C for 16 h. (d) Entire photo of alginate bead from diet FG. Abbreviation: diet FG (fishmeal + grape marc).

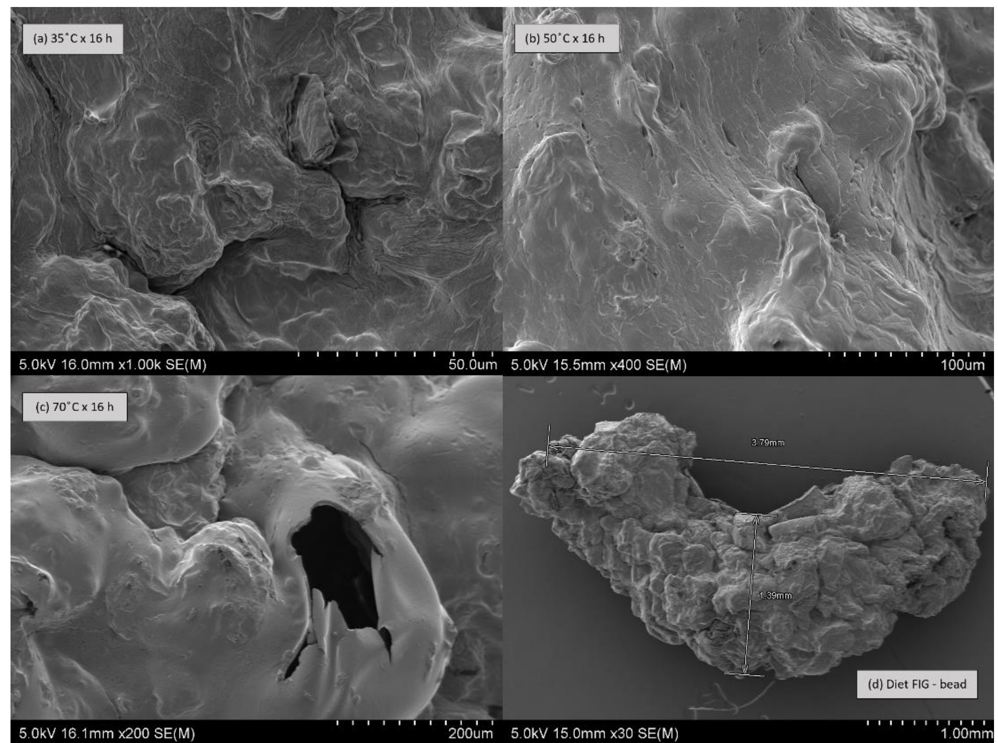

Figure 4.5 The SEM photographs of diet FIG. (a) diet FIG dried at 35 °C for 16 h (b) diet FIG dried at 50 °C for 16 h and (c) diet FIG dried at 70 °C for 16 h. (d) Entire photo of alginate bead from diet FIG. Abbreviation: diet FIG (fishmeal + insect meal + grape marc).

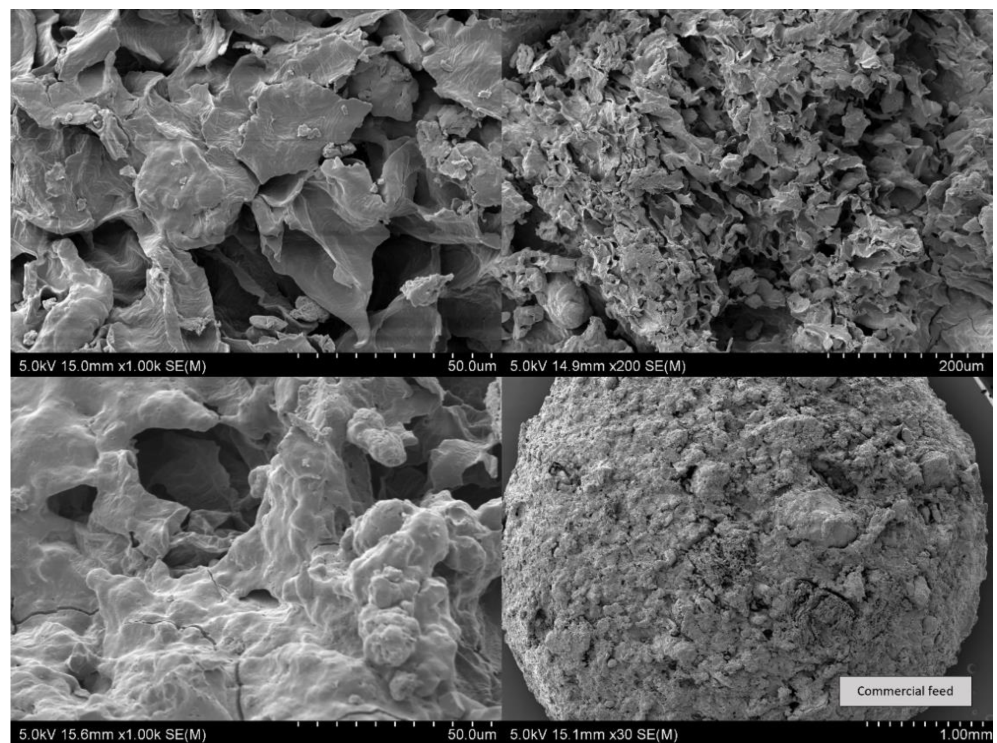

Figure 4.6 The SEM photographs of commercial feed (CF). Abbreviation: commercial feed (CF).

Texture profile analysis (TPA)

Overall, the texture profile analysis of the experimental diets and CF showed that the time of immersion in seawater affects the hardness, gumminess, and chewiness of the diets. When experimental diets were fresh, the inclusion of IM or GM resulted in significantly higher gumminess and the inclusion of IM significantly increased the hardness of the baseline diet F (Figure 4.7a). When experimental beads were refrigerated (2–8 °C), diets that included GM resulted in significantly higher hardness, gumminess, and chewiness compared to diets without GM (Figure 4.7b). Compared to fresh beads, gumminess increased almost twice during refrigerated condition, particularly diet F and FI. After 16 h in seawater, the experimental diets resulted in lower hardness, gumminess, and chewiness compared to the CF, but there were no significant differences among the experimental diets (Figure 4.7c). After 24 h in seawater, the CF showed almost 10 times more hardness, gumminess, and chewiness compared to the experimental diets (Figure 4.7d). There were no significant changes among the experimental diets F, FI, FIG, and FG. After 48 h in seawater, the value of hardness, gumminess, and chewiness were not significantly different among experimental diets but were significantly different between commercial diet and experimental diets (Figure 4.7e). Hardness, gumminess, and chewiness of the CF remained quite similar between 24 and 48 h.

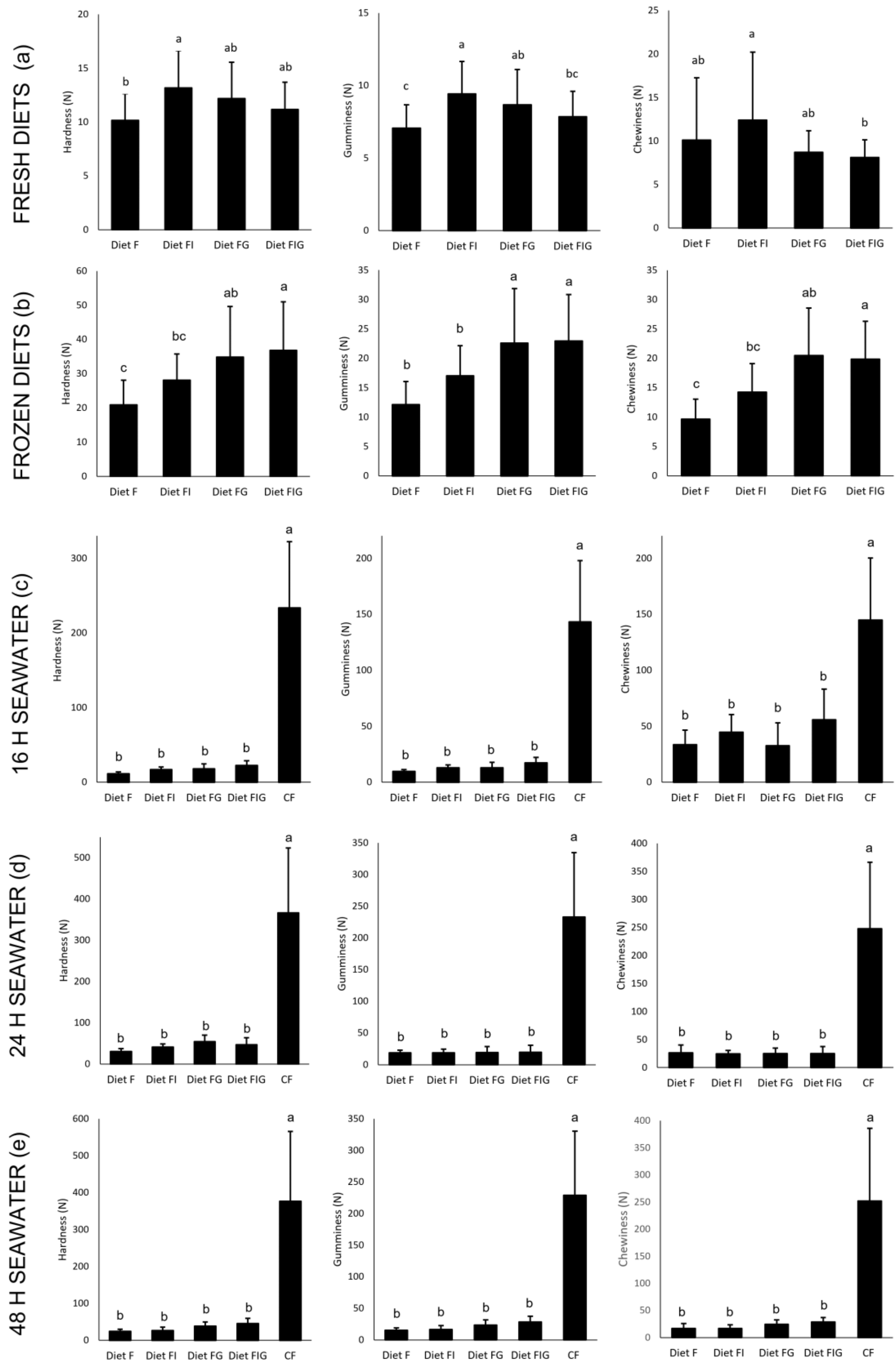

Figure 4.7 Texture profile analysis of fresh experimental diets and commercial feed at different storage conditions (fresh and frozen) and at different time points of immersion in seawater (16, 24, and 48 h). Data are presented as means ± SD (n = 25). Bars with the same superscript (a, b, c) are not significantly different from Tukey post hoc tests ($p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

Durability index

The durability index showed the ability of experimental diets and commercial feed to withstand mechanical handling. The results showed that after 20 min of shaking, most of the beads from diet F and FI (no GM) were less than 2 mm but more than 1 mm in size with 62.48% and 71.06%, respectively (Table 4.3). The diet FI was the most affected among all the experimental feeds during shaking with 2.35% of solids less than 1 mm but more than 0.6 mm in size. Contrarily, diets with GM (FG and FIG) showed better durability compared to diets without GM with 43.22% and 59.03% of beads remaining in the 2 mm sieve, respectively. All the experimental diets had at least 97% of particles bigger than 1 mm after 20 minutes of shaking indicating that the inclusion of IM and GM did not significantly influence the durability of the beads. CF had 99.92% pellets bigger than 2 mm and the effect of abrasion was minimum.

Table 4.3 Durability index of four experimental diets and a commercial feed according to pore size of sieves (mm). Values are expressed in grams.

	Diet				
Sieve (mm)	F	FI	FG	FIG	CF
2	36.08^{de} ± 10.57	26.27^{e} ± 6.34	43.22^{d} ± 2.66	59.03^{c} ± 2.82	99.92^{a} ± 0.07
1	62.48^{bc} ± 9.98	71.06^{b} ± 5.05	56.02^{c} ± 2.83	40.66^{d} ± 2.40	0.02^{f} ± 0.03
0.6	0.91^{f} ± 0.45	2.35^{f} ± 0.99	0.59^{f} ± 0.15	0.29^{f} ± 0.41	0.03^{f} ± 0.03
0.425	0.16^{f} ± 0.17	0.14^{f} ± 0.13	0.05^{f} ± 0.06	0.02^{f} ± 0.03	0.00^{f}
0.25	0.12^{f} ± 0.15	0.11^{f} ± 0.11	0.02^{f} ± 0.03	0.00^{f}	0.00^{f}
0.15	0.09^{f} ± 0.12	0.04^{f} ± 0.05	0.02^{f} ± 0.03	0.00^{f}	0.01^{f} ± 0.01
0.075	0.09^{f} ± 0.12	0.01^{f} ± 0.02	0.07^{f} ± 0.1	0.00^{f}	0.01^{f} ±0.01

Data represent means and standard deviation (n = 3). Means with the same superscript (a, b, c) in each column are not significantly different from Tukey post hoc tests ($p < 0.05$).
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

4.5.2 Proximate analyses of feeds

Proximate composition results of the experimental diets are shown in Table 4.4. The estimated energy content was in the range of 18.1–20.4 KJ/g for all experimental diets. The energy content of the CF was not disclosed by the manufacturer but was calculated according to the calories assigned for protein, carbohydrate, and lipid proportion (Table 4.4 legend 3). Experimental diets containing GM (FG and FIG)

contained more energy compared to other experimental diets (F and FI) considering the carbohydrate levels resulted from subtraction (Table 4.4 legend 1). Although this calculation is still used in food design to calculate energy from nutrients, values should be used with caution as GM contains high levels of non-digestible carbohydrates rather than digestible carbohydrates. All experimental diets had the same level of protein and the CF had significantly higher levels of protein. Experimental diets containing GM (FG and FIG) contained significantly lower levels of reducing sugars with 8.9 ± 1.9% and 9.1 ± 1.1%, and significantly higher dietary fibre levels with 16.1 ± 0.1% and 15.4 ± 0.3%, respectively. The CF had the lowest level of dietary fibre (3.7 ± 0.3%). Diets containing IM (FI and FIG) had significantly higher levels of lipid compared to the diets without it. The lowest lipid level was found in CF (1.2 ± 0.3%). Diet FG had significantly higher levels of ash (14.5 ± 0.3%) compared to the other experimental diets and CF had the lowest level (6.8 ± 0.4%). The CF had the highest level of moisture (10.7 ± 0.1%) and diet FI the lowest (3%).

Table 4.4 Proximate composition of four experimental diets and a commercial feed.

	Diet				
Proximate composition	F	FI	FG	FIG	Commercial feed[4]
Protein (%)	30.4 ± 0.1^{ab}	27.3 ± 0.3^{b}	30.8 ± 0.7^{ab}	26.4 ± 2.0^{b}	32.4 ± 2.7^{a}
Carbohydrate (%)[1]	47.9	50.8	45.4	49.3	46.34
Carbohydrate-Reducing sugars (%)[2]	28.9 ± 1.3^{b}	32.0 ± 3.1^{ab}	8.9 ± 1.9^{c}	9.1 ± 1.1^{c}	39.1 ± 4.7^{a}
Total dietary fibre (%)	9.0 ± 0.2^{b}	7.5 ± 0.1^{c}	16.1 ± 0.1^{a}	15.4 ± 0.3^{a}	3.7 ± 0.3^{d}
Lipid (%)	4.0 ± 0.2^{c}	7.0 ± 0.6^{a}	5.3 ± 0.5^{b}	7.2 ± 0.3^{a}	1.2 ± 0.3^{d}
Ash (%)	13.5 ± 0.1^{b}	11.9 ± 0.1^{c}	14.5 ± 0.3^{a}	12.9 ± 0.3^{b}	6.8 ± 0.4^{d}
Moisture (%)	4.2 ± 0.01^{b}	3.0 ± 0.01^{b}	3.9 ± 0.03^{b}	4.2 ± 0.8^{b}	10.7 ± 0.1^{a}
Energy (KJ per g)[3]	18.1	20.4	18.9	20.2	15.9

Data represent means and standard deviation. For each parameter, significant differences are shown by different superscripts (Tukey's test, $p < 0.05$).
[1]Carbohydrate proportion was calculated by difference 100 – (moisture + protein + lipid + ash).
[2]Carbohydrate was determined using reducing sugar method Anthrone.
[3] Total energy was calculated based on the physiological values at 5.6 kCal g^{-1} protein, 9.5 kCal g^{-1} lipid and 4.1 kCal g^{-1} carbohydrates (Cho et al., 1982).
[4] Commercial feed used was Marifeed S34.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

4.5.3 Chemical characterisation of the feeds.

Amino acid level of feeds

The amino acid profiles of the experimental diets and the CF at time 0, 24 and 48 h on seawater are shown in Table 4.5. Amino acids L-histidine, L-arginine and L-lysine were the only essential amino acids that were significantly different among diets. Experimental diets FI and FIG resulted in significantly higher levels of L-histidine compared to CF. All experimental diets had significantly higher levels of L-arginine and L-lysine compared to CF. Effects of time immersed in seawater showed that time significantly increased L-histidine, L-arginine in the first 24 h of immersion, while significantly reduced taurine, L-threonine, L-valine, L-lysine, L-phenylalanine, and L-tryptophan. The interaction diet*time showed that the levels of hydroxylysine and lysine varied significantly dependent on the type and time of immersion in seawater.

Table 4.5 Amino acid composition of four experimental diets and a commercial feed after immersion in seawater for 0, 24, and 48 h.

	Diet																	
Amino acid (mg/ g sample)	F			FI			FG			FIG			CF			ANOVA (*p* value)		
Time in seawater (h)	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	Diet (A)	Time (h) (B)	A x B
L-histidine*	7.8 ± 0.9	9.7 ± 0.1	8.8 ± 0.3	6.4 ± 0.3	13.9 ± 1.5	12.5 ± 1.5	6.7 ± 0.9	10.2 ± 0.9	13.02 ± 2.4	8.4 ± 2.4	13.4 ± 3.5	11.6 ± 1.4	6.7 ± 0.8	8 ± 1.1	9.9 ± 1.1	**0.015**	**<0.0001**	0.070
Hydroxy-L-Proline	3.6 ± 0.4	2.1 ± 0.1	2.0 ± 0.3	2.2 ± 0.1	2.3 ± 0.3	2.5 ± 0.5	4.1 ± 0.2	3.8 ± 0.3	4.7 ± 0.6	3.9 ± 1.3	3.4 ± 0.5	3.2 ± 0.2	2 ± 0.1	1.6 ± 0.1	1.7 ± 0.4	<0.0001	0.104	0.170
L-arginine*	14.7 ± 1.5	16.7 ± 0.5	18.8 ± 3.3	12.3 ± 1.3	23.1 ± 2.4	24.4 ± 2.5	15.9 ± 1.3	23.9 ± 3.4	30.2 ± 4.5	16.6 ± 6.8	24.5 ± 1.5	21.1 ± 3	12.5 ± 1	16.2 ± 0.9	15.2 ± 2.4	**0.0004**	**<0.0001**	0.102
Ethanolamine	0.3 ± 0.02	0.2 ± 0.01	0.2 ± 0.02	0.4 ± 0.1	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.01	0.2 ± 0.03	0.2 ± 0.05	0.5 ± 0.1	0.3 ± 0.03	0.4 ± 0.02	0.4 ± 0.05	0.2 ± 0.02	0.2 ± 0.004	<0.0001	<0.0001	0.915
L-serine	16.8 ± 2.5	11.4 ± 0.2	11.6 ± 0.4	14.1 ± 1.3	15.1 ± 0.8	14.8 ± 2.5	15.1 ± 0.8	12.5 ± 0.4	16.1 ± 2.6	17.2 ± 3.5	15.2 ± 2.8	13.5 ± 0.7	14.2 ± 3.4	11.5 ± 1.1	14.0 ± 2.3	0.420	0.089	0.302
Glycine	19.4 ± 1.7	10.3 ± 0.5	10.1 ± 0.6	16.1 ± 0.9	12.7 ± 1.7	13.2 ± 1.8	18.8 ± 0.6	13.3 ± 0.9	15.9 ± 2	21.1 ± 4.8	14 ± 1.5	13.6 ± 0.6	14.9 ± 2.3	9.8 ± 0.7	11.5 ± 1.7	0.003	<0.0001	0.240
L-aspartic acid	31.5 ± 3	20.1 ± 0.5	23.1 ± 1.5	26.2 ± 0.7	23.1 ± 0.4	23.2 ± 1.1	32.4 ± 1.4	24 ± 2.1	29.8 ± 1.6	35.1 ± 8.6	26.5 ± 1.1	25.4 ± 1.1	27.6 ± 2.2	24 ± 0.7	28.3 ± 0.1	0.017	<0.0001	0.145
Taurine*	1.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	1.8 ± 1.1	0.2 ± 0.05	0.3 ± 0.05	2 ± 1	0.4 ± 0.4	0.3 ± 0.05	2.4 ± 1	0.5 ± 0.3	1.5 ± 1.7	4.3 ± 0.5	0.3 ± 0.01	0.4 ± 0.2	0.076	**<0.0001**	0.081
b-alanine	0.2 ± 0.03	0.3 ± 0.01	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.1	0.3 ± 0.01	0.3 ± 0.01	0.2 ± 0.03	0.2 ± 0.02	0.3 ± 0.1	0.2 ± 0.03	0.3 ± 0.01	0.3 ± 0.05	0.3 ± 0.1	0.3 ± 0.05	0.205	0.345	0.433
L-threonine*	17.5 ± 1.5	10.5 ± 0.2	11 ± 0.5	14.3 ± 0.4	12.4 ± 1.4	12.7 ± 1.3	17.3 ± 0.8	11.9 ± 0.5	15.1 ± 1.7	19.4 ± 5	13 ± 0.8	12.8 ± 0.3	14.4 ± 1.7	10.6 ± 0.4	13 ± 1.5	0.077	**<0.0001**	0.197

	Diet																	
Amino acid (mg/ g sample)	F			FI			FG			FIG			CF			ANOVA (*p* value)		
Time in seawater (h)	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	Diet (A)	Time (h) (B)	A x B
L-glutamic acid	49 ± 3.8	19.9 ± 1.2	22.5 ± 1.6	40.2 ± 1.4	23.3 ± 0.7	24.1 ± 1	49.2 ± 2.1	23.7 ± 2.2	29.4 ± 1.8	52.6 ± 12.6	25.7 ± 1	25 ± 0.6	44.3 ± 1.6	25.3 ± 0.2	29.3 ± 1	0.105	<0.0001	0.184
Citruline	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.03	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.04	0.2 ± 0.02	0.1 ± 0.04	0.1 ± 0.01	0.5 ± 0.3	0.2 ± 0.05	0.1 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.391	0.094	0.467
L-alanine	18.4 ± 1.9	12.7 ± 0.1	14.1 ± 0.5	16.5 ± 0.2	16 ± 0.2	16.7 ± 0.7	18.4 ± 0.5	14.4 ± 1.2	18.5 ± 1.4	21.1 ± 5.2	16.9 ± 0.6	16.1 ± 0.5	16.1 ± 1.4	14.4 ± 0.7	16.4 ± 0.6	0.066	0.002	0.151
GABA	0.4 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	0.4 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.4 ± 0.01	0.1 ± 0.01	0.05 ± 0.01	0.4 ± 0.01	0.1 ± 0.02	0.05 ± 0.01	0.4 ± 0.01	0.1 ± 0.08	0.1 ± 0.01	0.690	<0.0001	0.954
L-proline	13.8 ± 0.9	9.3 ± 0.3	10 ± 0.6	12.8 ± 0.3	11.5 ± 0.6	12.4 ± 1	13.4 ± 0.7	10.5 ± 0.8	12.8 ± 1.1	16.2 ± 4	11.9 ± 0.4	11.4 ± 0.1	13.4 ± 0.7	12.2 ± 0.3	14 ± 0.9	0.100	0.0002	0.101
delta-Hydroxylysine	0.9 ± 0.1	-	-	0.7 ± 0.1	-	-	1 ± 0.2	-	-	0.9 ± 0.2	-	-	0.5 ± .04	-	-	0.005	-	-
L-ornithine	3.7 ± 1.9	1.1 ± 0.2	1.3 ± 0.5	3.2 ± 1.5	1.1 ± 0.3	1.2 ± 0.1	2.1 ± 0.8	0.8 ± 0.2	0.5 ± 0.1	2 ± 0.5	0.4 ± 0.2	0.4 ± 0.1	3.7 ± 2.3	0.2 ± 0.2	0.1 ± 0.04	0.341	<0.0001	0.831
L-valine*	16.5 ± 1.3	11 ± 0.3	11.9 ± 0.5	14.2 ± 0.1	13.5 ± 0.8	13.7 ± 1	17 ± 0.8	12.1 ± 1.1	15.2 ± 1.3	20.5 ± 5.9	14.1 ± 1	14.2 ± 0.3	15.4 ± 1	13 ± 0.4	15 ± 1	0.094	**0.0002**	0.221
L-methionine*	0.5 ± 0.03	6.9 ± 0.1	6.7 ± 0.3	0.4 ± 0.02	7 ± 0.8	7.3 ± 0.9	0.4 ± 0.02	6.3 ± 0.2	8.3 ± 1.2	0.5 ± 0.2	6.1 ± 0.7	6 ± 0.4	0.3 ± 0.02	5.5 ± 0.2	6.7 ± 0.9	0.071	**<0.0001**	0.117
L-lysine*	23.1 ± 2.5	7.4 ± 0.2	9.0 ± 0.4	18.6 ± 0.2	7.6 ± 0.6	9.9 ± 1	24.7 ± 1.4	10.5 ± 1.7	9.5 ± 0.5	25.3 ± 5.5	7.3 ± 1.2	5.6 ± 0.7	16.7 ± 0.7	3.5 ± 0.9	2.4 ± 0.3	**<0.0001**	**<0.0001**	**0.006**
L-anserine	1.6 ± 0.9	-	-	1.1 ± 0.1	-	-	1.2 ± 0.4	-	-	1.1 ± 0.2	-	-	2.1 ± 0.8	-	-	0.410	-	-

	Diet																	
Amino acid (mg/ g sample)	F			FI			FG			FIG			CF			ANOVA (*p* value)		
Time in seawater (h)	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	Diet (A)	Time (h) (B)	A x B
L-cystine	1.4 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1	0.7 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.6 ± 0.2	1.1 ± 0.5	1.3 ± 0.3	0.6 ± 0.1	0.7 ± 0.2	1.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.362	<0.0001	0.073
L-tyrosine	12.2 ± 0.6	8 ± 0.2	7.7 ± 0.7	11.5 ± 0.4	10.5 ± 1.6	10.9 ± 1.9	11 ± 0.5	7.6 ± 0.2	9.8 ± 1.6	15.8 ± 5.3	10.5 ± 1.8	10.5 ± 1.2	9.8 ± 0.9	8 ± 0.5	9.7 ± 2	0.036	0.003	0.467
L-leucine*	22.2 ± 1.4	19.2 ± 1.1	20.9 ± 1.4	18.9 ± 0.6	23 ± 1.3	23.8 ± 1.7	21.3 ± 1.1	20.5 ± 1.7	25.9 ± 2.4	24.4 ± 6.3	23.2 ± 1.2	22.7 ± 0.5	20.6 ± 1	23.4 ± 0.5	28 ± 2.47	0.180	**0.020**	0.068
L-isoleucine*	13.7 ± 0.6	10.8 ± 0.5	11.8 ± 0.7	11.5 ± 0.2	12.5 ± 0.8	12.8 ± 1	14.5 ± 0.9	11.7 ± 1.2	14.7 ± 1.2	16.7 ± 4.9	13.3 ± 0.9	13.3 ± 0.3	13 ± 0.6	12.7 ± 0.3	14.9 ± 1.1	0.103	0.087	0.221
L-phenylalanine*	16.4 ± 0.5	11.2 ± 0.2	10.3 ± 1.1	12.5 ± 0.3	11.9 ± 1.5	12.4 ± 1.8	15 ± 0.7	11 ± 0.5	13.8 ± 2.3	18 ± 5.9	12.3 ± 1.9	12.4 ± 1.2	14.9 ± 1	12.3 ± 0.5	14.7 ± 3.1	0.490	**0.004**	0.360
L-tryptophan*	0.3 ± 0.1	0.1 ± 0.01	0.1 ± 0.02	0.2 ± 0.07	0.1 ± 0.05	0.1 ± 0.02	0.2 ± 0.04	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.03	0.1 ± 0.01	0.709	**0.001**	0.462

Data are represented by means of three replicates ± standard deviation (n = 3).
For each parameter, homogeneous groups and significant differences are indicated by alphabetic superscripts ($p < 0.05$).
*Values in bold are essential amino acids in abalone.
(-) Data not detectable.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

Carbon, nitrogen, and phosphorus levels in feeds

For both experiments, diets with both fishmeal and insect meal (FI and FIG) resulted in less amount of phosphorus compared to diets that included fishmeal only (F and FG) at time 0 h. Time did not play a significant role in variations of P levels in the tanks without abalone (Table 4.6). However, time played a significant role in variations of P levels in tanks with abalone (Table 4.7). In tanks with and without abalone, P levels were significantly affected by the interaction of diet and time in seawater indicating an additive effect.

Diets with inclusion of either IM or GM produced higher C:N ratio at time 0 h. Diet F had the lowest value at time 0h, indicating more N in the formulation. The two-way ANOVA showed that diet was the most critical factor that affects the C: N ratio in both tanks with (Table 4.7) and without abalone (Table 4.6). Similarly, the time of immersion in seawater played a significant role in the C: N ratio of diets in tanks with and without abalone. The interaction of diet and time of immersion seawater played a significant role in both P levels and C:N ratio, in tanks without abalone, while it did not have a significant role in the C:N ratio in diets trialled in the tanks with abalone.

Table 4.6 Phosphorus (P) and carbon: nitrogen (C:N) leaching values (tanks without abalone) of four experimental diets and a commercial feed.

	Diet																	
	F			FI			FG			FIG			CF			ANOVA (*p* value)		
Time in seawater (h)	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	Diet (A)	Time (h)(B)	A x B
Phosphorus *	1076.2 ± 99.8	1048.8 ± 35.2	1106.9 ± 15.9	786 ± 1.3	830.6 ± 22.4	823.9 ± 6.2	943.5 ± 13.4	804.2 ± 20.1	901.6 ± 34.0	666.5 ± 9.3	706.3 ± 23.4	745.0 ± 16.6	876.6 ± 8.7	1031.8 ± 90.7	988.4 ± 18.6	**<0.001**	0.058	**0.003**
C:N ratio	6.5 ± 0.1	7.8 ± 0.2	8 ± 0.2	8.6 ± 0.3	8.2 ± 0.1	7.9 ± 0.3	7.5 ± 0.2	7.2 ± 0.1	7.2 ± 0.1	8.2 ± 0.4	8.1 ± 0.6	7.7 ± 0.06	7.6 ± 0.1	8.6 ± 0.06	8.1 ± 0.04	**<0.001**	**0.047**	**0.004**

Data represent means and standard deviation (n = 3). A significant level of p < 0.05 was used for all statistical tests.
*Phosphorus in ppm per g diet.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

Table 4.7 Phosphorus and carbon: nitrogen leaching (C:N) values (tanks with abalone) of four experimental diets and a commercial feed.

	Diet											
	F				FI				FG			
Time in seawater (h)	0 h	24 h	48 h	96 h	0 h	24 h	48 h	96 h	0 h	24 h	48 h	96 h
Phosphorus*	1446.8 ± 60.5	2506.5 ± 33.2	2334.3 ± 87	2403.2 ± 53	908.9 ± 42.5	2022.6 ± 22.3	1940.8 ± 119.9	1891.5 ± 67	1110.5 ± 20.6	1217.4 ± 47.6	1220.7 ± 37	1192.9 ± 23
C:N ratio	6.5 ± 0.1	6.2 ± 0.2	6 ± 0.2	5.7 ± 0.2	8.6 ± 0.3	8.1 ± 0.2	7.9 ± 0.2	8.2 ± 0.2	7.5 ± 0.2	7 ± 0.1	6.8 ± 0.3	7 ± 0.9

	FIG				CF				ANOVA (*p* value)		
Time in seawater (h)	0 h	24 h	48 h	96 h	0 h	24 h	48 h	96 h	Diet (A)	Time (h) (B)	A x B
Phosphorus*	466.2 ± 42.4	631.3 ± 30.4	676.4 ± 20.2	615.4 ± 41.7	795.4 ± 23.3	1740.6 ± 29.2	699.6 ± 83.5	666.2 ± 13.7	**<0.0001**	**<0.0001**	**<0.0001**
C:N ratio	8.2 ± 0.4	8.7 ± 0.7	8.2 ± 0.4	7.5 ± 0.2	7.6 ± 0.1	8 ± 0.03	7.5 ± 0.06	7.4 ± 0.02	**<0.0001**	**0.003**	0.290

Data represent means and standard deviation (n = 3). A significant level of p < 0.05 was used for all statistical tests.
*Phosphorus in ppm per g diet.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

4.5.4 Growth performance and enzyme activity

Due to the length of the feeding trial (14 days), evaluation of abalone growth was limited. However, the acceptance and feed intake were assessed to evaluate experimental diets. Results showed no significant differences in FCR among experimental diets and CF (Table 4.8). In terms of enzyme activity, there was a significant difference between diet FI and FG, and FG and CF in trypsin. There was significant increase of amylase activity in diets which contained GM (FG and FIG) compared to the other experimental diets which did not contain GM. There were no significant differences among experimental diets and CF in the lipase activity.

Table 4.8 Growth performance and nutrient utilisation of *H. iris* fed four experimental diets and a commercial feed.

	Diet				
	F	FI	FG	FIG	CF
Growth performance					
Difference weight (g)	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.3	0.2 ± 0.1
Difference Shell length (mm)	1 ± 0.6	0.8 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	1.5 ± 1
Difference Shell width (mm)	0.3 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.5
Feed conversion ratio	0.9 ± 0.4	0.8 ± 0.2	1.7 ± 1.4	1.2 ± 1.1	0.9 ± 0.2
Enzyme activity					
Trypsin (U mg protein $^{-1}$)	0.0008 ± 0.003^{ab}	0.007 ± 0.001^{b}	0.011 ± 0.004^{a}	0.009 ± 0.004^{ab}	0.007 ± 0.001^{b}
Amylase (U mg protein $^{-1}$)	4.01 ± 1.24^{b}	5.01 ± 1.28^{b}	18.02 ± 4.56^{a}	14.01 ± 5.52^{a}	13.79 ± 3.41^{a}
Lipase (U mg protein $^{-1}$)	0.013 ± 0.09	0.014 ± 0.011	0.010 ± 0.006	0.007 ± 0.004	0.006 ± 0.003
Protein in viscera (mg/ g sample)	294.9 ± 55.4^{b}	314.2 ± 29.1^{ab}	313 ± 34.7^{ab}	349.3 ± 29.6^{ab}	366.8 ± 39.1^{a}

Data represent means of three replicates and standard deviation. For each parameter, significant differences are shown by different superscripts (Tukey's test, $p < 0.05$).
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc), and commercial feed (CF).

4.6 DISCUSSION

Physical characterisation

Our study shows the potential of encapsulation for the delivery of nutrition and the inclusion of two alternative ingredients for the New Zealand abalone. The physical characteristics of the experimental diets suggest that these diets perform better compared to the CF. The particle size of the experimental diets was not significantly different from the CF. This finding suggest that the experimental diets were suitable for ingestion for juvenile abalone. The particle weight showed that the CF had the heaviest particles. The diets that included GM (FG and FIG) were heavier compared to diets without GM and this is supported by the values from the sinking rate test. Diets containing GM had significantly longer sinking rates than diets without GM, indicating that GM provides a certain 'structural' characteristic that creates more density in the particle, yet particles do not sink faster. A possible explanation of this phenomenon is the formation of a complex lignin-alginate formed inside the particle. Lignin is an insoluble polymer highly present in grape marc and can create a higher stable polymer complex with calcium alginate matrix, which has a high capacity of water absorption (Song et al., 2020). This absorbent capacity is supported by the values of water absorption index. In fact, GM has been used in industrial applications to adsorb pigments from aqueous solutions (Perez-Ameneiro et al., 2014). Although the experimental diets in their fresh and dried form had longer sinking rates than the CF, this was improved when they were frozen. This might indicate that the frozen delivery is the most adequate due to the bottom feeding nature of abalone and the risk of feeds being washed away. The frozen form might provide additional advantages, such as the delivery of probiotics in the gastrointestinal tract of abalone (Masoomi et al., 2021) promoting more viable bacteria compared to room temperature formulations (Rosas-Ledesma et al., 2012; Sousa et al., 2012).

Our study shows that our experimental diets were more stable in seawater, less resistant to abrasion compared to CF and yet promoting growth in abalone. The solid loss in the CF was significantly higher compared to experimental diets in both 24 and 48 h. These results support the fact that the encapsulation method is more efficient at keeping the particles inside the capsule compared to extruded pellets. In fact, alginate beads are recognized for their water stability due to the calcium crosslinker, which provides a semi-permeable bead allowing the containment of the particles during prolonged times (Moreira et al., 2006). The CF showed lower stability in seawater due to the higher solubility (WSI), a

higher porous structure (microscopy), and the higher density of powder reflected in the high weight of the CF compared to the experimental diets. Each pellet of CF of 3.9 ± 0.3 mm size contained 16.6 ± 2.3 g of powder, almost double that of the weight of the experimental diet beads. The durability index showed that the experimental diets with GM were more resistant to the mechanical handling than diets without GM. Particles with high durability form fewer small particles during bagging, storage, and finally show low degradation in pneumatic feeding devices when fed to aquatic animals (Kaliyan & Morey, 2009; Thomas & Van der Poel, 1996). The CF has a completely different physical structure than the experimental diets, as evident from the microscopic analysis. The CF showed more predominant scaffolds and more chains extended through the surface, suggesting a more absorbing capacity as supported by the water absorbent index (WAI) and the water solubility of the powder (WSI).

Another finding in our study was the non-significant variation of the texture profile of our experimental diets as time went on. Experimental diets that included IM and GM were more stable in seawater after 24 and 48 h. However, their hardness, gumminess, and chewiness were not significantly different after 16, 24 and 48 h of immersion in seawater. This observation is of particular interest as water stability does not jeopardize texture particle characteristics for abalone ingestion. Interestingly, the main variations among experimental diets occurred when they were in the fresh or frozen form. In the fresh form, the inclusion of GM and/or IM produced diets with significantly higher gumminess compared to diets without GM and/or IM, but hardness nor chewiness were affected. In the frozen form, these traits were amplified showing that the inclusion of GM resulted in diets with significantly higher hardness, gumminess, and chewiness compared to diets without GM. Conversely, the inclusion of IM did not cause significant variations. The texture analysis from the encapsulated diets with GM showed higher hardness, gumminess, and chewiness, particularly in the frozen form. This fact reinforces the presence of the highly stable 'lignin-calcium alginate matrix' in the experimental beads (Song et al., 2020), which may trap water and ions from the water column. This matrix may resemble the 'egg box model' cross linking matrix present in alginate beads (Shilpa et al., 2003). Although quite stable, this structure is debilitated while immersion in seawater extends due to calcium sequestration from the hydrogel (Smidsrød & Skja, 1990). This structure is created by L-guluronate and D-mannuronate residues and provides support along with calcium via cross-linking. Once this structure is formed, the substances inside the 'box' are slowly released from the gel depending on the divalent ions on the surroundings and pH. The rapid diffusion of small molecules through the alginate membrane (Lee & Mooney, 2012) allows more particles and water to be absorbed. Due to these properties and the high ability of calcium alginate beads

to ‘protect’ molecules from the harsh environments of the stomach (Chen et al., 2004), they are of high interest for inclusion of symbiotics, probiotics and post-biotics.

Chemical characterisation

The proximate composition of the diets suggests that the inclusion of insect meal and grape marc affected the lipid, carbohydrate, dietary fibre, and ash composition. Diets containing only fishmeal as a protein source (F and FG) were relatively higher in protein compared to diets that included a mixture of fishmeal and insect meal (FI and FIG), although the changes were not significant. These variations are caused by the reduced levels of protein of the insect *Tenebrio molitor* (around 50%) (Ramos-Elorduy et al., 2002) compared to fishmeal (around 53%) (data not showed in this study). In addition, IM contains a higher lipid proportion (Ramos-Elorduy et al., 2002) compared to fishmeal, reflected in the significantly higher lipid proportion of diets that included IM diets FI (7.0 ± 0.6 %) and FIG (7.2 ± 0.3 %). The most remarkable difference among experimental diets was in dietary fibre. Diets FG and FIG, which contained GM, resulted in significantly lower levels of digestible carbohydrates (reducing sugars) and higher levels of dietary fibre. The GM non-digestible proportion is made of complex carbohydrates, such as oligosaccharides (OS) (Tian et al., 2023), which positively influence gut health by acting as prebiotics (Sinrod et al., 2021). Usually, the beneficial effect of GM has been attributed mainly to polyphenols and antioxidants, such as tocopherol. However, recent research has showed that oligosaccharides from grape seeds have a significant prebiotic activity on *Lactobacillus acidophilus* (Bordiga et al., 2019).

Our results suggest that the ingredients in the diet formulation affected the phosphorus level and C:N ratio over time. Diets that contained significantly higher levels of protein (F, FG, and CF) had lower C:N ratios. Diets that contained IM as a supplement and therefore, significantly lower levels of protein, had lower levels of C:N ratios. This finding supports the inclusion of IM to reduce the nitrogen load while still promoting growth of abalone. Therefore, the inclusion of IM would reduce the over-enrichment of N of the water column compared to fishmeal (Fleming et al., 1996). Although the inclusion of IM successfully increased the C:N ratio, there are no data on the preferred C:N ratio for abalone feeds to promote growth. Most of the feeds used in aquaculture have a low C:N ratio of around 7–10:1 (Asaduzzaman et al., 2010; Xu et al., 2018), which implies low levels of C and high levels of N as it is the main element for protein formation.

The increased levels of P and C:N ratio in both, tanks with and without abalone, showed that these levels are significantly affected by the diet composition rather than the time of immersion in seawater (0, 24 and 48 h). In addition, the P levels, and the C: N ratio are strongly affected by the presence of abalone in the tanks, contributing to higher levels of P and N in uneaten feed as time went on. This finding suggests that abalone excretion plays a role in the C and N levels of uneaten feed (Avnimelech, 1999; Galasso et al., 2017) where possibly dissolved nitrogen is absorbed in the capsule/pellet. As uneaten feed waste is partially discharged with the effluent water in farms (Amirkolaie, 2011), it corroborates the potential environmental risk from enriched N-uneaten feed waste.

The amino acid profile of diets showed that the essential amino acids L-histidine, L-arginine, and L-lysine were mainly affected by the type of diet. From these amino acids, L-lysine is the most important as it is considered the first limiting amino acid in abalone (Bansemer, Qin, Harris, Howarth, et al., 2016; Shipton, 1999). The findings in this study suggest that the experimental diets would have provided L-lysine equally or more than to what CF provided at least in the first 24 h. Although the lysine levels for the CF at time 0h were the lowest, they promoted good growth over time. The L-lysine values decreased significantly in the first 24 h for all diets supporting the fact that lysine supplementation might be needed to improve growth performance (Shipton et al., 2002). Another interesting finding was the significantly increased levels of arginine in the experimental diets which included IM and/or GM compared to the CF and diet F (only fishmeal as protein source) at time 0 h. Along with lysine, arginine has been considered a limiting amino acid in abalone feeds (Mai et al., 1994). Interestingly, our study showed that arginine was found to significantly increase in the first 24 h along with L-histidine, L-methionine, and L-leucine. The increase of amino acids in the uneaten feed can be explained by the adsorbent capacity of the pellets/capsules according to their morphology and nutrient composition. The encapsulated diets may possibly adsorb compounds from the water column such as peptides and free amino acids from dead cell material (Braven et al., 1995; Singleton & Skerman, 1973). The capacity of the encapsulated diets to adsorb methionine, arginine, and histidine after 48 h can be utilised for waste removal. The adsorbent capacity along with the improved seawater stability of encapsulated diets (only 7-8% disintegration after 48 h) facilitates uneaten feed waste removal with a sieve of 1 mm.

The essential amino acids taurine, L-threonine, L-valine, L-phenylalanine, and L-tryptophan were significantly reduced after 24 h of immersion in seawater in all diets. Among these amino acids, taurine was the most reduced with almost 80 % reduction. For this

reason, some commercial formulations add taurine. Taurine is a recognized feed enhancer along with methionine or glutamine to promote faster growth (Lunger et al., 2007; Salze & Davis, 2015).

Growth performance and enzyme activity

Our results from the 14-day feeding trial provided limited information for growth evaluation. Daily growth for abalone is around 0.1 mm, therefore, providing only a total of 1.4 cm approximately for comparison. A longer feeding trial is recommended to expect more significant differences among dietary treatments considering providing a settling period with the new introduced diets to avoid biases. However, growth data are presented here as a reference point for future research. Weight gain and growth indicated that the experimental diets were accepted and promoted growth in abalone which was one of the outcomes of this research. There was a high variability in diet FIG weight gains, suggesting that a bigger sample size of abalone should be considered in the future. High variability of weight and shell length is expected particularly in juvenile populations (Stone et al., 2013). The FCR results suggest that the feed consumption of the experimental diets, in particular diet FG and FIG, were quite variable. This phenomenon might be explained by the gradual adaptation of abalone to GM. The FCR was not significantly different among the experimental diets and CF.

Our results corroborate that the digestive enzyme activity of abalone is modulated by the feed consumed. Previous studies have shown that enzyme adaptation takes place depending on the diet consumed in *Haliotis laevigata* (Bansemer, Qin, Harris, Schaefer, et al., 2016) and *Haliotis fulgens* (García-Carreño et al., 2003). The presence of lignin in the diets with GM might have promoted a more stable polymer, yet not highly digestible. The high content of dietary fibre in diets that included GM may have caused abalone to spend more energy in digesting the fibre, thus, slowing down the allocation of energy for growth. As a result, the shell length was slightly reduced compared to diet F (only fishmeal as protein source) and the CF. In addition, the increased activity of amylase in diets with GM support the prebiotic nature of GM which similar to other feed supplements, such as probiotics, increases enzyme activity (Assan et al., 2022), therefore possibly increasing food absorption and efficiency (Yu et al., 2022).

4.7 CONCLUSIONS

The inclusion of alternative ingredients into aquatic feeds requires a holistic evaluation of many factors attributed to the feed, animal, and environment. In this study, we focused on the physical and chemical characteristics, feed delivery form, nutritional value, and feed intake of experimental diets that included IM and GM as a supplement. To our knowledge, this is the first study to formulate abalone feeds in an alginate bead form and compare performance with a commercial feed in terms of physico-chemical characteristics and abalone growth. The experimental diets delivered in alginate beads were more stable in seawater over 24 and 48 h compared to the CF. The inclusion of GM resulted in significantly harder particles when they were dried, thus increasing the capacity to resist abrasion during longer periods of storage and manipulation. However, the inclusion of grape marc and a combination of insect meal and grape marc resulted in frozen particles significantly harder, gummier, and chewier compared to diets without IM and GM. Interestingly, hardness, chewiness and gumminess were reduced significantly in experimental diets with GM as time went on. Our results indicate that the appropriate delivery for the experimental diets is frozen beads, which reduce the sinking rate and add formulation advantages such as the inclusion of bioactives or thermolabile compounds. Due to less solid loss after 24 h and 48 h, alginate beads may improve efficiency in farms allowing less cleaning which can be reduced from 48 to 96 h. The growth data need further examination as there were no significant differences among treatments in 145 days. However, experimental diets were consumed by abalone. The inclusion of GM and the combination of GM and IM significantly increased amylase activity but did not increase trypsin and lipase activity compared to the diet without GM and IM and the CF. Overall, the present study showed two viable alternative ingredients, IM and GM for abalone feeds that if introduced at 10% and 30%, promoted growth, feed intake, and modulation in digestive enzymes. Longer studies are required to evaluate the long-term effect of both alternative ingredients along with the inclusion of micronutrients, such as vitamins and minerals for further feed optimisation.

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Chapter

5

Effect of insect meal and grape marc in the nutritional profile, growth, and digestibility of juvenile New Zealand farmed abalone (*Haliotis iris*)

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5.1 ABSTRACT

Generally, one of the most significant bottlenecks of land-based abalone aquaculture is the high cost of the feed, which is associated with the use of wild fish stocks to produce fishmeal. Fishmeal is a key ingredient used in aquaculture feeds due to its nutritional profile and palatability. Alternative ingredients, such as insect meal (IM) and grape marc (GM) are potential candidates to be included in aquafeeds due to their suitable nutritional profile and more sustainable production. This study aimed to evaluate the effect of IM and GM on growth and nutritional profile using abalone as an animal model in a feeding trial for 165 days. For the feeding trial, four experimental diets containing graded levels of IM and GM were developed. A commercial feed was used as a control. Proximate analyses were used to identify nutritional variations in abalone tissue and faecal matter, and liquid chromatography-mass spectrometry and gas chromatography mass-spectrometry analyses were utilised to characterise amino acid and fatty acid composition. Results showed a 95% survival for all treatments. The inclusion of IM and GM did not affect the protein proportions of abalone tissues, but differences were found in the carbohydrate and lipid proportions. The inclusion of IM and GM significantly affected levels of amino acids L-histidine, L- methionine, and L-phenylalanine, and fatty acids oleic acid, linoleaidic acid, palmitic acid, palmitoleic acid, and α-linolenic suggesting a correlation between the feed source and fatty acid tissue composition. This work provides promising results for future optimisation of abalone diets including more affordable and sustainable alternative ingredients.

5.2 INTRODUCTION

Abalone (*Haliotis iris*) represent a valuable export product in New Zealand, contributing between NZD $50–60 million annually (Prime Minister's Chief Science Advisor, 2021) towards the country's export economy. Most of this production is from wild harvest, mainly collected from Fiordland in the Southland and the Chatham Islands. A small aquaculture production in land-based facilities is projected to grow by 100% by 2035 (The New Zealand Government, 2019).

Generally, one of the most significant bottlenecks of land-based abalone farming is the high cost of the feed, which can be up to 50% of the production cost (Fleming et al., 1996). The high price has been attributed to fishmeal (FM), which is a key ingredient used in aquaculture feeds due to its excellent amino acid profile and palatability. However, the use of FM in aquafeeds has been questioned since it usually requires 4–5 kg of forage fish input to generate 1kg of FM (Péron et al., 2010). Thus, the use of wild fish to feed farmed fish is thought to be unsustainable with dramatic effects on already depleting marine fish resources. New trends in aquaculture practices intend to develop feed formulations that use more sustainable replacements for FM, thus reducing the proportion of FM in the final feed. Currently, aquaculture uses almost 60% of the total world production of fishmeal (Péron et al., 2010). Examples of successful alternative ingredients incorporated in abalone trials are steam distilled grape marc meal (Currie et al., 2019), tuna by-product meal (Jung et al., 2016), seaweed meal (Viera et al., 2015), soy meal and silkworm pupae meal (Cho, 2010).

Insect sources have recently been considered to be potential candidates for FM replacement due to their excellent nutritional profile with 42–63% crude protein content (Fasolin et al., 2019), rich in amino acids, lipids, vitamins, and minerals (Makkar et al., 2014; Nogales-Mérida et al., 2018). The five major groups of insects used in feeds are black soldier flies (*Hermetia illucens*), the house flies (*Musca domestica*), mealworm beetles (*Tenebrio molitor*), locusts, grasshoppers, crickets, and silkworm. *T. molitor* larvae contain all essential amino acids (EAA) in sufficient quantities to meet the dietary requirements of most aquatic species. The essential amino acid profile of the mealworm larval meal is comparable to those of fish and soy meals (Azagoh et al., 2016) and most of the EAA contents are higher than the requirements suggested by the FAO/WHO/UHU (Li et al., 2013). Insect meal (IM) is considered to be a more sustainable ingredient for aquafeeds due to the lower requirements for land and water resources compared to soybean meal (Ferrer et al., 2019). In addition, IM contributes to a substantial reduction in greenhouse emissions since insect

rearing has lower waste and waste products can be used for insect feeding (Nugroho & Nur, 2018). Some examples of successful inclusion of insects into aquafeeds include houseflies (*M. domestica*) as partial replacement, which promotes growth performance for tilapia (*Oreochromis niloticus*) (Wang et al., 2017). Mealworm (*T. molitor*) inclusion in rainbow trout (*Oncorhynchus mykis*) diets, which generates an increase in antioxidant intestinal activity and a reduction of lipid peroxidation (Henry et al., 2018). Black soldier flies (*H. illucens*) were found to enhance gut microbial diversity and the amount of beneficial lactic acid bacteria in rainbow trout (*O. mykis*) microbiota (Terova et al., 2019). To our knowledge, no study has been conducted to use mealworm meal as a replacement for FM in abalone diets.

Another alternative ingredient for aquafeeds is grape marc (GM), which is an underutilised biowaste product from the viticulture industry comprising the skins and seeds remaining after pressing. Due to the large amount of waste produced annually in vineyards throughout New Zealand, GM disposal has become problematic. It has been estimated that almost 18–20% of all harvested grapes used for wine production end up as GM (Spanghero et al., 2009), and the Marlborough region alone produced approximately 50,000 tonnes of GM in 2020 (Massey University, 2020). GM is commonly used as fertilizer, but no use has been assigned to the aquaculture feed industry. GM nutritional properties include low levels of digestible carbohydrates, polyphenols (anthocyanins, catechins, flavonols, and phenolic acids), large amounts of resveratrol present in the skin and pulp of red grapes, as well as high levels of polyunsaturated fatty acids (over 60%), and more than 2% being omega-3 fatty acids (Habeanu et al., 2015). These properties might positively influence the glycaemic load, energy intake, and antioxidant capacity in abalone. GM has been mainly included in feeds for ruminants, showing no detrimental effects in growth, with an improved environmental impact (Moate et al., 2014). However, the dietary application of GM in aquatic species is very limited. To our knowledge, GM derivatives have been used only in feeds for *Haliotis laevigata* with great success (Currie et al., 2019). Seam distilled GM (Acti- Meal®) has resulted in better growth rates and feed conversion ratios of *H. laevigata* compared to commercial diets and feed without GM supplement (Currie et al., 2019).

The aim of the present study is to evaluate the effect of different inclusion levels of IM and GM on aquafeeds to improve growth performance, feed utilization, proximate composition, amino acid, and fatty acid profiles of juvenile New Zealand farmed abalone.

5.3 MATERIALS AND METHODS

5.3.1 Experimental animals

This study was conducted within a commercial abalone (*Haliotis iris*) farm (The New Zealand Abalone Company) in Bluff, Invercargill, New Zealand. Juveniles with initial mean weight and shell length of 1.1 ± 0.5 g and 21.5 ± 3.3 mm, respectively, were used in the feeding trials. The animals were 17-months old, and they were randomly selected from the farm stock. A total number of 3000 animals were used for the feeding trial.

5.3.2 Diet preparation

Four experimental diets were formulated to contain different levels of fishmeal (FM), corn meal (CM), insect meal (IM) and grape marc (GM). The source of energy came from carbohydrates either in the CM or GM and the source of protein was from FM or IM or both. Seaweed and starch served as attractant and binder, respectively. The composition of the experimental diets is shown in Table 5.1. The commercial feed Marifeed®, was used as the control. The procedures for diet preparation were as follows:

Experimental diets were prepared by mixing pre-weighed finely ground ingredients in a commercial food mixer. All ingredients were mixed, except the starch (Native Maize flour). Starch syrup at 10% was prepared and sequentially added to the dried mixture to form a dough with even consistency which was then placed on a flat tray to be dried at 65 °C for 16 h. The dried dough was ground using a commercial blender (Nutri Bullet 600 household mixer) for 30 s, and the powder was sieved two times using a kitchen sieve.

Table 5.1 Percentage (dry weight) composition of the experimental diets (g/100g)

	Diet			
Ingredients (g/100g diet)	F	FI	FG	FIG
Fishmeal[1]	35	25	35	25
Insect meal[2]	-	10	-	10
Corn meal	30	30	-	-
Grape marc[3]	-	-	30	30
Seaweed (dry) *Macrocystis pyrifera*[4]	4	4	4	4
Starch (Native Maize flour)[5]	10	10	10	10

[1] Fishmeal supplied by Sandford, NZ.
[2] Insect meal supplied by Mahurangi Technical Institute (MTI).
[3] Grape marc supplied by Bragato Research Institute, NZ.
[4] Seaweed (*Macrocystis pyrifera*) supplied by Southern Clams.
[5] Starch supplied by New Zealand Starch.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc) and FIG (fishmeal + insect meal + grape marc).

5.3.2.1 Encapsulation of experimental diets

Encapsulation was performed using calcium chloride solution (0.1M) and alginate solution (1% w/v). Alginate solution (1%) was prepared overnight to promote good incorporation of the powder. Briefly, for encapsulation, 70 g of feed powder were mixed with 250 mL of distilled water until hydrated completely. A total of 750 mL alginate solution (1% w/v) was added and mixed right before extrusion. For the extrusion, 400 mL of calcium chloride were placed in a 1L beaker to extrude 100 mL of alginate solution (which contained the feed powder). The formed beads were rinsed with 100–200 mL distilled water and placed in an oven tray covered with aluminium foil. Beads were air dried in a commercial oven (Piron PF8906, Italy) at 65 °C for 16 h. The dried beads were then packed in a vacuum-sealed bag to avoid contamination. Dried beads were hydrated in seawater for 8–12 hours before abalone feeding.

5.3.3 Feeding trial and sample collection

Fifteen plastic tanks containing 90 L of filtered seawater (100-micron filter) were stocked with 200 abalone juveniles each. These tanks were specifically allocated for the feeding trial within the farm location, following the same conditions. From the 200 animals, 50 were tagged for allometric measurements. For tagging, abalone were removed from the

tanks, measured in shell length, width, and weight (baseline), and tagged with one colour plastic tags (numbered) which were adhered to the shells with cyanoacrylate glue. The tagging process was performed on one day and the water temperatures during this process fluctuated from 12.1 to 13 °C.

Five dietary treatments were randomly allocated to three replicate tanks per treatment. A flow-through water system was maintained at a rate of 1.5 L/min, which equates to a total water exchange of 40 times per day. The tanks were drained and cleaned as per farming procedure every other day. Briefly, the stopper at the bottom of the tank was pulled off from the tank, and the tanks were completely drained. Faeces, debris, and uneaten food residues were flushed out with the help of water supply. Animals remained undisturbed in the tank while cleaning was performed. Water temperature and dissolved oxygen were measured electronically before and after cleaning and fluctuated from 12.4–19.7 °C. Water quality dissolved oxygen was measured before and after cleaning using a dissolved oxygen meter (Handy Polaris TCP, Denmark). The dissolved oxygen meter was calibrated before use in 'air-saturated' seawater according to manufacturer's instructions. The dissolved oxygen was maintained between 86.3–104.1 % oxygen saturation.

Abalone were fed 1.2–2.2% of their body weight per day. The daily ration was given once in the late afternoon (about 1600 h). The experiment lasted 165 days. Abalone were evaluated for proximate analyses, fatty acid, and amino acid composition before (baseline) and after the feeding trial. Before the feeding trial, 105 abalone were randomly collected from the cohort for proximate analyses, and 20 abalone for fatty acid and amino acid composition analyses. After the feeding trial, 70 abalone were collected from each tank for proximate composition and 15 abalone for fatty acid and amino acid composition analyses. The whole soft bodies were placed into 2 mL cryovial (Biostor™) and quenched in liquid nitrogen for 10 min and then stored in dry ice (-80 °C) for transportation to the -80 °C freezer located at the Auckland University of Technology (Auckland, New Zealand) where samples were stored until further analyses.

5.3.4 Growth parameters and feed consumption

Growth measurements of tagged animals were recorded on animals collected at the start of the experiment (baseline) and after 165 days of treatment. This was done by removing the animals from the water with a blunt knife. Then, the animals were dried with paper towels, and their maximum shell lengths and widths (mm) and total animal wet weights (g) were recorded. Lengths were measured with a vernier calliper (Mitutoyo 0–125mm,

Warwickshire, UK) to the nearest 0.1 mm, and weights were measured with a digital balance to the nearest 0.1 g.

Abalone specific growth rate (SGR, in % days $^{-1}$) was calculated according:

Specific growth rate total weight

$$SGR\ TW\ \%\ day^{-1}\ = 100\ [(ln_f\ \ TW - ln_i\ \ TW)\ \div 165\ days\ ]$$

Specific growth rate shell length

$$SGR\ SL\ \%\ day^{-1}\ = 100\ [(\ln final\ SL - \ln initial\ SL)\ \div 165\ days]$$

Specific growth rate shell width

$$SGR\ SW\ \%\ day^{-1}\ = 100\ [(\ln final\ SW - \ln initial\ SW)\ \div 165\ days]$$

Where ln_f is the natural log of the final total weight (TW), final shell length (SL), final shell width (SW) and ln_i is the natural log of the initial total weight (TW), initial shell length (SL), and initial shell width (SW).

Daily increment in shell length (DISL, in µm d^{-1}) was calculated according to (Dlaza et al., 2008)

$$DISL\left({}^{\mu m}/_{day}\right) = 1000\ x\ \left(\frac{SL_f - SL_i}{165\ days}\right)$$

Condition factor

The condition factor (CF, in g mm^{-1}), which is an index that was developed to account for the relationship between the weight of abalone per unit shell length, was calculated following Britz (1996)

$$CF = [BW/\ SL^{2.99}\ ]\ \times\ 5\ 575$$

Where BW is the mean body weight (g) and SL the mean shell length (mm); 2.99 and 5.575 are constants.

Muscle yield (%):

$$= (muscle\ weight\ \ \div\ \ total\ body\ weight)x\ 100\%$$

5.3.5 Faecal sample collection

Faecal collection occurred at week 12 of the feeding trial and was performed for two weeks. All samples were pulled together per treatment due to insufficient biomass for analysis. For the faecal collection, the feeding regime changed from night-time (normal time of faecal deposition) to daytime to facilitate faecal matter collection not contaminated with feed particles. Briefly, animals were fed in the early morning and tanks were completely cleaned of feed residues in the afternoon. After the cleaning, animals deposited faeces until the next day when tanks were cleaned, faeces collected, and animals fed again. The faecal matter was dried for 16 h at 50 °C and weighed after. Dried faecal matter was storded in air-tight container in the freezer at 2–8 °C until proximate and fibre analyses.

5.3.6 Proximate Analyses

Proximate composition analyses, including crude protein, crude lipid, ash, and moisture content were conducted on the experimental diets, animal soft tissues and faecal samples following AOAC (1985). Moisture was determined in three replicates using a convection oven at 135 °C for 3 h for feeds and faecal samples. For abalone soft tissues, 105 animals were collected at the beginning of the trial and 70 animals at the end of the trial. Abalone soft tissues were freeze-dried (Christ alpha series freeze dryer, Osterode am Harz, Germany) for 48 h and moisture calculated as the weight difference. After moisture determination, dried diets, abalone tissues and faecal samples were ground up to powder using a grinder (IKA A11 model analytical mill, Germany) with the addition of liquid nitrogen to avoid denaturation/oxidation of metabolites before other analysis analyses.

5.3.6.1 Protein analyses

Ground samples (2–4 mg) were weighed and nitrogen content was determined by a CE-440 Elemental Analyser (Exeter, Chelmsford, Massachusetts, USA). The combustion and reduction temperatures were 980 and 700 °C respectively with pure oxygen as the combustion gas and pure helium as the carrier gas. The purge and combustion times were 15 and 20 s respectively. The percentage of crude protein in the samples was calculated multiplying the total nitrogen in the samples by the conversion factor 5.8 (faecal matter and abalone tissues) according to the marine organisms described by Gnaiger and Bitterlich (1984) and 6.25 for nitrogen conversion (feed samples).

5.3.6.2 Lipid analyses

Two different methods were used. For lipid determination in feeds, Soxhlet extraction with Petroleum ether for 2.5 h was used. For lipid determination in animal tissues and faecal samples, crude fat extraction was performed according to Bligh and Dyer (1959) for small samples. Briefly, 0.1 g of dried samples were hydrated in 1 mL chloroform and 2 mL methanol and 800 µL distilled water. The mixture was vortexed for 2 min and 1 mL chloroform was added. The solution was vortexed again for 30 s and 1 mL distilled water was added to separate layers. The mixture was vortexed again for 30 s and centrifuged at 3000 rpm for 5 min. The lower organic solvent layer was collected into a pre-weighted 8 mL glass tube. A nitrogen stream was used until all solvent was vaporized. Finally, the net weight of the lipids in the sample was recorded.

5.3.6.3 Ash analyses

The ash content was obtained by complete combustion in a furnace at 550 °C for 6 h.

5.3.6.4 Carbohydrate analyses

Carbohydrate contents were determined using the Anthrone method (Yemm & Willis, 1954). Briefly, 30 mg of sample (diets, abalone tissue or faecal samples) were digested with 2.5 N HCl for 3 h. The homogenate was centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was collected and diluted 50 times for better determination. Four mL of Anthrone reagent were added to each tube having 1 mL sample (diluted if needed for better determination). All the tubes were capped and incubated at 100 °C for 15 min. After cooling down, the colour developed was read against glucose standard and blank at 620 nm in a UV–visible spectrophotometer using a quartz cuvette. The values obtained were expressed as mg of glucose/g wet weight of tissue.

5.3.7 Digestibility of feeds

5.3.7.1 Acid insoluble ash

Acid-insoluble ash analyses were performed on the experimental diets and faeces as described by Montaño-Vargas et al. (2002) using small samples due to low faecal matter biomass (0.2 g or less) as described by Liu (2022). Briefly, ashes were obtained after using a furnace at 550 °C x 16 hours. Then, 0.1 g ash sample from either feed or faeces were

weighed and digested in 20 mL 2N HCl. The mixture was heated in a water bath at 85 °C for 30 min and centrifuged at 4000 rpm x 10 min. The supernatant was separated and washed twice with distilled water by using centrifugation method (4000 rpm x 10 min). The final precipitate was dried in a forced air oven at 95 °C for 2 h and the residues weighed over a blank.

The feed digestibility coefficient of diets using acid insoluble ash (apparent digestibility) was calculated as follows:

$$AD\ (\%) = 100 - (100 \times \frac{\%\ marker\ in\ the\ feed}{\%\ marker\ in\ the\ faeces})$$

Marker: acid insoluble ash

AD is also called Apparent digestibility coefficient (in dry matter)

5.3.8 Amino acid composition

The total amino acid extraction of diets and animal bodies were conducted in accordance with Paramás et al. (2006). Three replicates of 50 mg dried powdered samples of abalone soft bodies and diets were mixed with 1.5 mL ultra-pure water in a 4 mL screw-top glass tube. Each tube was placed in a sonicator (Elma sonicator S30H, Elmasonic, Germany) for 1 min to promote dispersion of loads. To each sample, 1.5 mL 12M concentrate hydrochloric acid was added. Air was removed with the help of nitrogen stream and tubes were capped tightly and heated using a heating block at 110 °C for 22 h. Once the samples digested, the tube content was filtered through filter paper number and rinsed with 10 mL ultra-pure water. Each solution was neutralised to pH 4–6 with NaOH 1M. Once neutralised, all volumes were adjusted to 20 mL with ultra-pure water. Each solution was centrifuged at 3000 rpm for 5 mins and kept frozen until further analysis.

The amino acid standard for these analyses contained 37 amino acids (A9906 amino acid standard Sigma 485845-1G). After extraction, samples were derivatised using the AccQ-Tag (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) method for liquid chromatography–mass spectrometry (LC–MS). Briefly, 40 µL of samples were placed in a 1.5 mL centrifuge tube and 40uL of Internal standard was added (D-4 alanine). The internal standard was prepared using 1 mg d-4 alanine in 100 mL methanol. Samples were vortexed and subsequently 10 µL of AccQ-Tag reagent was added. Samples were vortexed immediately after AccQ-Tag addition, capped and incubated at 55 °C for 15 min in a heating

block. After this time, 400 µL of neutralising solution (10 mL formic acid in 90mL miliQ water) was added to each vial.

Samples were processed on a LC-MS system using an Agilent 1260 Infinity Quaternary LC System (Santa Clara, CA 95051 USA). The system consisted of the following components: 1260 quaternary pump (model number: G1311B), 1260 infinity ALS sampler (model number: G1329B), 1200 series autosampler thermostat FC/ALS/Therm (model number: G1330B), 1260 infinity TCC column component (model number: G1316A), 1260 infinity diode array detector (DAD) (model number: G4212B), connected to a 6420 triple quadruple LC/MS system with electrospray ionisation (ESI) source operating in positive electrospray ionisation mode. Multiple reaction monitoring (MRM) was used for quantification.

Phenomenex Kinetex evo C18 (2.1 x 150mm, 1.7µm) was used for this analysis. The mobile phase was composed of water containing 0.1 %(v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The initial gradient condition was 99:1 (A:B). From 3 to 8 min the B was increased to 13 %, from 8 to 15 min the B was increased to 17 %, from 15 to 16 min the B was increased to 80% and from 16 to 17.5 min B was decreased to 1 %. The total run time was 30 min.

5.3.9 Fatty acid composition

The fatty acid extraction of diets and animal bodies were conducted in accordance with Lepage and Roy (1986). Briefly, 20mg of samples was weighed into 8mL screw-cap glass culture tubes (Kimax). To each sample, 2 µL of the surrogate solution (containing 20 mg Tridecanoic acid in 50 mL toluene, analytical grade, Merck) and 200 µL of the extraction solution (2 mL of methanol: toluene 1:1, Analytical grade, Merck) containing the internal standard (Nonadecanoic acid, 72332-56-F, Sigma Aldrich). Due to the reactive nature of acetyl chloride, the solution was added slowly in a dropwise manner over a period of 1 min to avoid excessive heating. Each tube was closed tightly and sealed with teflon tape around the cap to avoid leakage. Tubes were incubated at 100 °C for 1 h. Once incubated, the tubes were left to cool before the addition of 4 mL of 6 % potassium carbonate (K_2CO_3). Tubes were vortexed and centrifuged at 25 rpm for 5 min at room temperature to separate the clear upper toluene phase. This phase was transferred to a 4 mL culture tube and a scoop (0.1–0.2 g) of sodium sulphate was added to remove remaining water. The remaining liquid on the vial was pipetted with a glass pipette and placed in an amber autosampler vial. If the liquid was below 1 mL final volume, toluene was used to top up the final solution.

The samples were analysed using the Agilent 6890N GC equipped with 5973 mass spectrometer detector (MSD) with an electron impact ionisation source. An Agilent DB-Fast FAME column (20m x 0.18mm x 0.2µm) was used. The inlet temperature was set at 250 °C and the samples were injected in split mode with a split ratio of 20:1. The initial column temperature was held at 85 °C for 0.74 minutes and then ramped at 43.84 °C/min to 175, then ramped at 6.75 °C/min to 185 °C and held for 3 minutes, then ramped at 10 °C/min to 230 °C and held for 1.5 minutes, then ramped 40 °C/min to 28 °C and held for 2 minutes. The total run time was 13.39 minutes. MSD transfer line was held at 280 °C, ion source at 250 °C and the quad at 150 °C and the solvent delay was 1.12 min. The data were acquired using selective ion monitoring (SIM) mode.

5.4 STATISTICAL ANALYSES

Growth and nutritional composition data (dietary treatments and abalone) were analysed by one-way analysis of variance (ANOVA) when a normal distribution was found (Kolmogorov–Smirnov test, $p < 0.05$) followed by pairwise comparisons with Tukey's post hoc test. Non-parametric Kruskal Wallis test was used when data did not follow a normal distribution ($p < 0.05$) followed by Dunn post hoc test for multiple comparison. Homogeneity of variances was analysed using Levene's test when samples followed a normal distribution ($p < 0.05$). The baseline measurements (before feeding trial) were not considered in statistical computation. The fixed factor was dietary treatment (diet F, FI, FG, FIG, and commercial feed), and the random factor was tank. Principal Component Analysis (PCA) was further applied to the dataset of one dependent variable (fatty acid) to further explore the relationship between the means of the samples. All univariate and multivariate analysis were carried out using MetaboAnalysit 5.0 (California, USA) and XLSTAT 2022.3.1 (Addinsoft, New York, USA).

5.5 RESULTS

5.5.1 Proximate composition, amino acid, and fatty acid profile of dietary treatments

Proximate compositions of the experimental diets are shown in Table 5.2. The estimated energy content was in the range of 18.1–20.4 KJ/g for all diets. The experimental diets were isonitrogenous with similar protein content. Higher levels of protein (%) were detected in commercial diet (32.4 ± 2.7%) compared to experimental diets F (fishmeal based), FI (fishmeal and insect meal based), FG (fishmeal and grape marc based), and FIG

(fishmeal, insect meal and grape marc based). Commercial feed had the highest carbohydrate-reducing sugars levels (39.1 ± 4.7%), whereas diet FIG and FG had the lowest values (9.1 ± 1.1% and 8.9 ± 1.9%, respectively). Diet FIG had the highest lipid level (7.2 ± 0.3%) followed by diet FI (7.0 ± 0.6%). The lowest lipid level was in the commercial feed (1.2 ± 0.3%). Diet FG had the highest ash level (1.4 ± 0.3%) followed by diet F (13.5 ± 0.1%). The commercial feed had the highest level of moisture (10.7 ± 0.1%) and diet FI the lowest (3%). The acid insoluble ash was the highest in diet FG (8.4 ± 0.7%) and lowest in diet F (1.6 ± 0.2%) and FI (1.9 ± 0.1%).

Table 5.2 Proximate composition of four experimental diets and a commercial feed.

	Diet				
Proximate composition	F	FI	FG	FIG	Commercial feed[5]
Protein (%)	30.4 ± 0.1^{ab}	27.3 ± 0.3^{b}	30.8 ± 0.7^{ab}	26.4 ± 2.0^{b}	32.4 ± 2.7^{a}
Carbohydrate (%)[1]	47.9	50.8	45.4	49.3	46.3
Carbohydrate-Reducing sugars (%)[2]	28.9 ± 1.3^{b}	32.0 ± 3.1^{ab}	8.9 ± 1.9^{c}	9.1 ± 1.1^{c}	39.1 ± 4.7^{a}
Total dietary fibre (%)	9.0 ± 0.2^{b}	7.5 ± 0.1^{c}	16.1 ± 0.1^{a}	15.4 ± 0.3^{a}	3.7 ± 0.3^{d}
Lipid (%)	4.0 ± 0.2^{c}	7.0 ± 0.6^{a}	5.3 ± 0.5^{b}	7.2 ± 0.3^{a}	1.2 ± 0.3^{d}
Ash (%)	13.5 ± 0.1^{b}	11.9 ± 0.1^{c}	14.5 ± 0.3^{a}	12.9 ± 0.3^{b}	6.8 ± 0.4^{d}
Moisture (%)	4.2 ± 0.01^{b}	3.0 ± 0.01^{b}	3.9 ± 0.03^{b}	4.2 ± 0.8^{b}	10.7 ± 0.1^{a}
Acid insoluble ash[3]	1.6 ± 0.2	1.9 ± 0.1	8.4 ± 0.7	2.7 ± 0.3	2.9 ± 0.04
Energy (KJ per g)[4]	18.1	20.4	18.9	20.2	15.9

Data represent means and standard deviation of dry matter (n = 3). Means with the same superscript (a, b, c) in each column are not significantly different from Tukey post hoc tests ($p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).
[1]Carbohydrate proportion was calculated by difference 100 – (moisture + protein + lipid + ash).
[2]Carbohydrate was determined using reducing sugar method Anthrone.
[3]Acid insoluble ash in dry matter content.
[4] Total energy was calculated based on the physiological values at 5.6 kCal g^{-1} protein, 9.5 kCal g^{-1} lipid and 4.1 kCal g^{-1} carbohydrates (Cho et al., 1982).
[5] Commercial feed used was Marifeed S34.

Amino acid composition of the dietary treatments is shown in Table 5.3. Significant differences were detected in hydroxy-proline, taurine, hydroxy-lysine and cysteine. Fatty acid composition of the dietary treatments showed a significant variation of 20 fatty acids among the diets. Fatty acid composition of the dietary treatments showed a significant variation of 20 fatty acids among the diets (Table 5.4). The inclusion of IM and GM promoted

higher levels of myristic acid, palmitic acid, palmitoleic acid, heptadecanoic acid, oleic acid, linoleaidic acid, α-Linolenic acid (ALA), arachidic acid, behenic acid, and tricosanoic acid. The decreased levels of fishmeal in diets FI and FIG reduced the contents of fatty acids cis-11-Eicosenoic acid, arachidonic acid (ARA), 5,8,11,14,17-Eicosapentaenoic acid (EPA), and 4,7,10,13,16,19-Docosahexaenoic acid (DHA).

Table 5.3 Amino acid composition of four experimental diets and a commercial feed.

	Diet				
Amino acid (mg/ g sample)	F	FI	FG	FIG	Commercial feed ®
L-histidine*	7.8 ± 0.9	6.4 ± 0.3	6.7 ± 0.9	8.4 ± 2.4	6.7 ± 0.8
Hydroxy-L-Proline	3.6^{ab} ± 0.4	2.2^{ab} ± 0.1	4.14^{a} ± 0.2	3.9 ^{ab} ± 1.3	2.0^{b} ± 0.1
L-Arginine*	14.7 ± 1.5	12.3 ± 1.3	15.9 ± 1.3	16.6 ± 6.8	12.5 ±1
Ethanolamine	0.3	0.4 ± 0.1	0.3	0.5 ±0.1	0.4
L-Serine	16.8 ± 2.5	14.1 ± 1.3	15.1 ± 0.8	17.2 ± 3.5	14.2 ± 3.4
Glycine	19.4 ± 1.7	16.1 ± 0.9	18.8 ± 0.6	21.1 ± 4.8	14.9 ± 2.3
L-Aspartic acid	31.5 ± 3.0	26.2 ± 0.7	32.4 ± 1.4	35.1 ± 8.6	27.6 ± 2.2
Taurine*	1.2^{b} ± 0.1	1.8^{ab} ± 1.1	2.0^{ab} ± 1.3	2.4^{ab} ± 1.3	4.3^{a} ± 0.5
b-Alanine	0.2	0.3	0.3	0.3 ± 0.1	0.3
L-Threonine*	17.5 ± 1.5	14.3 ± 0.4	17.3 ± 0.8	19.4 ± 5	14.4 ± 1.7
L-Glutamic acid	49.0 ± 3.8	40.2 ± 1.4	49.2 ± 2.1	52.6 ± 12.6	44.23 ± 1.6
Citruline	0.2 ± 0.1	0.1	0.1	0.1	0.1 ± 0.1
L-Alanine	18.4 ± 1.9	16.5 ± 0.2	18.4 ± 0.5	21.1 ± 5.2	16.1 ± 1.4
GABA	0.4	0.4	0.4	0.4	0.4
L-Proline	13.8 ± 0.9	12.8 ± 0.3	13.4 ± 0.7	16.12 ± 4	13.4 ± 0.7
delta-Hydroxylysine	0.9^{a} ± 0.1	0.7^{ab} ± 0.1	1.0^{a} ± 0.2	0.9^{ab} ± 0.2	0.5^{b}
L-Ornithine	3.7 ± 1.9	3.2 ± 1.5	2.1 ± 0.8	2.0 ± 0.5	3.7 ± 2.3
L-Valine*	16.5 ± 1.3	14.2 ± 0.1	17.0 ± 0.8	20.5 ± 5.9	15.4 ± 1
L-Methionine*	0.5	0.4	0.4	0.5 ± 0.2	0.3
L-Lysine*	23.1 ± 2.5	18.6 ± 0.2	24.7 ± 1.4	25.3 ± 5.5	16.7 ± 0.7
L-Anserine	1.6 ± 0.9	1.1 ± 0.1	1.2 ± 0.4	1.1 ± 0.3	2.1 ± 0.8
L-Cysteine	1.4^{ab} ± 0.1	1.0^{b}	1.2^{ab} ± 0.1	1.3^{ab} ± 0.3	1.7^{a} ± 0.1
L-Tyrosine	12.2 ± 0.6	11.5 ± 0.4	11.0 ± 0.5	15.8 ± 5.3	9.8 ± 0.9
L-Leucine*	22.2 ± 1.4	18.9 ± 0.6	21.3 ± 1.1	24.4 ± 6.3	20.6 ± 1
L-isoleucine*	13.7 ± 0.6	11.6 ± 0.2	14.5 ± 0.9	16.7 ± 4.9	13.0 ± 0.6

L-Phenylalanine*	16.4 ± 0.5	12.5 ± 0.3	15.0 ± 0.7	18.0 ± 5.9	14.9 ± 1
L-Tryptophan*	0.3 ± 0.1	0.2 ± 0.1	0.2	0.1	0.2 ± 0.1

*Values in bold are essential amino acids in abalone.
Data are represented by means of three replicates ± standard deviation. Significant differences are shown by different superscripts (Tukey's tests; $p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

Table 5.4 Fatty acid composition of four experimental diets and a commercial feed.

		Diet				
Fatty acid (ug/g sample)	Abbreviation	F	FI	FG	FIG	Commercial feed®
Hexanoic acid	C6:0	-	-	-	-	-
Octanoic acid	C8:0	-	-	-	-	-
Decanoic acid	C10:0	-	-	19.7 ± 1.3	20.3 ± 0.4	-
Undecanoic acid	C11:0	-	-	-	-	-
Dodecanoic acid	C12:0	-	30.7 ± 2.18	29.6 ± 1.4	55.7 ± 1.6	13.9 ± 0.7
Tridecanoic acid	C13:0 (reference)	265.4 ± 11.4	267.4 ± 17.9	265.5 ± 12.4	265.8 ± 6	280.8 ± 22.7
Myristic acid	C14:0	301.3 ± 11.4[a]	493.4 ± 41.6[b]	336.4 ± 15[a]	550.5 ± 30[b]	745.4 ± 38.7[c]
Myristoleic Acid	C14:1(n-5)	-	-	-	-	-
Pentadecanoic acid	C15:0	50.1 ± 3	54.9 ± 5.7	53.9 ± 2.4	62.1 ± 5.2	52.9 ± 2.9
cis-10-Pentadecenoic acid	C15:1 (n-5)	-	-	-	-	-
Palmitic acid	C16:0	3048.2 ± 161.3[a]	4518.2 ± 460.9[bc]	3638 ± 225.2 [ab]	5506.7 ± 479.1[c]	4021.5 ± 200.8[ab]
Palmitoleic acid	C16:1 (n-7)	345.4 ± 15.1[a]	473.9 ± 49.3 [ab]	402.6 ± 23.6[a]	562.6 ± 62.6[b]	963.9 ± 51.4[c]
Heptadecanoic acid	C17:0	54.9 ± 3.1[a]	65.3 ± 6[abc]	61.6 ± 4.7[ab]	76.2 ± 7.7[bc]	81.5 ± 4[c]
cis-10-Heptadecenoic acid	C17:1 (n-7)	-	-	-	-	-
Stearic acid	C18:0	750.9 ± 49.8[a]	1024.7 ± 112.2[a]	1069.3 ± 85.5[a]	1506 ± 174.2[b]	822.1 ± 35.8[a]
Oleic acid	C18:1 (n-9)	3443.5 ± 168.5[b]	5431.5 ± 481.8[c]	3496.6 ± 269.3[b]	5414.7 ± 489.6[c]	1827.5 ± 75.1[a]
Linoleic acid	C18:2 (n-6)	-	-	-	-	-
Linoleaidic acid	C18:2 (n-6,9)	2924.2 ± 133.2[a]	6384.6 ± 723.3[b]	5601.6 ± 768.5[b]	8906.9 ± 853.2[c]	2288.5 ± 114[a]
γ-Linolenic acid	C18:3 (n-6)	-	-	-	-	-
α-Linolenic acid	C18:3 (n-3)	179.5 ± 9.8[a]	319.3 ± 39[b]	285.8 ± 22.8[ab]	496.8 ± 58.2[c]	242.3 ± 6.7[ab]
Arachidic acid	C20:0	31 ± 1.2[a]	41.1 ± 5.6[a]	78.2 ± 10[bc]	98 ± 12.7[c]	53.1 ± 2[ab]
cis-11-Eicosenoic acid	cis-C20:1 (n-9)	714.8 ± 40.2[bc]	525.3 ± 64.8[b]	737.6 ± 88.1[c]	586.8 ± 82.9[bc]	171.8 ± 11[a]
cis-11,14-Eicosadienoic acid	cis-C20:2 (n-6)	50.2 ± 1.7[b]	48.6 ± 4.1[b]	52.1 ± 5.1[b]	54.8 ± 5.1[b]	35.5 ± 1.9[a]
cis-8,11,14-Eicosatrienoic acid	cis-C20:3 (n-9)	22.5 ± 1.1[a]	20.5 ± 1.5[a]	22.7 ± 1.3[a]	19.6 ± 1.3[a]	27.7 ± 1.4[b]
11,14,17-Eicosatrienoic acid	C20:3 (n-3)	18.5 ± 2.4	13.7 ± 1.1	18.2 ± 2.4	14.3 ± 1.7	-

		Diet				
Fatty acid (ug/g sample)	Abbreviation	F	FI	FG	FIG	Commercial feed®
Arachidonic acid	C20:4 (n-6)	242.2 ± 11.7^{b}	177.7 ± 21.7^{a}	213.4 ± 24.2^{ab}	186.6 ± 20.6^{ab}	165.7 ± 5.4^{a}
5,8,11,14,17-Eicosapentaenoic acid	C20:5 (n-3)	879.9 ± 51.4^{b}	590.3 ± 71.8^{a}	825.6 ± 91.6^{ab}	661.5 ± 89.5^{ab}	2203.8 ± 101.3^{c}
Heneicosanoic acid	C21:0	3.4 ± 0.6^{a}	3 ± 0.3^{a}	8.8 ± 1.1^{b}	9.4 ± 2^{b}	4.9 ± 0.6^{a}
Behenic acid	C22:0	9.6 ±0.6^{a}	10.3 ± 2.5^{a}	71.3 ± 8.6^{b}	83.9 ± 14.7^{b}	17.5 ± 0.8^{a}
Erucic acid	C22:1 (n-9)	98.8 ± 15.3^{b}	61.1 ± 12.1^{ab}	80.9 ± 13.5^{b}	75.3 ± 19.1^{b}	26.1 ± 2.9^{a}
cis-13,16-Docasadienoic acid	C22:2 (n-6)	-	-	-	-	30.9 ± 1.3
Lignoceric acid	-	-	-	-	57.8	58.7
4,7,10,13,16,19-Docosahexaenoic acid	C22:6 (n-3)	2817.7 ± 178.9^{b}	1800 ± 221^{a}	2528.8 ± 345.4^{ab}	2011.3 ± 278.1^{a}	2069.8 ± 104.2^{a}
Tricosanoic acid	C23:0	14.8 ± 0.8^{a}	15.6 ± 0.6^{a}	24.1 ± 1.6^{b}	25.1 ± 1.9^{b}	18.8 ± 1^{a}
Cis-15-tetracosenoic acid	-	265.4 ± 20.9^{b}	193 ± 8.6^{ab}	235 ± 39.8^{ab}	210.5 ± 26.8^{ab}	155.2 ± 19^{a}

Data are represented by means of three replicates ± standard deviation. For each parameter, significant differences are shown by different superscripts (Tukey's test, $p < 0.05$).
(----) not detected.
FAME (fatty acid methyl ester), diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

5.5.2 Feeding trial

5.5.2.1 Survival

Abalone survival was high during the feeding trial with an overall average of 99% among all dietary treatments.

5.5.2.2 Growth parameters

Specific growth rates determined from allometric measurements prior and post to the feeding trial revealed that the inclusion of IM and GM did not significantly affect the growth performance compared to the IM/GM free diet (diet F). A significant increased growth in abalone fed with the commercial feed compared to the experimental diets was observed (Figure 5.1)

The growth parameters showed that the inclusion of IM and GM did not significantly affect the condition factor, muscle yield and soft body/shell ratio (Table 5.5). The inclusion of IM and GM significantly affected the shell length increment showing higher shell growth in abalone fed diet FG compared to diet FI. Significant differences in shell length and condition factor were also observed between experimental diets and commercial feed.

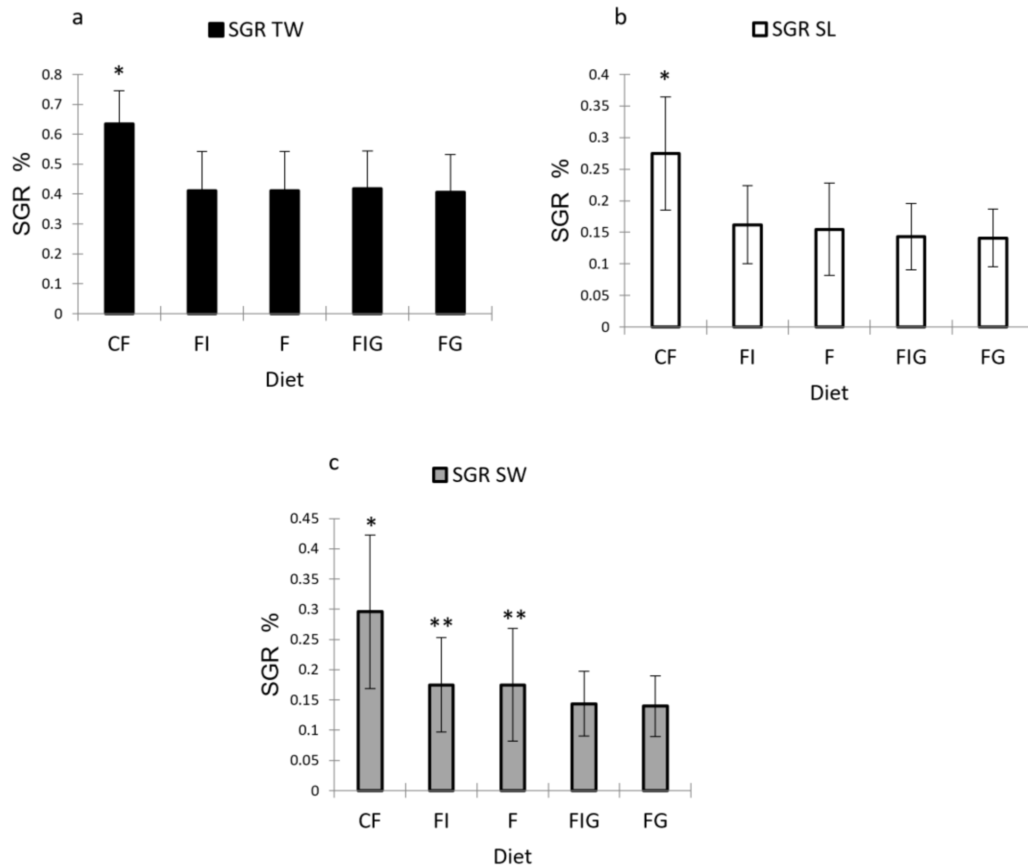

Figure 5.1 (a) Specific growth rate for total weight (SGR TW), (b) Specific growth rate for shell length (SGR SL) and (c) Specific growth rate for shell width (SGR SW) of juvenile *H. iris* grown on four different experimental diets and a commercial feed. Error bars denote ± standard deviation. Means with the same asterisk in each bar are not significantly different from Tukey post hoc tests ($p < 0.05$).

Table 5.5 Growth and its indicators in *H. iris* fed on four experimental diets and a commercial feed.

	Diet				
Growth indicators	F	FI	FG	FIG	Commercial feed
Length increment (μm day $^{-1}$)	37.65 ± 16.23[bc]	33.83 ± 11.32[c]	42.11 ± 17.51[b]	33.31 ± 12.12[c]	72.55 ± 32.49[a]
Condition factor	0.46 ± 0.05[a]	0.49 ± 0.34[a]	0.45 ± 0.04[a]	0.46 ± 0.08[a]	0.44 ± 0.15[a]
Muscle yield	68.49 ± 2.10[a]	68.49 ± 2.10[a]	68.60 ± 2.12[a]	68.77 ± 2.67[a]	67.76 ± 1.96[a]
SB/S ratio	2.11 ± 0.19[a]	2.19 ± 0.22[a]	2.20 ± 0.22[a]	2.23 ± 0.37[a]	2.11 ± 0.19[a]
Feed ratio (% body weight)	1.2 -2.2 %	1.2 -2.2 %	1.2 -2.2 %	1.2 -2.2 %	1.2 -2.2 %

Data are represented by means ± standard deviation. For each parameter, significant differences are shown by different superscripts (Dunn post hoc test, $p < 0.05$). Abbreviations: SB/ S ratio = soft body/ shell ratio, diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF). Initial weight, length and width were 1.2 g, 21.6 mm, and 14.5 mm, respectively at the beginning of the study (n = 750).

5.5.2.3 Abalone proximate composition

There were no significant differences in protein levels among dietary treatments. The overall protein mean was 58.1 ± 0.2 (SE). There was a significant difference in carbohydrate content in the soft body of abalone among all diets with the highest value found in animals fed on commercial feed (4.9 ± 0.5 %) followed by FI (3.3 ± 0.2 %), FG (3.3 ± 0.5 %), F (3.1 ± 0.2 %) and FIG (3.0 ± 0.3 %). Animals in the baseline showed 4.6 ± 0.3 % carbohydrate content. Lipid content was significantly lower in animals fed diets containing grape marc (FG and FIG) compared to diets that did not contain grape marc (F and FI). The lowest lipid value was found in animal fed commercial feed (5.7 ± 0.9 %). Moisture and ash content was not significantly different among treatments. The overall mean for moisture was 81.8 ± 0.2 % (SE) and for ash content 13.3 ± 0.1 % (SE).

Table 5.6 Proximate composition of *H. iris* fed on four experimental diets and a commercial feed.

	Diet					
Proximate composition	Baseline	F	FI	FG	FIG	Commercial feed
Protein (%)	61.8 ± 0.5	58.0 ± 3.4[a]	57.3 ± 2.5[a]	57.6 ± 2.8[a]	57.7 ± 2.2[a]	59.1 ± 2.4[a]
Carbohydrate (%)	4.6 ± 0.3	3.1 ± 0.2[b]	3.3 ± 0.2[b]	3.3 ± 0.5[b]	3.0 ± 0.3[b]	4.9 ± 0.5[a]
Lipid (%)	6.0 ± 0.7	7.7 ± 0.9[a]	7.2 ± 1.3[a]	6.0 ± 0.9[b]	6.1 ± 1.0[b]	5.7 ± 0.9[b]
Ash (%)	12.3 ± 0.5	13.3 ± 0.4	13.0 ± 0.5	13.6 ± 0.6	14.3 ± 0.4	12.3 ± 0.3
Moisture (%)	82.7 ± 0.7	81.9 ± 0.4[a]	81.9 ± 0.5[a]	81.7 ± 1.0[a]	82.3 ± 0.7[a]	81.4 ± 0.5[a]

Data are represented by means ± standard deviation. For each parameter, significant differences are shown by different superscripts (Tukey's test, $p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

5.5.2.4 Amino acid profiles

In our study, 27 amino acids were identified in abalone soft tissues, including 11 essential amino acids (valine, leucine, isoleucine, threonine, phenylalanine, lysine, histidine, methionine, tryptophan, taurine, and arginine) and 16 non-essential amino acids (hydroxyproline, ethanolamine, serine, glycine, aspartic acid, alanine, glutamic acid, citrulline, GABA, proline, hydroxylysine, ornithine, anserine, cysteine and tyrosine).

After the feeding trial, the amino acid composition of abalone soft tissues was not greatly impacted by the experimental feeds or commercial diet (Table 5.7). Significant differences were only seen in eight amino acids including L-histidine, ethanolamine, glycine, L-aspartic acid, taurine, L-threonine, and L-methionine. The inclusion of IM (diet FI) did not produce significant differences in abalone compared to the IM/GM free diet (diet F). The inclusion of GM (diet FG) produced abalone with significant lower levels of L-histidine. The inclusion of both, IM and GM, produced abalone with significant lower levels of L-histidine, glycine, L-methionine, and L-phenylalanine.

The commercial feed produced abalone with significant higher ethanolamine, glycine, L-aspartic acid, taurine, and threonine compared to animals fed the IM/GM free diet

(diet F). The more similar AA composition in abalone soft tissues came from animals fed the commercial diet and diet FIG and the least were the commercial feed and diet F.

Table 5.7 Amino acid composition of abalone soft bodies fed on four experimental diets and a commercial feed.

		Diet				
Amino acid (mg/g sample)	Baseline	F	FI	FG	FIG	Commercial feed
L-histidine*	9.3 ± 1.8	7.4^{a} ± 2.1	9.2^{ab} ± 2.1	9.6^{b} ± 1.2	9.8^{b} ± 1.2	7.9^{ab} ± 2.6
Hydroxy-L-Proline	10.5 ± 1.4	9.4 ± 1.7	9.8 ± 1.4	9.8 ± 1.4	10.6 ± 1.2	10.6 ± 1.3
L-arginine*	38.3 ± 9.4	36.3 ± 7.0	36.2 ± 7.4	34 ± 5.1	36.2 ± 7.4	38.8 ± 9.4
Ethanolamine	1.3 ± 0.1	1.1^{a} ± 0.1	1.1^{ab} ± 0.1	1.1^{ab} ± 0.1	1.2^{ab} ± 0.1	1.2^{b} ± 0.1
L-serine	29.0 ± 2.1	24.4 ± 3.5	26 ± 3.7	26.3 ± 2.7	27.8 ± 2.3	31.8 ± 14.9
Glycine	44.9 ± 3.1	38.1^{a} ± 6.0	39.4^{ab} ± 5.3	40.8^{abc} ± 3.7	43.9^{bc} ± 4.1	46.1^{c} ± 7.0
L-aspartic acid	62.2 ± 1.9	51.5^{a} ± 7.7	55.1^{ab} ± 7.1	55.6^{ab} ± 6.4	59^{ab} ± 4.7	60.3^{b} ± 8.7
Taurine*	59.2 ± 4.9	48.1^{a} ± 5.1	53^{ab} ± 9.2	54^{ab} ± 9.8	55.9^{ab} ± 6.5	58.7^{b} ± 6.1
β-alanine	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
L-threonine*	25.8 ± 1.6	21.3^{a} ± 2.9	22.3^{ab} ± 2.8	23.2^{ab} ± 2.5	24.3^{ab} ± 1.9	25.3^{b} ± 4.5
L-glutamic acid	87.6 ± 3.3	76.5 ± 10.6	80.3 ± 10.2	80.2 ± 9.5	84.9 ± 7.0	84.9 ± 9.6
L-citrulline	-	0.2 ± 0.1	0.3 ± 0.4	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
L-alanine	30.0 ± 1.3	26.9 ± 4.0	28.4 ± 3.9	27.5 ± 3.2	28.8 ± 2.2	29.8 ± 4.4
GABA	0.1 ± 0.03	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.1
L-proline	27 ± 1.5	23.9 ± 2.9	24.9 ± 3.0	24.9 ± 3.0	26.1 ± 1.9	27.2 ± 3.0
L-alpha-Amino-n-butyric acid	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
delta-hydroxylysine	6.6 ± 0.1	6.7 ± 0.8	6.5 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.5 ± 0.1
L-ornithine	0.6 ± 0.3	1.1 ± 0.7	0.8 ± 0.5	0.7 ± 0.4	0.7 ± 0.4	0.6 ± 0.4
Cystathionine	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1
L-valine*	22.7 ± 1.1	20 ± 2.8	20.9 ± 2.5	21.3 ± 2.7	22.3 ± 1.8	22.4 ± 2.9
L-methionine*	11.2 ± 0.7	10.5^{a} ± 1.4	10.9^{ab} ± 1.4	11.3^{ab} ± 1.2	12.2^{b} ± 1.0	11.6^{ab} ± 1.4
L-lysine*	34.4 ± 1.2	31.8 ± 4.8	32.8 ± 4.5	31.7 ± 4.1	33.1 ± 2.7	34.1 ± 4.5
L-anserine	1.6 ± 0.8	1.6 ± 1.3	1.5 ± 1.1	2 ± 0.9	1.7 ± 0.9	1.4 ± 1.0
L-cystine	3.0 ± 0.4	2.6 ± 0.3	2.6 ± 0.3	2.6 ± 0.4	2.9 ± 0.5	2.9 ± 0.5
L-tyrosine	1.2 ± 0.6	1.2 ± 1.0	1.1 ± 0.8	1.5 ± 0.7	1.3 ± 0.7	1.3 ± 1.1

	Diet					
Amino acid (mg/g sample)	Baseline	F	FI	FG	FIG	Commercial feed
L-leucine*	39.1 ± 1.8	34.6 ± 4.8	35.8 ± 4.2	35.2 ± 5.4	38.6 ± 2.9	38 ± 3.9
L-isoleucine*	21.0 ± 1.2	18.5 ± 2.6	19.5 ± 2.5	19.5 ± 2.5	20.7 ± 2.0	20.4 ± 2.5
L-phenylalanine*	20.1 ± 1.3	16.7[a] ± 2.7	18.1[ab] ± 3.8	18[ab] ± 1.9	19.7[b] ± 1.7	19.6[b] ± 2.4
L-tryptophan*	**-**	**-**	**-**	**-**	**-**	**-**

Values in bold are essential amino acids in abalone. Data are represented by means replicates ± standard deviation (n = 75). Significant differences are shown by different superscripts (Tukey's tests; $p < 0.05$).
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

5.5.2.5 Fatty acids profiles

The inclusion of IM and GM significantly affected the fatty acid composition of abalone (Table 5.8). Diet F produced animals with significant lower levels of α-linolenic acid (ALA), linoleaidic acid, cis-11,14-eicosadienoic acid, cis-8,11,14-eicosatrienoic acid and arachidonic acid. The experimental diet with IM inclusion (diet FI) produced animals with significant higher levels of oleic acid, linoleaidic acid, α-linolenic acid (ALA), and arachidonic acid. The experimental diet with inclusion of both, IM and GM (diet FIG), produced animals with significant higher levels of palmitic acid, palmitoleic acid, heptadecanoic acid, stearic acid, oleic acid, α-linolenic acid (ALA), arachidonic acid and tricosanoic acid. In addition, significant higher levels of EPA, palmitoleic acid, and myristic acid were observed in abalone fed on commercial feed. Differences among the commercial feed and experimental diets were greatly noted whereas no significant differences were observed among the experimental diets (Figure 5.2).

Table 5.8 Fatty acid composition of abalone soft bodies fed on four experimental diets and a commercial feed.

			Diet					
Fatty acid (ug/g sample)	Abbreviation	Baseline	F	FI	FG	FIG	Commercial feed
Hexanoic acid	C6:0	-	-	-	-	-	-
Octanoic acid	C8:0	-	-	-	-	-	-
Decanoic acid	C10:0	-	-	-	-	-	-
Undecanoic acid	C11:0	-	-	-	-	-	-
Dodecanoic acid	C12:0	-	-	-	-	-	-
Tridecanoic acid	C13:0 (reference)	241.5 ± 10.6	246 ± 10.7	249.3 ± 8.4	251 ± 10.7	257 ± 11	269.2 ± 16.2
Myristic acid	C14:0	348.5 ± 14.3	277.1[b] ± 11.1	297.3[b] ± 11	266.8[b] ± 8.8	285.7[b] ± 12.5	494.9[a] ± 34.6
Myristoleic Acid	C14:1 (n-5)	-	-	-	-	-	-
Pentadecanoic acid	C15:0	64 ± 2.8	50.4[b] ± 2	52.1[ab] ± 1.8	57[ab] ± 2.3	60.9[a] ± 2.8	56.6[ab] ± 3.1
cis-10-Pentadecenoic acid	C15:1 (n-5)	-	-	-	-	-	-
Palmitic acid	C16:0	2755.5 ± 132.4	2315.8[b] ± 110.5	2747.1[ab] ± 100.7	2359.6[ab] ± 97.7	2854.9[a] ± 145.9	2727.6[ab] ± 167.3
Palmitoleic acid	C16:1 (n-7)	187.2 ± 16.7	107.2[c] ± 5.2	142.1[abc] ± 7.2	119.4[bc] ± 7.0	158.8[ab] ± 11.9	186.3[a] ± 23
Heptadecanoic acid	C17:0	128.4 ± 6.4	104.8[b] ± 4.8	122.6[ab] ± 4.9	112.5[ab] ± 4.2	127.2[a] ± 5.8	117.3[ab] ± 6.5
cis-10-Heptadecenoic acid	C17:1 (n-7)	-	-	-	-	-	-
Stearic acid	C18:0	1016 ± 58.6	842.6[b] ± 42.1	1000.8[ab] ± 39.3	919.7[ab] ± 33.1	1084.8[a] ± 53.1	961.4[ab] ± 56.0
Oleic acid	C18:1 (n-9)	1471.7 ± 140.4	1216.8[bc] ± 84.8	2279.6[a] ± 152.9	1399.3[b] ± 102.1	2019.1[a] ± 145.6	825.8[c] ± 70.5
Linoleic acid	C18:2 (n-6)	-	-	-	-	-	-
Linoleaidic acid	C18:2 (n-6,9)	768.3 ± 51.9	806.7[c] ± 57.7	2171.4[a] ± 157.7	1357.8[b] ± 99.2	2639[a] ± 220.7	520.8[c] ± 50.7
γ-Linolenic acid	C18:3 (n-6)	-	-	-	-	-	-
α-Linolenic acid	C18:3 (n-3)	224.4 ± 13.9	73.7[c] ± 2.7	125.9[ab] ± 6.9	109.9[b] ± 6.0	149.5[a] ± 9.4	129.6[ab] ± 7.5
Arachidic acid	C20:0	-	-	-	-	-	-
cis-11-Eicosenoic acid	cis-C20:1 (n-9)	188.7 ± 22.1	214.4[a] ± 16.1	236.9[a] ± 15.7	183.7[a] ± 13.3	193[a] ± 14	102.7[b] ± 9.1
cis-11,14-Eicosadienoic acid	cis-C20:2 (n-6)	123.7 ± 6.9	91.9[c] ± 4.6	166[ab] ± 9.6	142.3[b] ± 6.9	182.1[a] ± 13.7	97.4[c] ± 5.9
cis-8,11,14-Eicosatrienoic acid	cis-C20:3 (n-9)	70.4 ± 4.5	42.5[b] ± 2.3	62.4[a] ± 3.5	60.7[a] ± 2.6	74.8[a] ± 5.5	67.7[a] ± 3.7

Fatty acid (ug/g sample)	Abbreviation	Baseline	Diet				
			F	FI	FG	FIG	Commercial feed
11,14,17-Eicosatrienoic acid	C20:3 (n-3)	-	-	-	-	-	-
Arachidonic acid	C20:4 (n-6)	1105.4 ± 71.4	787.5[b] ± 39.2	1012.2[a] ± 44.8	888.1[ab] ± 37.9	1023.5[a] ± 56.1	899.9[ab] ± 68
5,8,11,14,17-Eicosapentaenoic acid	C20:5 (n-3)	2251.4 ± 144.7	1423[b] ± 5.5	1467.1[b] ± 54.8	1363[b] ± 71.9	1592.8[b] ± 95.5	2228.8[a] ± 161.6
Heneicosanoic acid	C21:0	-	-	-	-	-	-
Behenic acid	C22:0	-	-	-	-	-	-
Erucic acid	C22:1 (n-9)	6.6 ± 1.8	-	-	-	-	-
cis-13,16-Docasadienoic acid	C22:2 (n-6)	-	-	-	-	-	-
4,7,10,13,16,19-Docosahexaenoic acid	C22:6 (n-3)	1358.9 ± 105.9	787.8 ± 36.0	785.2 ± 44.7	702.5 ± 34.4	891.9 ± 60.5	806.4 ± 71.9
Tricosanoic acid	C23:0	15.7 ± 0.6	12.6[c] ± 0.2	14.7[b] ± 0.3	14.7[b] ± 0.3	16.8[a] ± 0.5	15.4[ab] ± 0.5

Data are represented by means ± standard error of the mean (n = 75). For each parameter, significant differences are shown by different superscripts (Tukey's test, $p < 0.05$).
(----) not detected.
Abbreviations: FAME (fatty acid methyl ester), diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

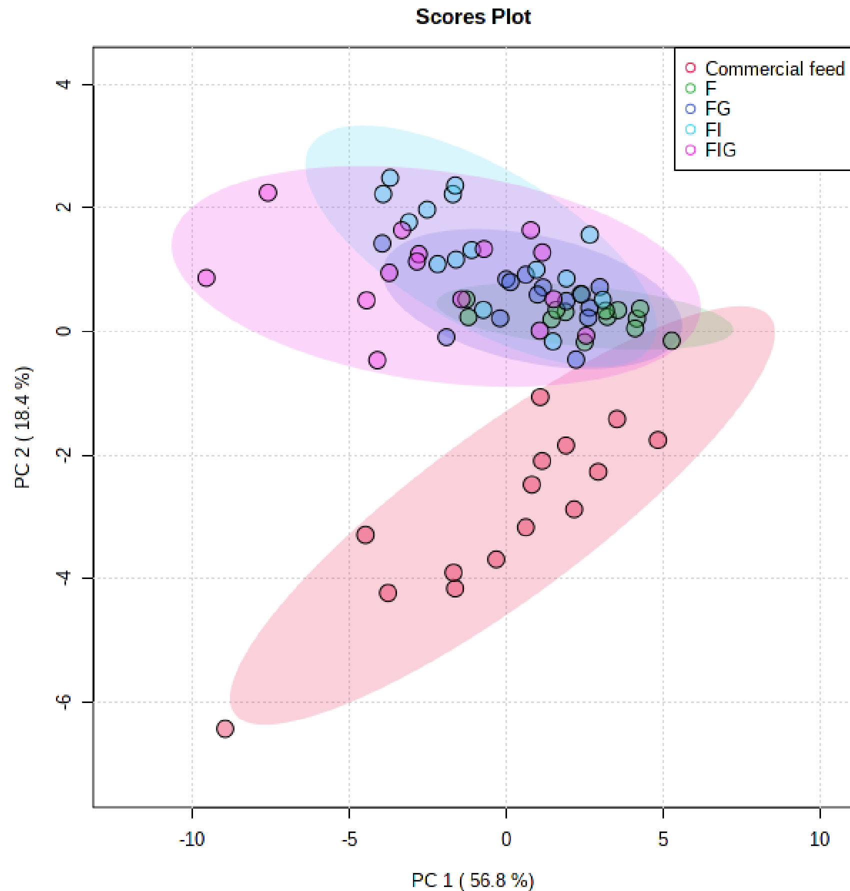

Figure 5.2 Principal component analysis plot based on the fatty acid composition of abalone grouped by dietary treatment. Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

5.5.2.6 Faeces proximate composition

On a dry basis, the crude protein of faecal matter from animals fed diets F (23.4 ± 1.7%) and FI (25.3 ± 1.9%) was slightly superior to diets FG (21.9 ± 3.1%), FIG (16.6 ± 2.2%), and commercial feed (18.8 ± 2.5%) (Table 5.9). The lipid proportion of faecal matter from animals fed diets containing GM FG (4.1 ± 1.0%) and FIG (4.5 ± 0.8%) was higher than diets F (1.8 ± 0.6%), FI (1.7 ± 0.6%), and commercial feed (2.5 ± 0.7%). There was significant difference in carbohydrate content in the faeces from abalone receiving diets containing GM compared to those which did not. Faecal matter from animals fed on diet FI (11.7 ± 0.4%) and diet F (10.1 ± 1.1%) contained higher levels of carbohydrates compared to animals fed on diets with GM, diet FG (4.4 ± 0.6%) and FIG (4.8 ± 0.6%). Ash levels indicated higher contents of organic matter in faeces from animals fed on diets F (48.3 ±

0.3%), commercial feed (47.6 ± 1.2%) and diet FI (40.8 ± 0.3%) and lower values in animals fed on diets containing GM, such as diet FG (32.6 ± 0.1%) and FIG (40.8 ± 0.3%). Acid insoluble ash resulted significantly higher in diet FG (13.2 ± 0.2%) and lower in diet FIG (5.4 ± 0.3%) (Table 5.9).

Table 5.9 Proximate composition of faecal matter collected over 2–week feeding period from *H. iris* fed on four experimental diets and a commercial diet.

	Diet				
Proximate composition	F	FI	FG	FIG	Commercial feed
Crude protein (%)	23.4 ± 1.7[ab]	25.3 ± 1.9[a]	21.9 ± 3.1[b]	16.6 ± 2.2[c]	18.8 ± 2.5[c]
Crude lipid (%)	1.8 ± 0.6[b]	1.7 ± 0.6[b]	4.1 ± 1.0[a]	4.5 ± 0.8[a]	2.5 ± 0.7[b]
Carbohydrate (%)	10.1 ± 1.1[b]	11.7 ± 0.4[a]	4.4 ± 0.6[d]	4.8 ± 0.6[d]	7.1 ± 0.6[c]
Ash (%)	48.3 ± 0.3[a]	40.8 ± 0.3[b]	32.6 ± 0.1[c]	30.6 ± 0.5[c]	47.6 ± 1.2[a]
Acid insoluble ash (%)[1]	7.2 ± 0.4[bc]	7.6 ± 0.2[b]	13.2 ± 0.2[a]	5.4 ± 0.3[c]	8.8 ± 1.2[b]

Values indicate mean values of three replicates samples expressed as % w/w on a moisture free basis ± standard deviation. For each parameter, significant differences are shown by different superscripts (Tukey's test, $p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

[1]Acid insoluble ash in total ash matter.

5.5.2.7 Apparent nutrient digestibility coefficients

The mean apparent digestibility coefficients calculated using acid-insoluble ash from faecal matter and feeds are shown in Table 5.10. The digestibility coefficient in dry matter was significantly reduced when GM was included in the diet in diet FG (36.3 ± 0.9%). A similar trend was found in the digestibility coefficient based on organic matter, crude protein, and carbohydrate. The inclusion of IM in diet FI (93.6 ± 0.3%) did significantly affect the digestibility of the lipid proportion compared to commercial feed (39.3 ± 11.4%).

Table 5.10. Mean apparent digestibility coefficient for abalone fed on four experimental diets and a commercial feed.

	Diet				
Apparent nutrient digestibility coefficient (ADC %)	F	FI	FG	FIG	CF
Dry matter	77.2 ± 1.8^{b}	72.7 ± 1.0^{ab}	36.3 ± 1.2^{a}	47.3 ± 3.4^{ab}	64.8 ± 6.2^{ab}
Organic matter	85.9 ± 2.6^{b}	82.0 ± 0.8^{ab}	48.1 ± 5.1^{a}	59.1 ± 8.1^{ab}	80.2 ± 3.7^{ab}
Crude protein	81.8 ± 3.3^{b}	75.1 ± 1.1^{ab}	53.2 ± 4.6^{a}	67.8 ± 6.4^{ab}	79.5 ± 3.9^{ab}
Crude lipid	89.6 ± 1.9^{ab}	93.6 ± 0.3^{b}	49.9 ± 4.9^{ab}	67.8 ± 6.4^{ab}	39.3 ± 11.4^{a}
Carbohydrate	91.7 ± 1.5^{ab}	90.2 ± 0.4^{ab}	67.5 ± 3.2^{a}	72.9 ± 5.3^{ab}	93.6 ± 1.2^{b}

Values indicate mean values of three replicates samples ± standard deviation. For each parameter, significant differences are shown by different superscripts (Dunn's test, $p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

5.6 DISCUSSION

Growth performance of abalone was not significantly affected by the inclusion of grape marc (GM) and insect meal (IM) compared to the experimental diet without these ingredients (Diet F). This indicates that GM and IM are potential alternative ingredients in aquafeeds for abalone. Grape marc and IM inclusion did not significantly affect the protein, carbohydrate and ash levels of abalone after 165 days compared to an IM/GM free diet (diet F). In addition, the inclusion of GM and its combination with IM affected the fatty acid composition of abalone meat showing higher levels of polyunsaturated fatty acids (PUFAs) C18:2n-6,9, C18:3n-3 (ALA), C20:2n-6 and C20:3n-9. Grape marc has been successfully included in feed for ruminants (Moate et al., 2014), carp (Mocanu et al., 2022), and abalone (Currie et al., 2019) providing different results depending on the species. For instance, in carp, the GM inclusion did not change significantly the PUFA profile of the animal's meat and did not affect the growth rate but increased the feed conversion ratio in a feeding trial for 56 days duration (Mocanu et al., 2022). In abalone, the inclusion of up to 20% of Acti-Meal® (grape marc steam distilled) resulted in better growth and better feed conversion ratios compared to animals fed control diets (Currie et al., 2019). Furthermore, Acti- Meal®

inclusion resulted in higher amounts of n-3 LC-PUFA by more than 100% (Currie et al., 2019) compared to diets without Acti- Meal®, suggesting the potential role of grape marc as a supplement of LC-PUFA relevant for human dietary interest. In another study, GM inclusion has improved the amounts of certain polyunsaturated fatty acids in the liver of pigs, such as C20:5n-3 (EPA), C22:5n-3 9(DPA) and C22:6n-3 (DHA) (Habeanu et al., 2015) which are known for their protective effect against lipid peroxidation and health benefits (Voicu et al., 2017). Grape marc antioxidant properties can also be attributed to the large amounts of resveratrol, one of the strongest antioxidants existing mostly in the skin and pulp of red grapes, polyphenols (5–8%), such as anthocyanin, hydroxybenzoic acid, hydroxycinnamic acid, flavonols, and stilbenes (Kammerer et al., 2004; Voicu et al., 2017), and the large amount of PUFAs mainly C18:2n-6 (LA) (Gómez-Brandón et al., 2019; Tsiplakou & Zervas, 2008).

One of the main problems of grape marc inclusion in feeds is the presence of antinutrients, such as lignin that can interfere with nutrient absorption if the ingredient is not pre-treated. Grape marc is high in lignin (Moate et al., 2014). Lignin is a collection of phenolic polymers, and it is one of the major components found in lignocellulosic biomass, playing a critical role in the rigidity of the cell wall and plant structure. It has been demonstrated that native lignin constitutes a barrier to nutrient digestion, and it is resistant to microbial degradation in monogastric animals (Moate et al., 2014). High lignin content lowers the energy value of animal feed and leads to reduced feed intake (Amyot et al., 2018). However, some purification techniques for lignin (enzymatic and chemical treatments) can offer new alternatives for grape marc inclusion in aquafeeds. Treated lignin, which refers to low molecular weight mix of phenolic monomers, have been successfully used as prebiotic at an inclusion of 1% in feeds for Atlantic salmon (*Salmo salar*) without affecting body weight, survival and gut health compared to animals not receiving lignin supplementation (Yossa et al., 2018).

In the present study, abalone fed on commercial feed resulted in significant better growth compared to the experimental diets. Traditional commercial feeds usually incorporate growth enhancers, additional vitamins, and minerals to expedite growth, and therefore the comparison between commercial feed and experimental diets produced in this study should only be used as a reference. Results showed that both IM and GM inclusion can be included in aquafeeds for abalone and that further optimisation of the feed formulation of the experimental diets can be carried out to promote faster growth. Another possible explanation for the significant higher growth of abalone fed on commercial feed

compared to experimental diets may be attributed to the high levels of dietary lipids in experimental diets. Evidence suggests that abalone growth is impaired when fed with dietary lipids above 5–7% (Bautista-Teruel et al., 2011), which occurred in treatments FI and FIG, due to IM. Abalone are herbivores and as such, utilize carbohydrates rather than lipids as an energy source with carbohydrate: lipid ratios of 48:2 and 47:3 as optimal values for growth (Lee et al., 2019). Contrary to other aquatic species, abalone do not possess digestive enzyme lipases to metabolise high levels of dietary lipids, which might deteriorate digestive function and growth performance. The experimental diets with the highest proportion of lipids included insect meals, which have a high crude lipid proportion of 39–48% depending on the larval stage (Dreassi et al., 2017).

Apart from the crude lipid proportion difference found, the fatty acid composition differed significantly among commercial feed and experimental diets which may explain the relevance of these compounds for animal growth. Provision of essential fatty acids and fat-soluble nutrients, such as sterols and polar lipids are crucial for abalone growth due to their role in cell membrane structure (Bautista-Teruel et al., 2011). The variation of dietary polyunsaturated fatty acids (PUFAs), which are considered to be the most important fatty acids for abalone growth (Bautista-Teruel et al., 2011) can explain the difference in growth performance due to the inclusion of IM and GM. Insect meals have high level of lipids, mainly comprising polyunsaturated fatty acids (PUFAs), such as α-linolenic acid (C18:3n-3) and α-linoleic acid (C18:2n-6), saturated fatty acids (SFA), such as palmitic acid (C16:0) and stearic acid (C18:0), and monounsaturated fatty acids (MUFA), such as oleic acid (C18:1n-9) (Dreassi et al., 2017). Insects normally do not contain eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), which are highly elevated in the marine environment and found specially in diatoms, fishmeal, and seaweeds (De la Peña et al., 2016). These two key fatty acids have been associated with defence mechanisms (Kiron et al., 1995), reduced inflammation and disease resistance in humans (Stenberg et al., 2019).

The role of essential fatty acids in abalone has been barely studied. Previous studies have demonstrated that supplementation of certain fatty acids produce better growth; however, these requirements are species-specific and depend on the developmental stage of the animal. For instance, the supplementation of highly saturated fatty acids (HUFA) and the PUFAs C18:2n-6 and C18:3n-3 in *Haliotis asinina* have produced animals with higher weight gain compared to animals receiving only the essential fatty acid requirements (Bautista-Teruel et al., 2011). Results from this study suggest that abalone do not possess fatty acyl desaturases and cannot form the previously mentioned PUFA from the monoene

C18:1n-9, and therefore these must be obtained from the diet as essential fatty acids (Bautista-Teruel et al., 2011). Uki et al. (1986) reported that *Haliotis discus hannai* require n-3 and n-6 HUFA as essential fatty acids. Similarly, Mai et al. (1996) reported that C18:2n−6, C18:3n−3, C20:4n−6, and C20:5n−3 are important fatty acids for the growth of abalone *H. discus hannai*. Durazo-Beltrán et al. (2004) have shown that HUFAs C20:4n−6, C20:5n−3; and C22:5n−3 are essentials in the diet of *Haliotis fulgens*. The above-mentioned studies showed that under healthy conditions, essential fatty acids specially PUFAs and HUFAs have critical roles in chain elongation and desaturation processes of fatty acids resulting in the production of higher PUFAs for lipid metabolism (Bautista-Teruel et al., 2011). In this study, it was observed that the inclusion of IM promoted significant higher levels of essential fatty acids such as arachidonic acid, oleic acid, and α-linolenic acid, suggesting an increased presence of omega-6 fatty acids precursors and derivatives which may impact abalone growth and fatty acid synthesis. The inclusion of GM promoted significant higher levels of α-linolenic acid. However, the inclusion of both ingredients, IM and GM, promoted a synergistic effect on increasing pentadecanoic acid, palmitic acid, stearic acid, oleic acid. This finding may suggest that abalone can reaccommodate fatty acid products and metabolites according to the needs, while integrating carbohydrate metabolites (Wang W et al. 2009) without affecting growth.

Lipid metabolism is closely involved with that of carbohydrates where a pyruvate resulting from glycolysis is oxidised to a compound called acetyl CoA which is the substrate for synthesizing fatty acids. Abalone do not possess enzymes to metabolise fatty acids, and therefore an excess of dietary lipids might be translated into poor growth (Lee et al. 2019).

In our study, the fatty acid composition of the commercial feed showed higher levels of myristic acid (C14:0), palmitoleic acid (C16:1n-7), heptadecanoic acid (C17:0), cis-8,11,14-eicosatrienoic acid (C20:3n-9) and 5,8,11,14,17-eicosapentaenoic acid – EPA (C20:5n-3) compared to the experimental diets. From all these fatty acids, C20:5n-3 has been highlighted in abalone nutrition because some species such as *H. asinina* do not synthesize it de novo, and therefore it needs to be included in their diet. C20:5n-3 (EPA) is further elongated to C22:6n-3 (DHA), which plays an essential role in osmotic regulation and salinity changes (Dunstan et al., 1996). Our results show that C22:6n-3 (DHA) was not significantly affected by the diet, suggesting that this compound could have been used for growth or other living processes. However, C20:5n-3 (EPA) levels were affected by feed, having the highest values in animals fed the commercial feed. This finding concurs with previous studies suggesting the importance of C20:5n-3 (EPA) in feeds and its potential

inclusion to promote abalone growth. However, further studies to evaluate the role of its inclusion as a supplement will need to be conducted, with special attention to the effects on nutrition, health, and immunity. According to Mai et al. (1995) growth promotion of abalone is collectively affected by the combination of different essential fatty acids rather than a single effect of one particular nutrient. The importance of adding supplements to the final formulation such as fatty acids to optimise abalone feed becomes more relevant as terrestrial alternative ingredients are being used for feed formulations for marine animals.

Another possible explanation for the better growth of commercial feed versus the experimental diets is the difference in carbohydrate levels among diets. In the wild, abalone consume seaweeds which consist of 40–50% carbohydrates. Abalone metabolism is carbohydrate-based (Bautista-Teruel et al., 2011) and they manage to metabolise complex carbohydrates by excreting amylase, cellulase, laminarinase, carrageenase, and alginase (Lee et al., 2017). The introduction of terrestrial carbohydrate sources, such as IM and GM may result in the inability of abalone to degrade dietary fibre due to the lack of specific digestive enzymes. For example, IM contains chitin, which would need to be degraded by chitinase and GM degraded by lignin peroxidase to be used as an energy metabolite. To our knowledge, there are no studies on the native chitinase or lignin peroxidase activity on abalone and their use as energy source or prebiotic still needs further research. The diets including GM (Diet FG and FIG) had 16.1% and 15.4% fibre levels and the diets without GM (diets F and FI) had 9% and 7.5%; both groups with higher fibre levels compared to the commercial feed (3.7%). As fibre compounds are indigestible, abalone increased the faecal output by allocating energy to excretion rather than using the energy in growth. Abalone only use 5% of the ingested energy for growth (Barkai & Griffiths, 1988). Hence, if energy expenditure is allocated to additional processes, such as extra digestion of fibre, the risk of affecting growth performance is high.

The inclusion of GM and IM significantly affected the digestibility of the feed. GM mainly reduced the digestibility of the carbohydrates and protein, whereas IM had an effect on the lipid digestibility of the diets. This finding indicates that GM and IM can be included in the diet up to 100% and 25%, respectively, and although they have a synergistic effect on reducing digestibility, abalone growth performance is not negatively affected compared to diet F, which did not have IM nor GM. This finding indicates that GM and IM can be included in the diet up to 100% and 25%, respectively, and although they have a synergistic effect on reducing digestibility, abalone growth performance is not negatively affected compared to diet F, which did not have IM nor GM. Based on the digestibility coefficient of the commercial

feed, which was comparatively less than diet F and diet FI, it is suggested that animals needed less amount of commercial feed to grow compared to diets F and FI. Although diet F had the highest digestibility coefficient, growth in this group of animals was not greatly improved. Further investigation on the digestibility of diets including IM and GM needs to integrate feed intake, to conclude the absolute effect on digestibility in abalone.

The present study delivered the experimental feeds in an alginate matrix due to the well-known effect of alginate as an immunostimulant. However, immunostimulatory properties may be jeopardized by higher doses of alginate. A previous study has shown positive effects of alginate inclusion doses of ≤ 3.0 g per kg^{-1} in *Haliotis diversicolor supertexta* showing an improved immune response, increasing resistance to pathogens such as *Vibrio parahaemolyticus*, increased activity of phenoloxidase (PO) and super oxide dismutase (SOD) (Cheng & Yu, 2013). In our study, alginate doses were close to 100 g per Kg^{-1}, which is significantly more than the amount of immunostimulants added in the feed for other species, such as tilapia (*O. niloticus*) (Van Doan et al., 2016) and shrimp (*Penaeus monodon*) (Liu et al., 2006).

The overdose of immunostimulants, such as alginate, may be the cause of slow growth. However, there is not enough evidence to support this claim. Further studies of long-term effects or oral administration of immunostimulants in aquatic animals are required. There are possible negative feedback responses that can be activated once administration of immunostimulants is sustained for long periods of time, depending on species and doses.

This study aimed at providing data on two sustainable ingredients which can be included in aquafeeds to replace fishmeal at some extent. Although formulations with IM and GM inclusion did not outperform formulations free of these two ingredients, they do not jeopardize abalone growth or nutritional profile. Insect meal and GM represent cheaper alternatives compared to fishmeal by being reared on waste, low energy, and high efficiency (insects), and by contributing to reuse a biowaste that represents and environmental issue (GM). The optimisation of these two ingredients to reduce deterrents, antinutrients, and increase digestibility will allow to produce cheaper feed alternatives with less environmental costs.

5.7 CONCLUSIONS

This study reported the effect of insect meal from *Tenebrio molitor* and grape marc inclusion on *Haliotis iris* proximate, fatty, and amino acid composition, and apparent digestibility. The inclusion of insect meal and grape did not produce significant variations in protein and carbohydrate proportions of animal tissues among the experimental diets, but differences were observed in the lipid proportion. The amino acid composition of abalone was affected by the inclusion of insect meal and grape marc modifying levels of 8 amino acids, such as taurine, threonine, and methionine. The fatty acid composition was significantly modified among the experimental diets due to the inclusion of insect meal and grape marc suggesting a key role of fatty acids in growth. Increased levels of specific fatty acids in abalone were correlated with insect meal and grape marc fatty acid composition. The inclusion of insect meal increased the digestibility whereas the inclusion of grape marc decreased the digestibility of experimental diets in the lipid component. However, the inclusion of insect meal and grape marc decreased the digestibility of diets in both the protein and carbohydrate component. These changes in digestibility did not affect the proximate composition of abalone tissues in terms of protein and carbohydrate. However, the lipid proportion in abalone tissues was significantly affected. The insights gained from this study suggest that insect meal and grape marc are valuable ingredients for aquaculture feed in terms of producing an acceptable nutritional profile for human consumption. However, further studies with gradual inclusions of insect meal and grape marc are recommended to evaluate correlation of nutrients and presence/absence of specific amino acids and fatty acids.

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Chapter

6

Effect of dietary insect meal and grape marc inclusion on flavour volatile compounds and shell colour of juvenile abalone (*Haliotis iris*)

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6.1 ABSTRACT

Almost 60% of the fishmeal produced globally is used in aquaculture feeds. Fishmeal production relies on finite wild-marine resources and is considered an unsustainable ingredient. Insect meal (IM) is considered a sustainable source with high levels of protein suitable for growth promotion. Grape marc (GM) is a waste by-product of the winery industry rich in pigments with antioxidant capacity. However, the inclusion of both ingredients can affect the flavour of the meat of abalone and the colour of the shell due to different nutritional profiles. The aim of this study was to evaluate the effect of the dietary inclusion of IM and GM on the flavour volatile compounds and shell colour of the juvenile *Haliotis iris* in a 165-days feeding trial. Abalone were offered four experimental diets with different levels of IM and GM inclusion and a commercial diet (no IM or GM) that was used as a control. Soft bodies of abalone were used to characterise volatile compounds using solid-phase microextraction gas chromatography-mass spectrometry, and colour changes were analysed in ground powder of abalone shells using colour spectrophotometry 400-700 nm (visible). Results showed 18 volatile compounds significantly different among the dietary treatments. The inclusion of IM did not significantly affect the flavour volatile compounds detected, whereas the inclusion of GM reduced volatile compounds associated with lipid-peroxidation in abalone meat. The inclusion of IM and GM did not significantly affect the lightness nor the yellowness, blueness, redness, and greenness of the ground shells among experimental diets. The lightness of the shells was significantly higher in animals feed commercial feed. The supplementation of abalone feeds with grape marc can help to reduce off-flavour compounds which may extend shelf-life of raw abalone meat.

6.2 INTRODUCTION

In recent years, there has been a growing interest to replace fishmeal in aquafeeds across the aquaculture industry globally. The reasons behind this quest are the increasing fishmeal price (300% in the last 30 years) (Tacon & Metian, 2009) and the use of wild marine fish stocks to produce fishmeal which is deemed unsustainable. Alternative ingredients have been widely investigated to replace fishmeal in aquaculture feeds (Barroso et al., 2014). One example of an alternative ingredient is insect meal, which is seen as a good source of protein and lipids (Barroso et al., 2014). Insects have fast growth, reproduce easily, convert low-grade organic matter into high-value protein and fat, and do not require cultivable land (Henry et al., 2015). These characteristics make insect meal a favourable option, which is regarded as more sustainable than fishmeal (Ferrer et al., 2019; Makkar et al., 2014). Different insect species have been included in aquafeeds. Yellow mealworm (*Tenebrio molitor*) is one of the most studied species with great potential for industrialisation and high digestibility indexes (Fontes et al., 2019). *T. molitor* has been included in diets for Nile tilapia (*Oreochromis niloticus*) (Fontes et al., 2019), European sea bass (*Dicentrarchus labrax*) (Basto et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Gasco et al., 2014), pacific white shrimp (*Litopenaeus vannamei*) (Panini et al., 2017), and mandarin fish (*Siniperca scherzeri*) (Sankian et al., 2018). Aquafeeds including insect meal for abalone have included silkworm pupae meal (Cho, 2010) promoting a weight and shell gain compared to fishmeal-based diets. A study in our research group have included *T. molitor* in abalone feeds without causing signs of intestinal inflammation compared to fishmeal-based diets (Bullon et al., 2023b).

Another ingredient which has potential for aquafeeds is grape marc. Grape marc is an agro-industrial by-product which consists of grape skins, stalks, and seeds from the winemaking process of *Vitis vinifera*. Almost 18–20% of all harvested grapes used for wine production end up as grape marc (Spanghero et al., 2009). Due to the large amount of waste produced annually, grape marc disposal has become problematic (García-Lomillo & González-San José, 2017). Grape marc has been used as feed for ruminants (Gladine et al., 2007; Ianni et al., 2019; Jerónimo et al., 2012) due to the high dietary fibre content and elevated levels of bioactive compounds, such as tocopherol and polyphenols (Pulgar et al., 2021). Grape marc has high levels of polyunsaturated fatty acids (PUFAs), mostly high levels omega-6 fatty acids, such as linoleic acid (C18:2n-6), oleic acid (C18:1n-9) (Yi et al., 2009), and saturated fatty acids (SFA), such as stearic acid (C18:0) and palmitic acid (C16:0) (Yu & Ahmedna, 2013).

Changes in animal diet affect the physical (Hoffmann et al., 2021) and chemical characteristics of the meat (Fabrikov et al., 2021). These characteristics play an important role in consumer perception of quality and therefore influence purchase price (Carlucci et al., 2015; Mancuso et al., 2016). From these characteristics, the flavour profile, which is mostly shaped by the volatile compounds, is the key difference for more competitive market advantages (Jones et al., 2022), representing a signature for high-quality products. Volatile compounds are good indicators of meat shelf-life (Bruni et al., 2020; Jones et al., 2022; Turchini et al., 2004) and many of them are products of fatty acid oxidation. Dietary fatty acids directly influence the fatty acid composition in the fish muscle (Grigorakis et al., 2009; Habeanu et al., 2015; Melenchón et al., 2021), and therefore the inclusion of insect meals and grape marc can potentially affect the volatile compound composition of the meat. Studies on the effect of insect meal and grape marc on the flavour volatile compounds in aquatic animals are limited because research on volatile compounds has been mainly focused on the effect of cooking methods, fresh/frozen conditions, and storage time on the meat. Insect meal has been included in diets for chickens decreasing the levels fatty acid peroxidation products, such as alcohols and aldehydes suggesting less grassy, oily, fatty, and sweet odours in the meat (Gkarane et al., 2020). To our knowledge, studies on the effect of insect meal inclusion on the volatile composition of aquatic animals have been limited to Atlantic salmon, without significant changes (Bruni et al., 2020). Grape marc has been included in feeds for beef and lamb, reducing the meat levels of volatile compounds that are linked to fast meat spoilage (Ianni et al., 2019; Jerónimo et al., 2012). Grape marc has been included in feeds for rainbow trout (Pulgar et al., 2021), grass carp (Souza et al., 2019), and abalone (Currie et al., 2019), but the volatile compound profiles have been not elucidated in those studies.

The shell colour of molluscs is determined by organic pigments, such as pyrroles, polyenes (carotenoids), melanin, and porphyrin (De Oliveira et al., 2013), which mostly come from dietary sources. It is presumed that pigments are not synthesized *de novo* by molluscs (Canales-Gómez et al., 2010), yet full understanding of shell pigmentation is still limited. In abalone species, the provision of diets supplemented with dietary pigments has shown to produce a change in the shell colouration, which can be manipulated according to market requirements. The dietary inclusion of the seaweed promoted a brownish coloured shell in *Haliotis asinina* (Bautista-Teruel & Millamena, 1999) *and Haliotis discus hannai* (Ju et al., 2016). In addition, the supplementation of carotenoids (astaxanthin, canthaxanthin and β-carotene) promoted shells with more yellow coloration in *Haliotis rufescens.*

This study aimed to assess the effects of the inclusion of insect meal from *T. molitor* and grape marc on the flavour volatile compound profile and shell colour of the New Zealand abalone *H. iris*. The key hypothesis of the study was that the fatty acid profile in *T. molitor* and grape marc will contribute to a significant change in the flavour volatile profile of the meat of *H. iris*. In addition, the supplementation of grape marc, which has anthocyanins as main pigment, will contribute to a change of coloration in the shell.

6.3 MATERIALS AND METHODS

6.3.1 Animals, experimental setup, and sample collection

This study was conducted within a commercial abalone (*H. iris*) farm (The New Zealand Abalone Company) in Bluff, New Zealand. Healthy juveniles with initial mean weight and shell length of 1.1 ± 0.5 g and 21.5 ± 3.3 mm, respectively, were used in the feeding trial. The animals were 17-months old and were randomly selected from the farm stock. Fifteen plastic tanks containing 90 L of filtered seawater were stocked with 200 juveniles per tank. Abalone were randomly distributed among the tanks (three tanks per treatment) and were fed one of the experimental diets or commercial feed for 165 days. Abalone were fed in excess at 1.2–2.2% of their body weight per day which was supplied in the late afternoon (1600 h). A flow-through water system was maintained the same during the feeding trial (water rate 1.5 L/min, water exchange of 40 times/day). The tanks were cleaned as per farming procedure every other day. Faeces, debris, and uneaten food residues were flushed out with the help of a water supply. Animals remained undisturbed in the tank while cleaning was performed. Water temperature and dissolved oxygen were measured using a dissolved oxygen meter (Handy Polaris TCP, Denmark) before and after cleaning abalone tanks. The dissolved oxygen meter was calibrated before use in 'air-saturated' seawater according to manufacturer's instructions. During the feeding trial, the temperature ranged from 12.4–19.7 ˚C, and dissolved oxygen was maintained between 86.3–104.1 % oxygen saturation.

At the end of the feeding trial, 20 abalone were removed from each tank with the aid of a blunt knife and removed from their shells. For shell colour analyses, 60 shells per treatment were used, whereas for flavour analysis 6 animals per treatment were used. Shells were cleaned with distilled water, air-dried, and collected for colour measurement of the shells. For flavour volatile compound analysis, two animals were removed from each tank and then removed from their shells. The soft bodies of these animals were placed into 2 mL cryovial (Biostor™) and quenched in liquid nitrogen for 10 min and then stored in dry ice (-80 °C) until further analysis.

6.3.2 Diet preparation

Four experimental encapsulated diets and a commercial feed were used in the feeding trial. Experimental diets were developed following Bullon et al. (2023a). Briefly, different levels of fishmeal (FM), insect meal (IM) and grape marc (GM) were included as per Table 6.1. Diets were encapsulated and classified as: diet F (only containing fishmeal as source of protein), FI (fishmeal + insect meal), FG (fishmeal + grape marc) and FIG (fishmeal + insect meal + grape marc). A commercial feed (Marifeed S34) served as a control. Dietary composition of the experimental diets and commercial feed was determined according to Bullon et al. (2023a) and reported in Table 6.2.

Table 6.1 Percentage (dry weight) composition of the experimental formulated diets (g/100g)

	Diet			
Ingredients (g/100g diet)	F	FI	FG	FIG
Fishmeal[1]	35	25	35	25
Insect meal[2]	-	10	-	10
Corn Meal	30	30	-	-
Grape Marc[3]	-	-	30	30
Seaweed (dry) *Macrocystis pyrifera*[4]	4	4	4	4
Starch (Native Maize flour)[5]	10	10	10	10

[1]Fishmeal supplied by Sandford, NZ.
[2]Insect meal supplied by Mahurangi Technical Institute (MTI).
[3]Grape marc supplied by Bragato Research Institute, NZ.
[4]Seaweed (*Macrocystis pyrifera*) supplied by Southern Clams.
[5]Starch supplied by New Zealand Starch.

Table 6.2 Proximate composition of four experimental diets and a commercial feed

	Diet				
Proximate composition	F	FI	FG	FIG	Commercial feed[4]
Protein (%)	30.4 ± 0.1^{ab}	27.3 ± 0.3^{b}	30.8 ± 0.7^{ab}	26.4 ± 2.0^{b}	32.4 ± 2.7^{a}
Carbohydrate (%)[1]	47.9	50.8	45.4	49.3	46.34
Carbohydrate-Reducing sugars (%)[2]	28.9 ± 1.3^{b}	32.0 ± 3.1^{ab}	8.9 ± 1.9^{c}	9.1 ± 1.1^{c}	39.1 ± 4.7^{a}
Total dietary fibre (%)	9.0 ± 0.2^{b}	7.5 ± 0.1^{c}	16.1 ± 0.1^{a}	15.4 ± 0.3^{a}	3.7 ± 0.3^{d}
Lipid (%)	4.0 ± 0.2^{c}	7.0 ± 0.6^{a}	5.3 ± 0.5^{b}	7.2 ± 0.3^{a}	1.2 ± 0.3^{d}
Ash (%)	13.5 ± 0.1^{b}	11.9 ± 0.1^{c}	14.5 ± 0.3^{a}	12.9 ± 0.3^{b}	6.8 ± 0.4^{d}
Moisture (%)	4.2 ± 0.01^{b}	3.0 ± 0.01^{b}	3.9 ± 0.03^{b}	4.2 ± 0.8^{b}	10.7 ± 0.1^{a}
Energy (KJ per g)[3]	18.1	20.4	18.9	20.2	15.9

Data represent means and standard deviation. Means with the same superscript (a, b, c) in each column are not significantly different from Tukey post hoc tests ($p < 0.05$).
[1]Carbohydrate proportion was calculated by difference 100 – (moisture + protein + lipid + ash).
[2]Carbohydrate was determined using reducing sugar method Anthrone.
[3] Total energy was calculated based on the physiological values at 5.6 kCal g^{-1} protein, 9.5 kCal g^{-1} lipid and 4.1 kCal g^{-1} carbohydrates (Cho et al., 1982).
[4]Commercial feed used was Marifeed S34.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

6.3.3 Volatile compounds analysis

The volatile compounds in abalone meat were extracted using the solid phase microextraction (SPME) and a gas chromatography-mass spectrometry system. Briefly, two animals from each experimental tank (six per treatment) were randomly selected for volatile compositional analysis at the end of the feeding trial. Since the size and wet weight of the animals were small, the whole body was used for analysis. Samples were kept at room temperature for 1 h and chopped into small pieces. The chopped tissues from each individual abalone (0.50 g) were homogenised in 10 mL milliQ water using an UltraTurrax homogeniser (10000 rpm, 1 min). The samples (2 mL) were placed in 10 mL flat bottom headspace vials fitted with a PTFE/silicone septum and screw cap (Supelco, Inc., Bellefonte, USA). A total of 0.7 g NaCl was added to increase the detection sensitivity of the solid-phase microextraction technique due to the “salting-out” effect. A volume of sodium azide solution (100 µL) was added to each vial to obtain the final concentration of 0.02% w/v in each vial.

A working solution of internal standard (1 ppm, 1, 2-dichlorobenzene) was prepared using milliQ-water. A volume of 100 µL of internal standard working solution was added to each vial. The vials were capped and transferred to gas chromatography – mass spectrometry equipment. The volatile compounds are extracted with a SPME fibre coated with 50/30 µm layer of divinylbenzene–carboxen–polydimethylsiloxane (Supelco Co.,Bellefonte, USA) that was inserted into the vial headspace at 50 °C for 25 min. The SPME fibre was then removed from the headspace vial and injected into the GC-MS injector at 250 °C for 20 min.

A gas chromatography system Agilent Technologies 7890B (Agilent Technologies, Santa Clara, CA, USA) attached to an Agilent Technologies 5977B MSD detector was used. The Agilent G3903-63008 GC column (Phase DB-FATWAX Ultra Inert) with the dimensions of 30 m x 250 µm x 0.25 µm was used. The sample analysis was randomized and automated using a Gerstel multi-purpose sampler (MPS). The initial temperature of the column oven was held at 40 °C for 2 min, then further increased to 240 °C at a rate of 8 °C /min and held for 3 min. The inlet temperature was held at 250 °C with a total flow rate of 46.1 mL/min in splitless mode. The scanning range of mass spectra was 38–400 *m/z*, and electron ionization mode at 70 eV. Ion source temperature was 250 °C, MSD transmission line temperature was 250 °C and the quad set to 150 °C. Volatile compounds were identified using the NIST 14 library using the MassHunter Acquisition software program. An alkane series C7–C30 (Supelco Co.,Bellefonte, USA) was used to assist in compound identification.

6.3.4 Shell colour determination

The colour of the shell powder was characterised by direct readings on a colour Spectrophotometer (ColorFlex EZ, HunterLab, USA). Twenty abalone shells were cleaned after dissection with deionized water, air-dried, and ground using a grinder mill (Planetary Ball Mill PM100/ Germany) for 3 min. Approximately 8 g of the shell powder was weighed for measurements and three replicates were used for each dietary treatment. The instrument was calibrated with green ($L^* = 51.67$, $a^* = -26.62$ and $b^* = 13.29$) and white ceramic standard plates ($L^* = 93.94$, $a^* = -0.95$ and $b^* = 0.94$). The colour was reported in CIE system. L^*, a^* and b^* parameters indicate L^*= lightness, a^* = redness/greenness, and b^* = yellowness/blueness, respectively.

6.4 STATISTICAL ANALYSES

For shell colour data, the normality was analysed using the Shapiro-Wilk test. Data were statistically treated by one-way ANOVA test and Tukey post hoc test for multiple

comparison when data followed a normal distribution. Homogeneity of variances was analysed using Levene's test when samples followed a normal distribution. Nonparametric Kruskal–Wallis test was used when data did not follow a normal distribution (followed by Dunn post hoc test for multiple comparison. The statistical package XLSTAT 2022.3.1 (Addinsoft, New York, USA) was used for those purposes. For volatile compounds, data were analysed using the MetaboAnalyst 5.0 software. Before analysis through MetaboAnalyst, samples were normalised via auto scaling (mean centred and divided by the standard deviation of each variable) to make features more comparable. Comparison of feeding groups was made by one way analysis of variance (ANOVA) and Principal Component Analysis (PCA) was used to identify groupings of all samples based on the underlying structure of the data. Tukey's post hoc tests were used to compare the mean differences between dietary treatments ($p < 0.05$).

6.5 RESULTS

6.5.1 Volatile compounds analysis

The PCA results showed that the first component (PC1) and second component (PC2) explained 39.8% and 25.2% of the variance, respectively. The samples from different dietary treatments F and FIG were relatively separated according to their volatile profile in the PCA. Diet FIG and FG were not well separated, nor diets F and FI (Figure 6.1). Agreed with the results from Table 6.3, significant differences ($p < 0.05$) were seen in 18 volatile compounds among diets, and they contributed to the separation between diets with grape marc (FG and FIG) and without grape marc (F and FI). Significantly higher levels of 2,4- di-tert-butyl-phenol and cyclohexanone were observed in diet FIG compared to the rest of the diets. Diet F and FI had significantly higher levels of 1-octen-3-ol and 3-octanone compared to diet FG and FIG. Benzoic acid methyl ester was significantly higher in diet FIG compared to the other diets. Diet F had significantly higher values of pentanoic acid, 2-methyl anhydride; 2-pentanone; 2,3-pentanedione; 1-pentanol, and 2-penten-1-ol compared to diet FG and FIG. Diet FIG had significantly higher values of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate. The compound 1-hexanol was significantly higher in diet FI compared to diet FG.

Diet FIG had significantly higher values of propanoic acid 2-methyl, 3-hydroxy-2,2,4-trimethylpentyl ester and 3-heptanone compared to other experimental diets. Diet F and FI had significantly higher values of hexanal compared to commercial feed, but not to the other experimental diets. Diet FI had significantly higher values of 2-penten-1-ol compared to diet

FG. Heptanal was significantly higher in diet F compared to CF, and 2-hexanone was significantly higher in diet FIG compared to CF.

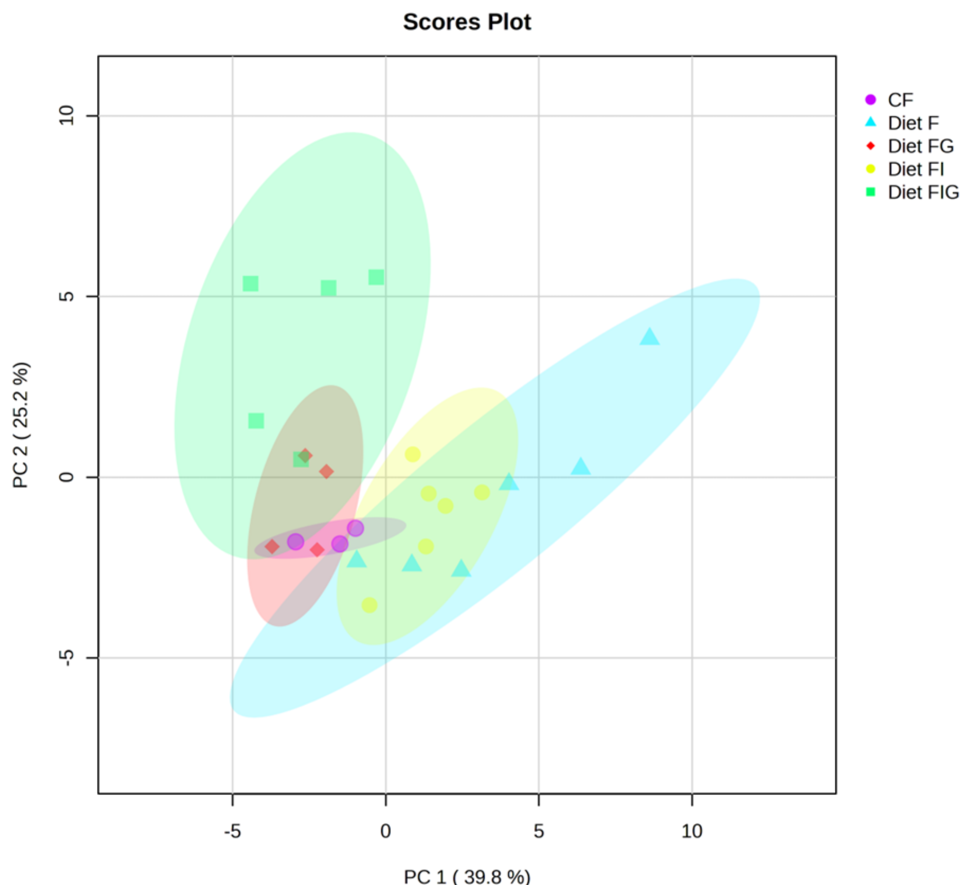

Figure 6.1 Principal component analysis plot based on the volatile compound profiles of abalone grouped by dietary treatment. Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

A heatmap of the volatile compounds showed that diet FIG had significant higher levels of ketones: 3-heptanone, 2-hexanone and cyclohexanone compared to diet F which had high levels of ketones: 2,3-pentanodione, 2-pentanone, and 3-octanone (Figure 2). All aldehydes and alcohols were elevated in diet F compared to the other diets. Two clusters were differentiated: Cluster A where samples fed diets F and FI were together, while cluster B showed samples fed diets FG, CF and FIG grouped together, which supported PCA ellipse separation. Cluster A was different from cluster B presenting elevated levels of alcohols, aldehydes, aromatic compounds, and ketones. Cluster B showed reduced levels of all compounds. Diet FIG, which was grouped under Cluster B, showed higher levels of ketones and esters compared to other diets in that cluster.

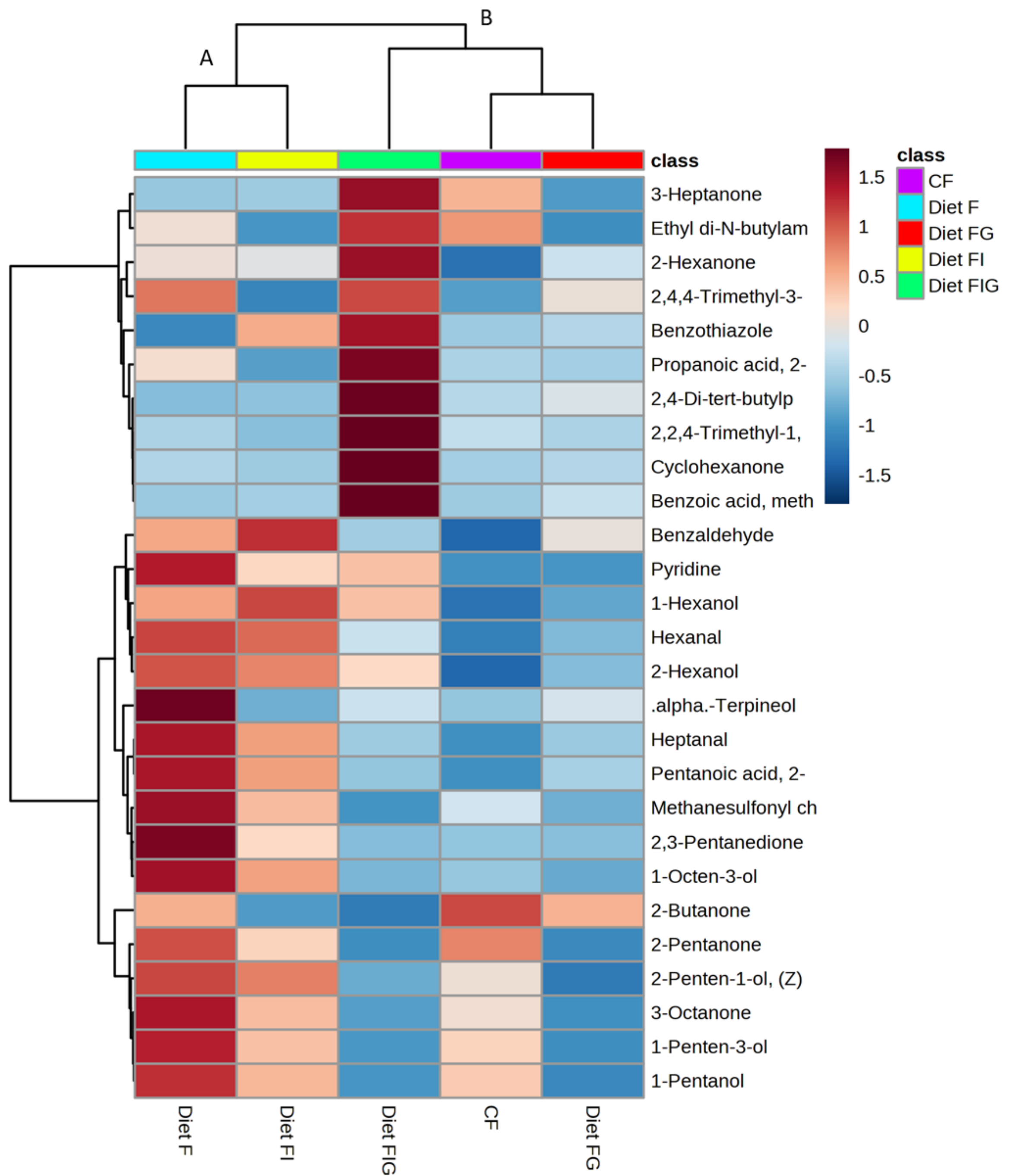

Figure 6.2 Heatmap of 27 volatile compounds found in abalone tissue. Only 18 compounds were found significantly different between the dietary treatments (One-way ANOVA, $p < 0.05$, FDR < 5%). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF). Metabolites abbreviations as per table 6.3.

Table 6.3 Significant different volatile compounds in abalone meat fed on four experimental diets and a commercial feed using SPME-GCMS.

Compound	R.I[ǂ]	CAS #	Aroma description[‡]	Tukey's HSD
Ketones				
2-hexanone	1067.4	591-78-6	Fruity, buttery	Diet FIG> CF
2,3-pentanedione	1046.0	600-14-6	Butter, caramel, sweet	Diet F>CF; Diet FG<Diet F; Diet FIG<Diet F
3-Heptanone	1140.1	106-35-4	Fruity, sweet	Diet FIG>Diet F; Diet FIG>Diet FG; Diet FIG>Diet FI
2-pentanone	962.9	107-87-9	Fruity	Diet FIG<CF; Diet FG<Diet F; Diet FIG<Diet F
3-octanone	1241.8	106-68-3	Mushroom, earthy	Diet F>CF; Diet FG<Diet F; Diet FIG<Diet F; Diet FI>Diet FG; Diet FIG<Diet FI
2- butanone	884.7	78-93-3	Fruity, green	n.s.
2,4,4-trimethyl-3-(3-methylbutyl) cyclohex-2-enone	1729.1	88725-82-0	n.d.	n.s.
Cyclohexanone	1278.8	108-94-1	n.d.	Diet FIG>CF; Diet FIG>Diet F; Diet FIG>Diet FG; Diet FIG>Diet FI
Aldehydes				
Hexanal	1067.7	66-25-1	Fish-like, grassy	Diet F>CF; Diet FI>CF
Heptanal	1172.1	111-71-7	Fish-like	Diet F>CF
Benzaldehyde	1507.9	100-52-7	Almond, nutty	n.s.
Alcohols				
1-octen-3-ol	1441.8	3391-86-4	Sweet, earthy	Diet F>CF; Diet FG<Diet F; Diet FIG<Diet F; Diet FI>Diet FG; Diet FIG<Diet FI
1-penten-3-ol	1160.6	616-25-1	Mushroom, fish-like	Diet FG<Diet F; Diet FIG<Diet F; Diet FI>Diet FG; Diet FIG<Diet FI
2-penten-1-ol, (Z)	1313.4	1576-95-0	Mushroom, fish-like	Diet FG<Diet F; Diet FIG<Diet F; Diet FI>Diet FG
1-pentanol	1247.4	71-41-0	Earthy, mushroom	Diet FG<Diet F; Diet FIG<Diet F
1-hexanol	1347.2	111-27-3	Green, fish-like	Diet F>CF; Diet FI>CF; Diet FI>Diet FG
2-hexanol	1216.8	626-93-7	Fruity, fatty, winey	n.s.
Ester				
Benzoic acid, methyl ester	1609.1	93-58-3	n.d.	Diet FIG>CF; Diet FIG>Diet F; Diet FIG>Diet FG; Diet FIG>Diet FI
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	1858.7	77-68-9	n.d.	Diet FIG>Diet FG; Diet FIG>Diet FI

Compound	R.I[ɨ]	CAS #	Aroma description[‡]	Tukey's HSD
Acid anhydride				
2,2,4-trimethyl-1,3-pentanediol diisobutyrate	1874.6	6846-50-0	n.d.	Diet FIG>CF; Diet FIG>Diet F; Diet FIG>Diet FG; Diet FIG>Diet FI
Pentanoic acid, 2-methyl-, anhydride	1311.0	63169-61-9	n.d.	Diet F>CF; Diet FI>CF; Diet FG<Diet F; Diet FIG<Diet F
Phenolic compounds				
Aromatics				
2,4-di-tert-butylphenol	2291.7	96-76-4	n.d.	Diet FIG>CF; Diet FIG>Diet F; Diet FIG>Diet FG; Diet FIG>Diet FI
Ethylbenzene	1105.0	100-41-4		n.s.
Benzothiazole	1941.7	95-16-9		n.s.
Pyridine	1181.0	110-86-1		n.s.
Terpenic derivatives				
Alpha-terpineol	1688.4	98-55-5		n.s.
Amine				
Ethyl di-N-butylamine	1769.1	4458-33-7		n.s.
Sulphur compound				
Methane sulfonyl chloride	1193.4	124-63-0		n.s.

[ɨ]Volatile compound identified by SPME-GCMS analysis based on comparison with the RI and the mass spectra of standard compounds.
[‡]Reference aroma description based on Wang et al. (2014) Jones et al. (2022) Fukami et al. (2002) Bai et al. (2019) and http://www.thegoodscentscompany.com
n.d: No documented.
n.s: No significant.
Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

6.5.2 Shell colour

Results showed that redness and greenness were not significantly different among dietary treatments; however, a more negative value in diet FIG (-0.7 ± 0.1) indicated more redness compared to CF (-0.4 ± 0.2) (Figure 6.3A). There were no significant variations in terms of yellowness and blueness among the experimental diets. However, experimental diet FG (3.0 ± 0.2) showed significantly less yellowness and more blueness compared to CF (3.4 ± 0.3) (Figure 6.3B). There were no significant variations in shell lightness among the experimental diets with or without insect meal and grape marc (Figure 6.3C). The lightness (also called brightness) of the shells was significantly higher in animals feed commercial

feed (CF) (52.2 ± 0.4) and FIG (51.5 ± 0.2) compared to other experimental diets F (51.4 ± 0.2), FG (51.5 ± 0.3), and FI (51.5 ± 0.3).

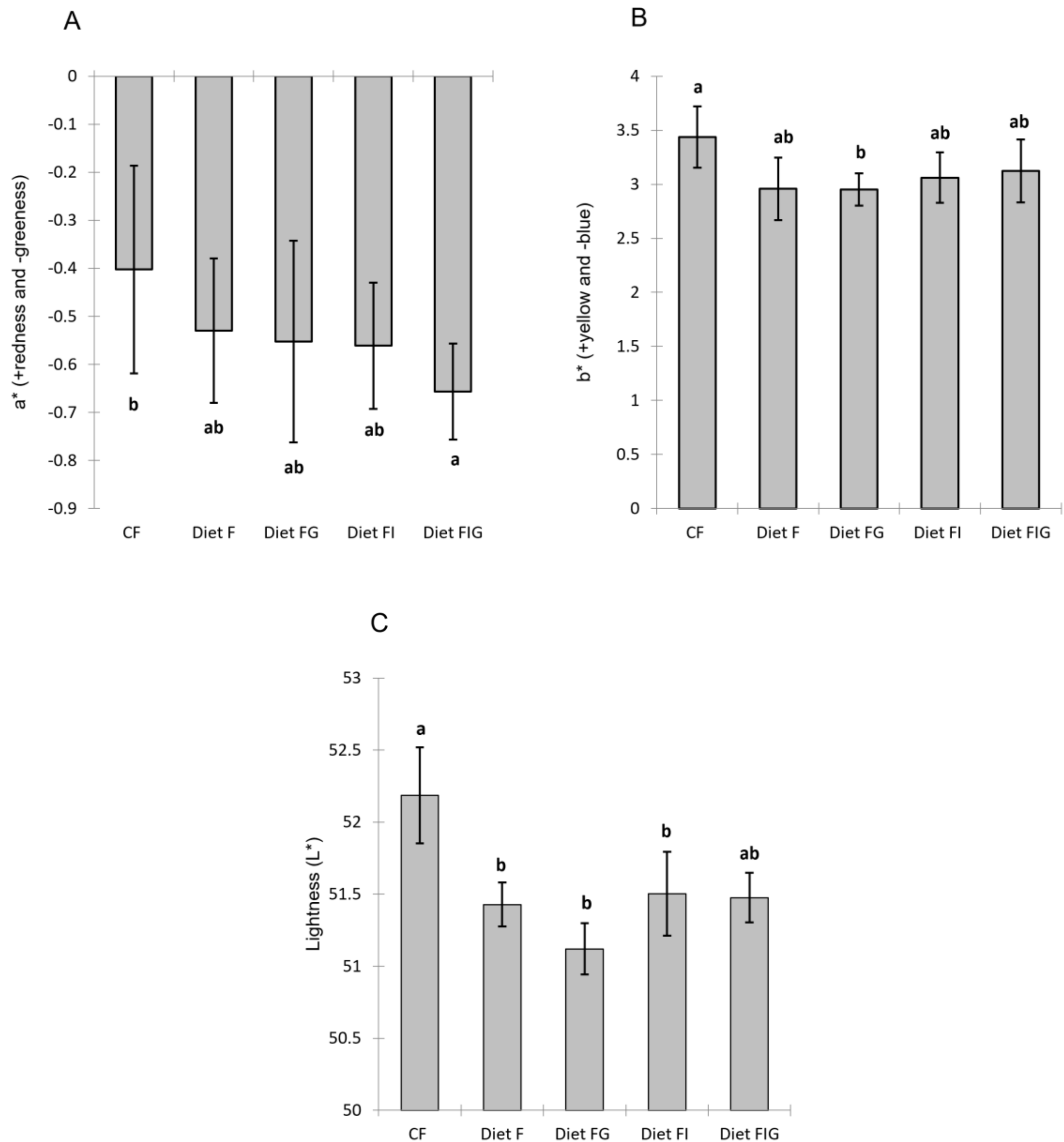

Figure 6.3 Shell colour in *H. iris* receiving different dietary treatments in a 165-feeding trial according to (A) redness /greenness, (B) yellowness / blueness, and (C) lightness. Data are presented as means ± SD of three technical replicates. Different letters on top of the error bars denote significant differences resulting from *Tukey* post hoc tests ($p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

6.6 DISCUSSION

H. iris is highly sought for the beauty of its shell colour, which is considered a traditional art decoration in New Zealand. *H. iris* is mostly fed commercial diets in the farms, which promote faster growth, with fishmeal being the main protein source. Fishmeal has been considered an excellent protein source due to its palatability and the increased growth it promotes compared to seaweeds. The current market of aquafeeds is seeking fishmeal replacements and alternative ingredients that can reduce the environmental impact, and simultaneously generate sustained economic profit by promoting the re-utilisation of food wastes.

H. iris shells are highly valued for their use in jewellery and across traditional and modern indigenous Māori art. In this study, our results suggest that the inclusion of grape marc and insect meal did not significantly affect the lightness of the shells, nor the colouration of the shells. Although the lightness comparison between experimental diets and the commercial feed is not possible due to the lack of knowledge about the ingredients in the commercial feed, it was noted that abalone fed with the commercial diets (CF) had lighter shells compared to the experimental diets. The white coloration, which indicates more lightness, is associated with the calcium carbonate crystals that form aragonite and calcite structures in the shell of abalone (De Oliveira et al., 2013). Sometimes, commercial feed producers add compounds, such as phosphorous in the feed (Coote et al., 1996), to promote faster growth and calcium carbonate formation, especially during the early stages of formation (Ajili et al., 2022), possibly increasing lightness, width, and height of the shell (Tung & Alfaro, 2011).

There were not significant differences among all experimental diets in terms of shell yellowness/blueness (b*) and redness/greenness (a*) coloration. Anthocyanins are water soluble pigments (Wang et al., 2022) and their colours can vary from red to blue depending on the pH value (Mainente et al., 2019). Studies have shown that anthocyanins from grape marc derivates modified the red and yellow colour in fish muscle (Sánchez-Alonso et al., 2008; Sánchez-Alonso et al., 2007). However, the effect of dietary components on molluscan shells is not well understood (De Oliveira et al., 2013; Hedegaard et al., 2006). The colours of molluscan shells are determined by organic pigments, such as pyrroles, polyenes (carotenoids), melanin, and porphyrin (De Oliveira et al., 2013) and their mechanism of affecting abalone has been observed but not elucidated (Bautista-Teruel & Millamena, 1999; Jin et al., 2020). It is known that abalone do not produce pigments *de*

novo, such as carotenoids, and instead these are obtained from the diet intensifying the yellow tones of *Haliotis rufescens* shells (Canales-Gómez et al., 2010). The aquamarine blue pigment is mostly present in farmed *Haliotis cracherodiii* (Cai et al., 2011) and *H. iris* (Mohammadi, 2017). This pigment is relatively stable, unless in the presence of metallic ions, such as Fe^{3+}, where the blue pigment become more yellow/green (Cai et al., 2011). It is possible that the amount of grape marc included in the experimental diets would not have been enough to produce a significant red colouration in the shells of abalone. More studies on this matter including photographic image and pigment extraction are recommended.

For consumers, one of the most appealing features of a snail meat is the flavour, which is an indicator of consumer acceptance and preference (Lasekan et al., 2018). Flavour is a complex phenomenon and starts with the visual perception and smell (Laing & Jinks, 1996). Once food is ingested, volatile compounds (odorants and irritants) from the food are transported to the odour receptors located in the nasopharynx (Laing & Jinks, 1996). Generally, the volatile compounds related to fish odour are generated by enzyme reaction, lipid oxidation, microbial action, and environmental reactions (Ma et al., 2020). Examples of these volatile compounds are volatile aldehydes, alcohols, ketones, acids, amines, and aromatic compounds produced by oxidation of fatty acids.

Our results identified volatile compounds in abalone meat that are usually related to the "fishy" odour, such as hexanal; benzaldehyde, 1-hexanol; 1-octen-3-ol; 2,3-octanedione; 2-penten-1-ol; 1-penten-3-ol; "fatty" odour, such as heptanal and "earthy" odour, such as 1-pentanol (Jones et al., 2022; Wang et al., 2014). Those volatile compounds were found among all diets and only 18 were significantly different. Abalone fed diet F (fishmeal as only source of protein) showed significantly higher levels of pentanoic acid, 2-methyl anhydride; 2-pentanone; 2,3-pentanedione; 1-pentanol; 2-penten-1-ol; 1-octen-3-ol, and 3-octanone compared to diets FG and FIG. This finding suggests that the presence of grape marc may have regulated the production of these lipid peroxidation products in abalone meat. Grape marc has natural antioxidant and antimicrobial properties due to the high tannin (Caetano et al., 2019) and polyphenol content (Guendez et al., 2005), mostly present in the skin and seeds of the grapes. Polyphenols and anthocyanins are free radical scavengers and prevent oxidation of polyunsaturated fatty acids, such as linoleic acid (Hung et al., 2014), which in our study resulted in a significant reduction of the compound 1-octen-3-ol. The inclusion of dietary grape marc to prevent the oxidation of animal meat is not novel. Turcu et al. (2020) included grape marc at 3% and 6% in feeds for broilers which decreased the amount of thiobarbituric acid-reactive substances (TBARS) in the meat. Similarly, Ianni et al. (2019)

included grape marc at 10% in feeds for calves producing meat with reduced levels of hexanal, which is a considered a marker of lipid peroxidation (Bennato et al., 2020; Shahidi & Pegg, 1994). Grape marc has shown to prevent microbial spoilage (Mattos et al., 2017) and the formation of contaminants in meat, such as nitrosamines (Li et al., 2013) acting as a natural food preservative (Saura-Calixto, 1998).

The addition of insect meal in diet FI did not produce a significant difference in flavour volatile compounds compared to diet F. Slight reductions of heptanal, 3-octanone, 1-penten-3-ol, and 1- pentanol in diet FI compared to diet F were observed, yet not significant. Typically, insect meal contains high levels of saturated fatty acids, and due to a slower oxidation compared to polyunsaturated fatty acids, insect meals have demonstrated to produce less peroxidation products compared to free-insect diets in the meat of chickens (Gkarane et al., 2020). One possible explanation for the absence of significant differences between diet F and FI is the fatty acid profile present in *T. molitor* meal, which resembles fishmeal and has been documented to be rich in PUFAs rather than saturated fatty acids (Dreassi et al., 2017), which is different from other insect meals. A previous study in our research group indicated that the dietary inclusion of insect meal by 10% significantly increased the levels of α-linolenic acid, linoleaidic acid, arachidonic acid, and oleic acid compared to a fishmeal-based diet in the meat of abalone (Bullon et al., 2023a). However, these changes may have not contributed to the increase of the lipid oxidation products in abalone meat. The lipid peroxidation products in animal meat have been mostly associated with higher contents of linoleic acid, which can cause the formation of more aldehydes (Ford et al., 1976). Although the inclusion of insect meal (diet FI) has promoted significantly higher levels of precursors of linoleic acid (oleic acid and stearic acid) in the meat of abalone free insect meal/ grape marc diet (Bullon et al., 2023a). However, in this study the volatile compounds products were not significantly altered. The inclusion of insect meal produced significantly higher levels of alcohols 2-penten-1-ol, 1-octen-3-ol, and 1-hexanol compared to diet FG in abalone meat. Of them, 2-penten-1-ol has been found to be more associated with buttery, fish-like, and green flavour, 1-octen-3-ol with mushroom, fermented, and potato flavour, and 1-hexanol with green and fish-like flavour (Jones et al., 2022). From these compounds, 1-octen-3-ol is mostly attributed to crustaceans, due to the high content of fats in those animals. The presence of these compounds suggests that the inclusion of 10% insect meal influenced significantly the fishy-buttery characteristics of the meat compared to the diet with grape marc (FG). Further studies need to be performed using gradual increments of insect meal and grape marc and a taste panel.

Two volatile compounds, heptanal and hexanal, were found to be significantly different between the experimental diets and commercial feed. Heptanal was significantly lower in CF compared to diet F, and hexanal was significantly lower in CF compared to diet F and diet FI. This finding indicates that abalone fed on diet F and FI (fishmeal + insect meal) may have a more intense green-plant-like aroma usually related to meat freshness (Edirisinghe et al., 2007; Wierda et al., 2006) similar to when fishes are immediately harvested (Josephson et al., 1984). In addition, the compounds 2-hexanone, 3-heptanone and 2-heptanone (all related to a fruity flavour) were significantly higher in abalone fed the diet FIG, which contained grape marc.

The findings in this study indicated that the inclusion of insect meal from *T. molitor* did not produce significant changes in terms of flavour volatile compounds in abalone meat, whereas the inclusion of grape marc significantly reduced the volatile compounds related to lipid peroxidation. In addition, the inclusion of both, insect meal and grape marc did not have an impact on the shell's lightness, yellowness/redness/greenness, and blueness.

6.7 CONCLUSIONS

This is the first study to report differences in the volatile composition of the meat and shell colour profile of *H. iris* after dietary treatment with diets that included insect meal from *T. molitor* and grape marc. The inclusion of insect meal did not significantly affect the volatile compound profile, whereas the inclusion of grape marc significantly reduced the production of most of the volatile compounds. Compounds such as 1-pentanol, 2-penten-1-ol, 2-pentanone, 2,3-pentanedione, 1-hexanol were found to be significantly reduced with the inclusion of grape marc indicating a possible antioxidant effect which prevented lipid peroxidation in the raw meat of abalone. The dietary inclusion of insect meal and grape marc did not affect the lightness, redness/greenness, and yellowness/blueness of the ground shells. The insights gained from this study suggest that insect meal and grape marc are valuable ingredients for aquaculture feed in terms of cost-effective and sustainable production. However, further studies including both dietary ingredients are needed to evaluate meat flavour (chemically or by using a taste panel) and shell colouration of adult market-size individuals.

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Chapter

7

Expanding the menu for the New Zealand farmed abalone: dietary inclusion of insect meal and grape marc (effects on gastrointestinal microbiome, digestive morphology, and muscle metabolome)

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7.1 ABSTRACT

Limited supply of sustainable feed ingredients is a significant concern for future aquaculture practices. Alternative ingredients, such as insect meal (IM) and grape marc (GM), are suitable for aquaculture nutrition due to their nutritional profile and more sustainable production methods. This study assessed the effect of dietary IM and GM on gut microbial composition, digestive system status, and muscle metabolome of *Haliotis iris*. Juvenile abalone were fed four encapsulated experimental diets with varying levels of IM/GM and a commercial feed for 165 days in a commercial farm. The gut health of abalone was evaluated through histopathology, and 16S rRNA amplicon libraries through Illumina *MiSeq* sequencing. The abalone metabolome was evaluated through gas chromatography-mass spectrometry (GC-MS). Results showed that abalone had good intestinal condition across all dietary treatments. Microbiome analysis revealed increased relative abundances of bacteria (*Firmicutes and Fusobacterium*) associated with the breakdown of polysaccharides in diets including IM and GM, suggesting an increase of beneficial bacteria species. Metabolite profile variations indicated that the inclusion of IM and GM favoured fatty acid metabolism and amino acid catabolism, respectively. However, increased growth was shown to be correlated with increased essential and non-essential fatty acids in the diet. It is concluded that the inclusion of both IM and GM at 10% and 30% respectively did not affect growth and intestinal health of abalone. This study provides foundation to produce more sustainable feeds including both ingredients where further optimisation is required.

7.2 INTRODUCTION

In the last two decades, significant efforts have been made to reduce the amount of fishmeal included in aquafeeds as a primary source of protein due to the unsustainability of wild pelagic stocks that provide this material (Tacon & Metian, 2008). Many alternative ingredients, such as plant proteins and insect meals, have been shown to promote comparable growth to fishmeal in aquatic species (Bautista-Teruel et al., 2003; Belghit et al., 2019; Bonvini et al., 2018; Cho, 2010; Sankian et al., 2018). However, the presence of antinutrients (substances that interfere with absorption of nutrients) in plant meals can jeopardize digestion and nutrient utilisation in aquatic species (Francis et al., 2001; X. Yu et al., 2022).

Abalone are herbivorous marine snails, which predominantly feed on seaweeds in the wild. Within land-based aquaculture farms, formulated feeds for abalone have generally included fishmeal as a main protein component (Troell et al., 2006; Bansemer et al., 2016), with around 30-40% to expedite growth. Some abalone formulated feeds have include seaweeds as the main source of protein, resembling the natural preference of this natural food for abalone. However, regulations in certain countries in terms of seaweed supply from natural resources and continuous availability throughout the year hinder the industrial use of seaweed for abalone formulated feeds.

Insect-based sources have gained interest due to their superior nutritional characteristics (Makkar et al., 2014), and their production is more sustainable than fish and plant production (Ferrer et al., 2019; Nugroho & Nur, 2018; Van Huis, 2015). Insect meals have shown acceptable digestibility (Basto et al., 2020), although the chitin level (the most available polysaccharide in the exoskeletons of insects) is relatively high in content (Marono et al., 2015; Stenberg et al., 2019). To date, insect meals have not been included in abalone commercial formulated feeds. However, they have been used in feeds for other aquatic animals, such as fish. Insect meals have been shown to improve immune function in diets for mandarin fish (*Siniperca scherzeri*) (Sankian et al., 2018), white shrimp (*Litopenaeus vannamei*) (Choi et al., 2018), and rainbow trout (Bruni et al., 2018; Rimoldi et al., 2021). To date, only five species of insects have been approved for use in animal feed in the European Union (European Comission, 2017). Among others, such as black soldier fly (*Hermetia illucens*) and house cricket (*Acheta domesticus*), mealworm (*Tenebrio molitor*) is considered to be the most promising species for industrial applications (Shin & Lee, 2021).

Grape marc is another alternative ingredient that has been demonstrated to have beneficial effects on the gut health and immune capacity of aquatic species, such as rainbow trout (Pulgar et al., 2021) and grass carp (*Ctenopharyngodon idella*) (Souza et al., 2019). Grape marc is a waste by-product from the winery industry (*Vitis vinifera*) comprising the skins and seeds of grapes. Grape marc disposal has become problematic and represents a current global environmental concern (Gómez-Brandón et al., 2019). Grape marc is characterised by high levels of non-digestible carbohydrates, such as oligosaccharides (OS), which act as prebiotics (Bordiga et al., 2019; Sinrod et al., 2021). The supplementation of grape by-products, such as grape seed extract has shown positive effects on abalone survival and gene expression of certain genes which are related to oxidative defences and innate immunity (Shiel et al. 2017). However, grape marc supplementation is still limited. The addition of grape marc in feeds for rainbow trout (Pulgar et al., 2021), grass carp (Souza et al., 2019), and abalone (*Haliotis laevigata*) (Currie et al., 2019) has shown positive effects on growth performance and immunity status. A previous study has shown that the inclusion of grape marc in feeds for abalone promotes comparable growth to formulated diets without grape marc (Bullon et al., 2023a).

Numerous studies in aquaculture nutrition have focused on the inclusion of feed supplements for growth improvement (Macey & Coyne, 2005; Ten Doeschate & Coyne, 2008). However, studies that comprehensively evaluate the multi-factorial effects of nutrition on overall health, such as gut health and metabolome, are still limited. Evaluating the effects on the gastrointestinal tract (GIT) is a crucial aspect when new dietary ingredients are included, as they indicate the capacity and effectiveness to absorb nutrients. The digestive gland is the major site of nutrient absorption in molluscs, while the intestinal epithelium, formed by columnal supporting cells and secretory cells, also contribute to nutrient absorption (Maguire, 1998). The intestinal epithelium cells of molluscs perform multiple tasks, such as endocytosis and intracellular digestion, absorption of small molecules, storage of glycogen and lipid, and transport of nutrients to the underlying tissues (Lobo-da-Cunha, 2019). The intestinal microbiome is another aspect of gut health which plays an important role in immunity, disease resistance, and growth of aquatic species (Hai, 2015). In aquaculture, the enhancement of gut colonization with beneficial bacteria is a common practice aimed at improving animal health and growth rates. The inclusion of black soldier flies in diets has been shown to benefit the lactic acid bacteria in the intestinal microbiota of salmonids (Bruni et al., 2018; Rangel et al., 2022). In addition, the inclusion of insect meal from *T. molitor* has increased the beneficial bacterial population of *Lactobacillus* and *Enterococcus* in the gut of rainbow trout (Józefiak et al., 2019). Beneficial bacteria in the gut

can produce bacteriocins which inhibit pathogens in the gastrointestinal tract (Dimitroglou et al., 2011; Ringø et al., 2005). The increase of these beneficial communities has also been associated with the presence of chitin in insect meals (Antonopoulou et al., 2019; Terova et al., 2021). Dietary chitin has demonstrated improvement in beneficial enzyme-producing bacterial communities with antibacterial activities (Assan et al., 2022) acting as a prebiotic (Rangel et al., 2022). Conversely, detailed information on the specific effects of dietary grape marc on the microbiome of aquatic animals is still limited. However, micro-encapsulated grape marc extracts have increased the population of *Acetobacteraceae* and *Lactobacillaceae* in the gut of rainbow trout, following a positive impact on growth (Pulgar et al., 2021).

Changes in the intestinal tissue morphology and microbiome can modify metabolic processes, which can further affect the animal's health (Levy et al., 2017). The metabolome provides insights on metabolite fluctuations while exposed to different nutrients (Venter et al., 2019). The presence or absence of certain metabolites in abalone can suggest enriched metabolic pathways that can affect growth (Masoomi et al., 2023; Venter et al., 2019), immunity (Nguyen et al., 2018), and feed efficiency (W. Yu et al., 2022). In abalone, higher feed efficiency has highlighted the role of amino acid metabolism, causing the upregulation of arginine, tyrosine, and tryptophan pathways (W. Yu et al., 2022). Increments of end-products, such as DL-methionine, tyramine, and indoleacetic acid metabolites have been linked to an increased abundance of *Proteobacteria* in *Haliotis discus hannai* with high feed efficiency (W. Yu et al., 2022)

This study aimed to assess the effect of insect meal and grape marc feed inclusion on gut microbial composition, digestive system morphology, and adductor muscle metabolome (indicative of nutritional and health stress conditions) of *H. iris*. The key hypothesis was that the dietary fibre content in grape marc and chitin in insect meal contribute to a better health status in abalone, evidenced by changes in microbiota and improved morphological state of the digestive tract including gills, foot, mantle, gonad, digestive tract, and kidneys. In addition, key signatures in metabolite profiles can reflect improved gut health.

7.3 MATERIALS AND METHODS

7.3.1 Experimental design and sample collection

This study was conducted within a commercial abalone (*H. iris*) farm (The New Zealand Abalone Company, Ocean Beach, Bluff, New Zealand). Juvenile abalones (17-month-old, n = 200) with initial mean weights (± SD) of 1.1 ± 0.5 g and shell lengths (± SD) of 21.5 ± 3.3 mm were randomly selected from the farm stock for the feeding trial. A total of 15 plastic tanks (3 tanks per treatment), each containing 200 juveniles, were stocked with filtered seawater (100-micron filter) at a volume of 90 L per tank. Out of the 200 abalone, 50 were tagged for the purpose of allometric measurements. For tagging, abalone were removed from the tanks with the aid of a flat, blunt knife and dried using paper towel. Numeric tags were attached to the dorsal exterior of the shells using a cyanoacrylate glue. The tagging process lasted approximately 5 minutes per animal and the water temperature during the tagging process was 13 °C.

Abalone were initially fed commercial feed (Marifeed S34) before being assigned to one of four experimental diets or the commercial feed, which were allocated in three replicates. The nutritional information of the commercial feed is shown in Table 7.1. The tanks were maintained using a flow-through water system with a water exchange of 40 times per day at a flow rate a rate of 1.5 L/min. Farming procedures were followed to clean the tanks by flushing out faeces, debris, and uneaten food residues with a water supply every other day. Animals remained with minimal disturbance in the tanks during cleaning and covered in total darkness during the whole feeding trial, except when feeding and cleaning activities were performed. During the feeding trial the water temperature ranged from 12.4–19.7 °C and the dissolved oxygen varied from 86.3–104.1 % oxygen saturation. Water temperature and dissolved oxygen were measured with a dissolved oxygen meter (Handy Polaris TCP, Denmark) which was calibrated before use using a 'air-saturated' seawater according to manufacturer's instructions.

During the 165-day feeding trial, abalone were fed 1.2–2.2% of their body weight per day in the late afternoon (~1600 h). A set of 50 animals per tank were tagged and measured for growth at the beginning and end of the feeding trial according to section 7.3.3. Prior to the dietary intervention, twenty individuals were selected, removed from their shells, and dissected for microbiome (gastrointestinal content) and histological analysis to provide baseline information on the initial condition of the animals. Samples for metabolomic

analyses were not collected at this point due to logistical constraints. At the end of the feeding trial, abalone were dissected for microbiome and metabolite profiling (n=9) and histological analysis (n=15) per dietary treatment. Gastrointestinal content (microbiome) and adductor muscle (metabolomic) samples were collected from the same animal. Microbiome samples were placed in 2 mL cryovials (Biostor™) that contained 0.5 mL RNA stabiliser (Qiagen, Germany) as per section 7.3.4. Metabolomic samples were collected and placed into 2 mL cryovials (Biostor™) following section 7.3.6. Both types of samples were then immersed in liquid nitrogen for 10 min and then stored in a -80 °C freezer until further analysis. Histological samples were collected and analysed according to section 7.3.5.

7.3.2 Diet preparation and encapsulation

Four encapsulated diets were used as experimental diets and a commercial feed (Marifeed S34) was used as a control. Experimental diets were formulated as per Bullon et al. (2023) to contain graded levels of fishmeal (FM), insect meal (IM) and grape marc (GM) and they were designed as follows: diet F (only containing fishmeal as source of protein), FI (fishmeal + insect meal), FG (fishmeal + grape marc), and FIG (fishmeal + insect meal + grape marc) (Table 7.1). The commercial feed was not encapsulated, and the nutritional profile is also detailed in Table 7.1. Experimental diets were encapsulated according to the protocol detailed by Masoomi et al. (2022) with small modifications as detailed in Bullon et al. (2023a). Dietary and proximate composition of the experimental diets is presented in Table 7.1. The nutritional determination method used has been detailed previously in Bullon et al. (2023a).

Table 7.1 Proximate composition of four experimental diets and a commercial feed, and ingredients used for experimental diets (g/100g diet).

	Diet				
Proximate composition (%)	F	FI	FG	FIG	CF[4]
Protein	30.4 ± 0.1[ab]	27.3 ± 0.3[b]	30.8 ± 0.7[ab]	26.4 ± 2.0[b]	32.4 ± 2.7[b]
Carbohydrate[1]	47.9	50.8	45.4	49.3	48.9
Carbohydrate- reducing sugars[2]	28.9 ± 1.3[b]	32.0 ± 3.1[ab]	8.9 ± 1.9[c]	9.1 ± 1.1[c]	39.1 ± 4.7[a]
Total dietary fibre	9.0 ± 0.2[b]	7.5 ± 0.1[c]	16.1 ± 0.1[a]	15.4 ± 0.3[a]	3.7 ± 0.3[d]
Lipid	4.0 ± 0.2[c]	7.0 ± 0.6[a]	5.3 ± 0.5[b]	7.2 ± 0.3[a]	1.2 ± 0.3[b]
Ash	13.5 ± 0.1[b]	11.9 ± 0.1[c]	14.5 ± 0.3[a]	12.9 ± 0.3[b]	6.8 ± 0.4[d]
Moisture	4.2 ± 0.01[b]	3.0 ± 0.01[b]	3.9 ± 0.03[b]	4.2 ± 0.8[b]	10.7 ± 0.1[a]
Energy (KJ per g)[3]	18.1	20.4	18.9	20.2	15.9
Ingredients (g/100g diet)					
Fishmeal[5]	35	25	35	25	-
Insect meal[6]	-	10	-	10	-
Corn meal	30	30	-	-	-
Grape marc[7]	-	-	30	30	-
Seaweed (dry) *Macrocystis pyrifera*[8]	4	4	4	4	-
Starch (Native maize flour)[9]	10	10	10	10	-

Data are represented by means ± standard deviation of three technical replicates. Significant differences are represented by different alphabetic superscripts (Tukey test, $p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

[1]Carbohydrate proportion was calculated by difference 100 – (moisture + protein + lipid + ash).

[2]Carbohydrate was determined using reducing sugar method Anthrone.

[3] Total energy was calculated based on the physiological values at 5.6 kCal g^{-1} protein, 9.5 kCal g^{-1} lipid and 4.1 kCal g^{-1} carbohydrates (Cho et al. 1982).

[4] Commercial feed used was Marifeed S34.

[5] Fishmeal supplied by Sandford, NZ.

[6] Insect meal supplied by Mahurangi Technical Institute (MTI).

[7] Grape marc supplied by Bragato Research Institute, NZ.

[8] Seaweed (*Macrocystis pyrifera*) supplied by Southern Clams.

[9] Starch supplied by New Zealand Starch.

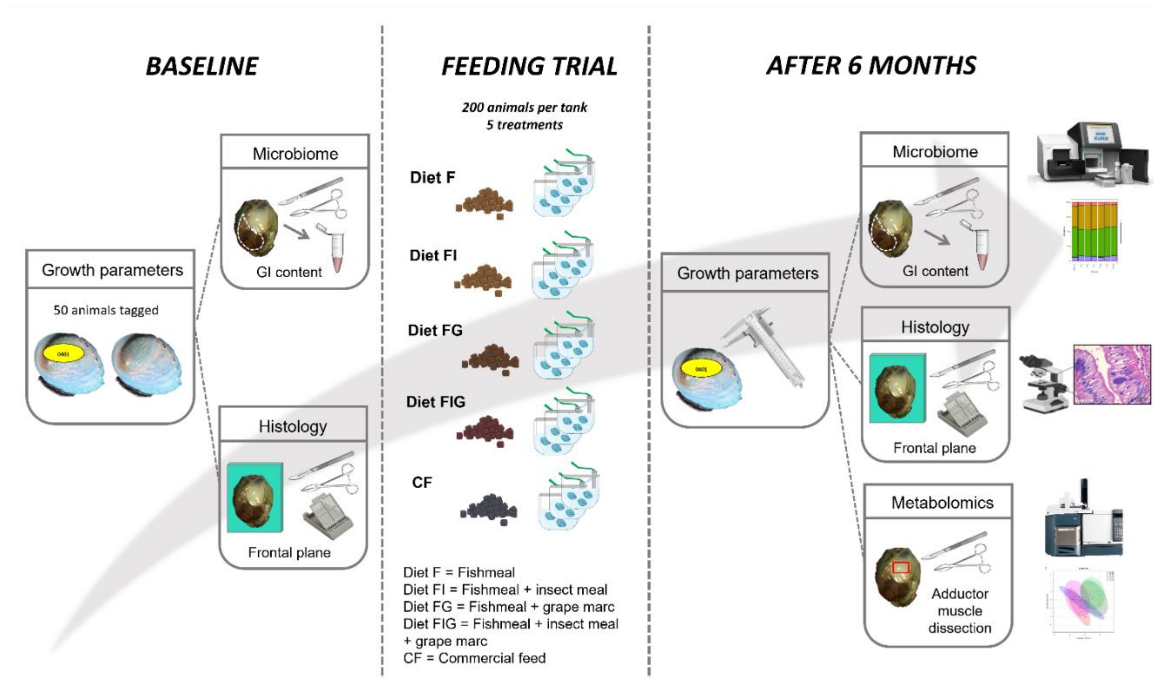


Figure 7.1 Summary of the experimental design and the experimental workflow

7.3.3 Growth parameters

Tagged abalone were measured in their maximum shell length and width using a vernier calliper (Mitutoyo 0–125mm, Warwickshire, UK) to the nearest 0.1 mm, and total and wet weights were measured with a digital balance to the nearest 0.1 g.

The growth performance was evaluated using the following parameters:

Abalone specific growth rate was calculated in total weight, shell length, and shell width as per equations:

Specific growth rate total weight

$$SGR\ TW\ \%\ day^{-1} = 100\ [(\ln final\ TW - \ln final\ TW) \div 165\ days\ ]$$

Specific growth rate shell length

$$SGR\ SL\ \%\ day^{-1} = 100\ [(\ln final\ SL - \ln initial\ SL) \div 165\ days]$$

Specific growth rate shell width

$$SGR\ SW\ \%\ day^{-1} = 100\ [(\ln final\ SW - \ln initial\ SW) \div 165\ days]$$

Where *ln final* is the natural log of the final total weight (TW), shell length (SL) or shell width (SW) and *ln initial* is the natural log of the initial TW, SL, and SW.

Daily increment in shell length (DISL, in μm d^{-1}) was calculated according to Dlaza et al. (2008) as per equation:

$$DISL\left({}^{\mu m}/_{day}\right) = 1000\ x\ \left(\frac{SL_f - SL_i}{165\ days}\right)$$

Where SL_f is the final shell length and SL_i is the initial shell length.

Muscle yield (%) as per the following equation:

$$Muscle\ yield\ (\%) = (muscle\ weight\ \div\ total\ body\ weight)\ x\ 100\%$$

Soft body: shell ratio (SB/S ratio) as per the following equation:

$$SB/S\ ratio = \ (Soft\ body\ weight\ \div\ shell\ weight)$$

7.3.4 Microbiome sampling

Sample collection

Abalone bodies were disinfected with ethanol 70% and phosphate buffer solution (PBS) to remove the surface biofouling. Stomach, digestive gland, and radula were aseptically excised from the abalone using sterile forceps and placed in 2 mL cryovials (Biostor™) with 0.5 mL RNA stabiliser. Vials were immersed in liquid nitrogen for 10 min and stored in -80°C freezer until processed for DNA analysis.

Genomic DNA extraction and Amplicon Sequencing

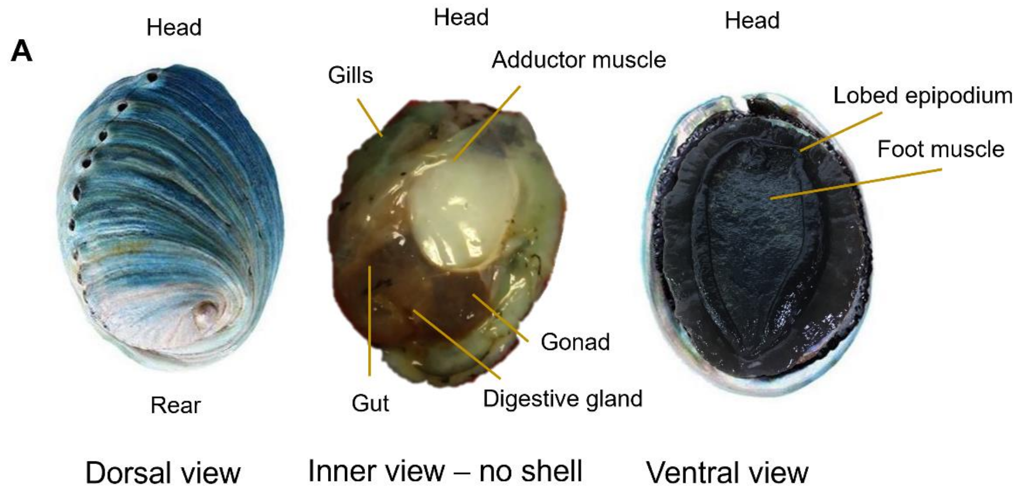
The laboratory workflow for 16S rRNA (V4 region of the gene) amplicon libraries followed the protocols published by Li et al. (2022) and consisted of four main steps. First, genomic DNA (gDNA) of abalone's gut content were extracted with the DNeasy PowerSoil Pro Kits (Qiagen, Category No. 12888-100, Germany) and quantified with a Qubit™ dsDNA HS Assay Kits (Thermo Fisher Scientific, Catalog No. Q32854). Second, gDNA was normalized to 4 nanograms/microliter (ng/µL) with molecular grade nuclease-free water and amplified through polymerase chain reactions (PCR) with a set of indexed 16S rRNA markers. The PCR amplification was conducted in triplicates, and subsequently they were pooled, purified with a customized bead solution, normalized to 2.5 ng/µL, and mixed into

one microcentrifuge tube for library quantification via the Qubit assays and the Bioanalyzer High Sensitivity DNA Kit (Agilent, Catalog No. 5067-4626). Finally, the quality-controlled sample libraries were sequenced on an Illumina *MiSeq* platform using the V3 (600-cycle) sequencing kits (Illumina, Catalog No. MS-102-3003).

7.3.5 Histological evaluation of intestinal samples

Specimens were sectioned transversally cutting approximately 5 mm thick slices to maximise the chances of obtaining the required tissues (gills, foot, mantle, digestive tract, digestive gland, and left and right kidney) (Figure 7.2B). The sections were placed in histology cassettes prior to fixation using a 4% formaldehyde solution (1:9 v/v, 37% formaldehyde: filtered seawater) for 48 h. Histological cassettes were then immersed and stored in 70% ethanol until the slides were processed. Samples were sent to Gribbles Veterinary pathology, Christchurch, New Zealand for histological processing. In brief, samples went through the routine process of dehydration, embedding in paraffin and sectioning using a rotary microtome, and staining using hematoxylin and eosin (H&E).

The tissues were examined under a compound light microscope, Olympus BX40, at magnifications of 40x, 200x, 400x and 1000x and images were captured using cellsens (Olympus cellsens Standard 3.1 [build 21199]). Each of the tissues was screened using semi quantitative criteria as described by Bignell et al. (2008); Costa et al. (2013) and Knowles et al. (2014) where deviation from normality was scored as: 0 No impact, 1: Mild, 2: Moderate, and 3: Severe impact on structural architecture of the tissue types when compared to baseline samples. For targeted gastrointestinal tract observations in histological section of abalone, observations were made on the mucosal layers' goblet cell types, alterations to the epithelial layers of the intestinal tract as well as the haemocytes in the connective tissues. The epithelial layer of the stomach and the posterior intestinal tract (rectum) were measured using the cellsens software (as above). A standardised section of stomach and rectum epithelium was selected and three measurements per individual were acquired. The three measurements were then averaged to produce a single measurement per individual (Figure 7.3B). Those individuals that did not have the same section in the same orientation were not selected or measured.



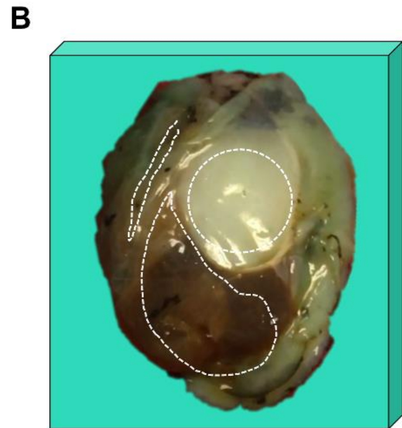
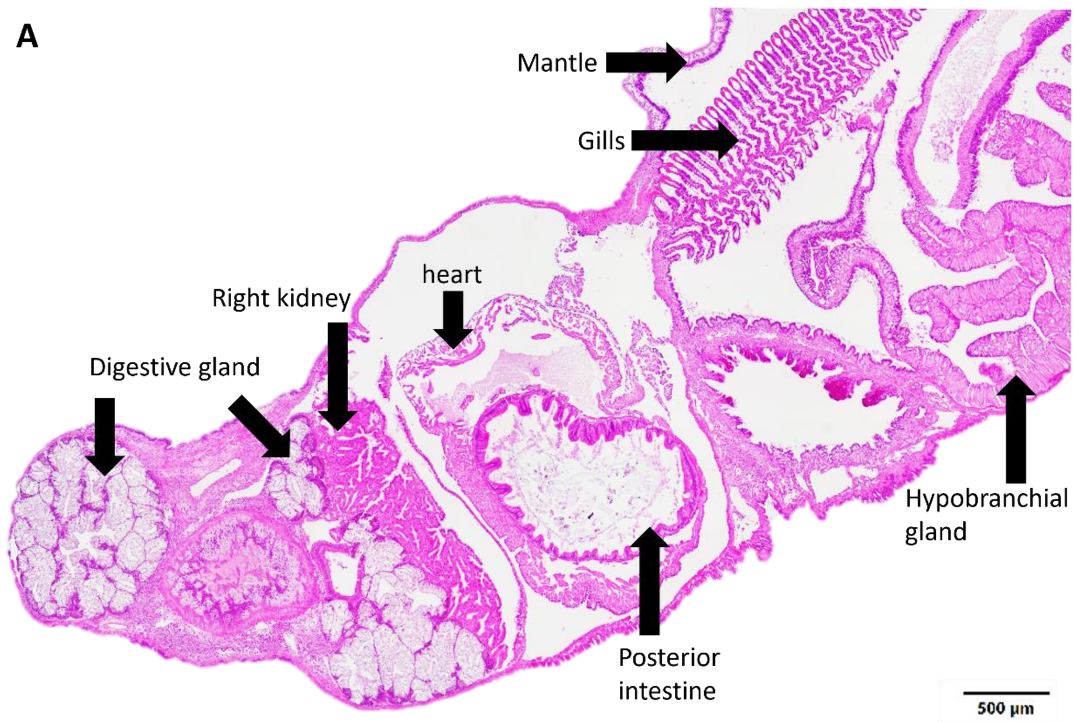


Figure 7.2 A) General diagram (juvenile abalone) indicating parts of interest for *H. iris*. B) Transversal cut plane for histological sectioning for *H.iris*.



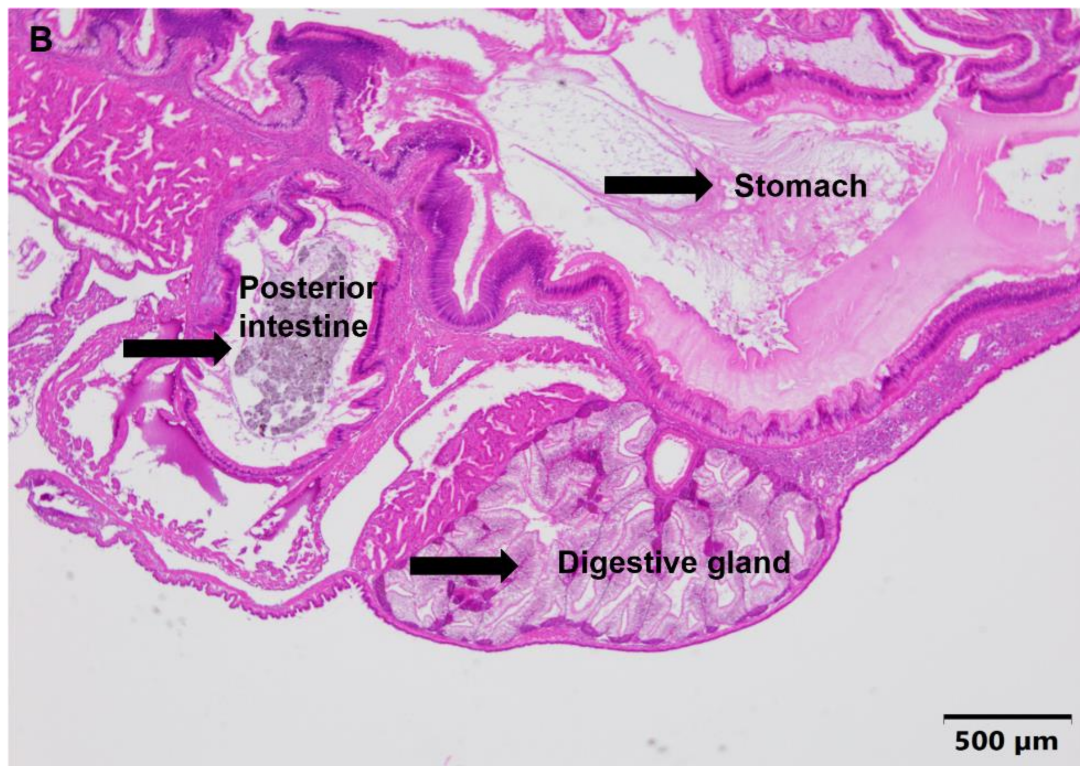


Figure 7.3 A) Histological image of tissues scored for *H. iris*, not shown in this image is the foot. B) location of epithelial measurements.

7.3.6 Metabolite profile

Adductor muscle tissue of approximately 1 cm^2 was obtained from abalone from each treatment. Before analysis, tissues were lyophilised for 48 h using a freezer dryer (-80 °C, 0.001 mbar, Christ alpha series freeze dryer, Osterode am Harz, Germany) and pulverised using a mortar and pestle.

Metabolite extractions were performed using the cold-methanol water method (Nguyen et al., 2018; Villas-Bôas et al., 2011). Briefly, 7-8 mg of powdered tissues were mixed with 20 uL of d_4-alanine (10 mM) as an internal standard. Extractions were performed using 400 µL of cold methanol-water solution (50% v/v, 80% v/v, respectively). The supernatants were collected and dried in a SpeedVac (Labconco, USA) concentrator for 6 hours.

Dried samples were derivatised using methyl chloroformate (MCF) (Nguyen et al., 2018). Derivatized samples were transferred into 2 mL amber GC-MS glass vials fitted with 300 µL inserts with bottom springs (Sigma-Aldrich, St. Louis, MO, USA) and then analysed on an Agilent 7890B gas chromatograph (GC) coupled to an Agilent MSD5977A mass spectrometer detector (Agilent Technologies, CA, USA), with an electron ionisation (EI) source operated at 70 eV. The system was equipped with a ZB-1701GC capillary column (30 m x 250 µm internal diameter x 0.15 µm film thickness with a 5 m guard column) (Phenomenex, Torrance, CA, USA). The instrument parameters were set according to Smart et al. (2010). Helium was used as the carrier gas and was held at a constant flow of 1 mL per min.

Different types of quality controls (QC) were used to guarantee reproducibility of GC-MS measurements, including d_4-alanine, blank samples, and pooled biological QC samples from all samples after extraction as previously described by Nguyen et al. (2021) with small modifications. Blank samples contained only 20 µL of 10 mM d_4-alanine. Blank samples and pooled QC samples were extracted and derivatized with the other samples. For QC purposes, chloroform solvent and non-derivatized n-alkanes (C10–C40) were injected at the beginning of the analysis, followed by pooled QC samples, and blank. A random fashion injection of samples after QCs was followed. Injections of pooled QCs were repeated after every 5 samples. On the final day of the analysis, all pooled QC samples were run again to compare with the previous days.

7.4 STATISTICAL ANALYSES

Growth data were analysed by one-way analysis of variance (ANOVA) when a normal distribution was found (Kolmogorov-Smirnov test, $p < 0.05$), followed by pairwise comparisons with Tukey's post-hoc test. Nonparametric Kruskal Wallis test was used when data did not follow a normal distribution ($p < 0.05$), followed by Dunn post-hoc test for multiple comparisons. Homogeneity of variances was analysed using Levene's test when samples followed a normal distribution ($p < 0.05$). The fixed factor was dietary treatment (diet F, FI, FG, FIG, and commercial feed), and the random factor was tank. Significant differences were evaluated using the statistical software XLSTAT 2022.3.1 (Addinsoft, New York, USA).

For histology, chi square test was used to determine the difference in the width of stomach epithelium and intestine epithelium ($p < 0.05$). For microbiome, data were processed using established amplicon DNA sequence analysis workflow (Archer et al. 2020). Briefly, quality DNA sequence data were generated following a modified DADA2 and cutadapt v3.4 (Martin 2011) pipelines. The SILVA nr v134 database was used for the taxonomic assignments of the unique amplicon sequence variants (ASVs). Comparisons of ASV and relative abundance at the microbial phylum level across dietary treatments before and after the feeding trial was performed using Mann-Whitney and Kruskal Wallis test ($p < 0.05$).

For metabolomic profiling, statistical analyses were performed using the integrated web-based platform MetaboAnalyst 5.0 (metaboanalyst.ca). Data were normalized by auto-scaling (mean-centred and divided by the standard deviation of each variable). Data were normalized by weight and internal standard. One-way ANOVA ($p < 0.05$; post-hoc analysis: Fisher's LSD) was used to compare effects of different dietary treatments on abalone metabolite profiles. Chemometric analysis via partial least squares-discriminant analysis (PLS-DA) was performed to assess the discrimination between dietary treatments and to facilitate visualization of the major trends. A heatmap of detected metabolites in adductor muscle samples was generated to visualize variations among treatments.

7.5 RESULTS

7.5.1 Growth performance

The inclusion of insect meal, grape marc, and both did not significantly affect the

specific growth rates in weight and length of abalone Table 7.2 compared to the diet free of insect meal and grape marc (diet F). However, there were significant effects on the shell width and shell length increment per day among the experimental diets. In addition, the inclusion of insect meal and grape marc did not significantly affect the muscle yield and the soft body/ shell ratio (SB/S).

Table 7.2 Growth indicators in *H. iris* fed on four experimental diets and a commercial feed.

	Diet				
Growth indicators	F	FI	FG	FIG	Commercial feed
SGR Total weight (%)	0.41 ± 0.1^{b}	0.41 ± 0.13^{b}	0.41 ± 0.13^{b}	0.42 ± 0.13^{b}	0.63 ± 0.11^{a}
SGR Shell length (%)	0.15 ± 0.07^{b}	0.16 ± 0.06^{b}	0.14 ± 0.05^{b}	0.14 ± 0.05^{b}	0.27 ± 0.09^{a}
SGR Shell width (%)	0.17 ± 0.09^{b}	0.17 ± 0.08^{b}	0.14 ± 0.05^{c}	0.14 ± 0.05^{c}	0.30 ± 0.13^{a}
Length increment (um day $^{-1}$)	37.7 ± 16.2^{bc}	33.5 ±11.3^{c}	41.9 ± 17.6^{b}	33.5 ± 11.3^{c}	72.6 ± 32.4^{a}
Muscle yield	68.5 ± 2.1^{a}	68.5 ± 2.2^{a}	68.6 ± 2.1^{a}	68.8 ± 2.7^{a}	67.8 ± 2.0^{a}
SB/S ratio	2.2 ± 0.2^{a}	2.2 ± 0.2^{a}	2.2 ± 0.2^{a}	2.2 ± 0.4^{a}	2.1 ± 0.2^{a}

Data are represented by means ± standard deviation. Means with the same superscript (a, b, c) in each column are not significantly different from Tukey post-hoc tests ($p < 0.05$).
Initial weight, length and width were 1.2 g, 21.6 mm, and 14.5 mm, respectively at the beginning of the study (n=750).
Note: Part of this data have been published previously in Bullon et al (2023a).

7.5.2 Microbiome analysis

The total number of ASVs generated from the sequencing reads was 3916 from 65 samples. Comparisons were made in two areas: between the baseline group and commercial feed (before and after the feeding trial) (Figure 7.4A), and among the dietary treatments after the feeding trial (Figure 7.4B). The baseline abalone group (before dietary treatment) possessed 2404 and 2174 total and unique ASVs, respectively. After the feeding trial, the abalone fed the commercial feed possessed 553 and 323 total and unique ASVs, respectively. The free-insect meal/grape marc diet (Diet F) showed the lowest amount of total and unique ASVs (458 and 194) while the diet including grape marc (Diet FG) had the highest number of total and unique ASVs (707 and 304). Although differences were

observed among dietary treatments, they were not significantly different. Only 100 ASVs were shared by all dietary treatments (Appendix Table 7.A.1).

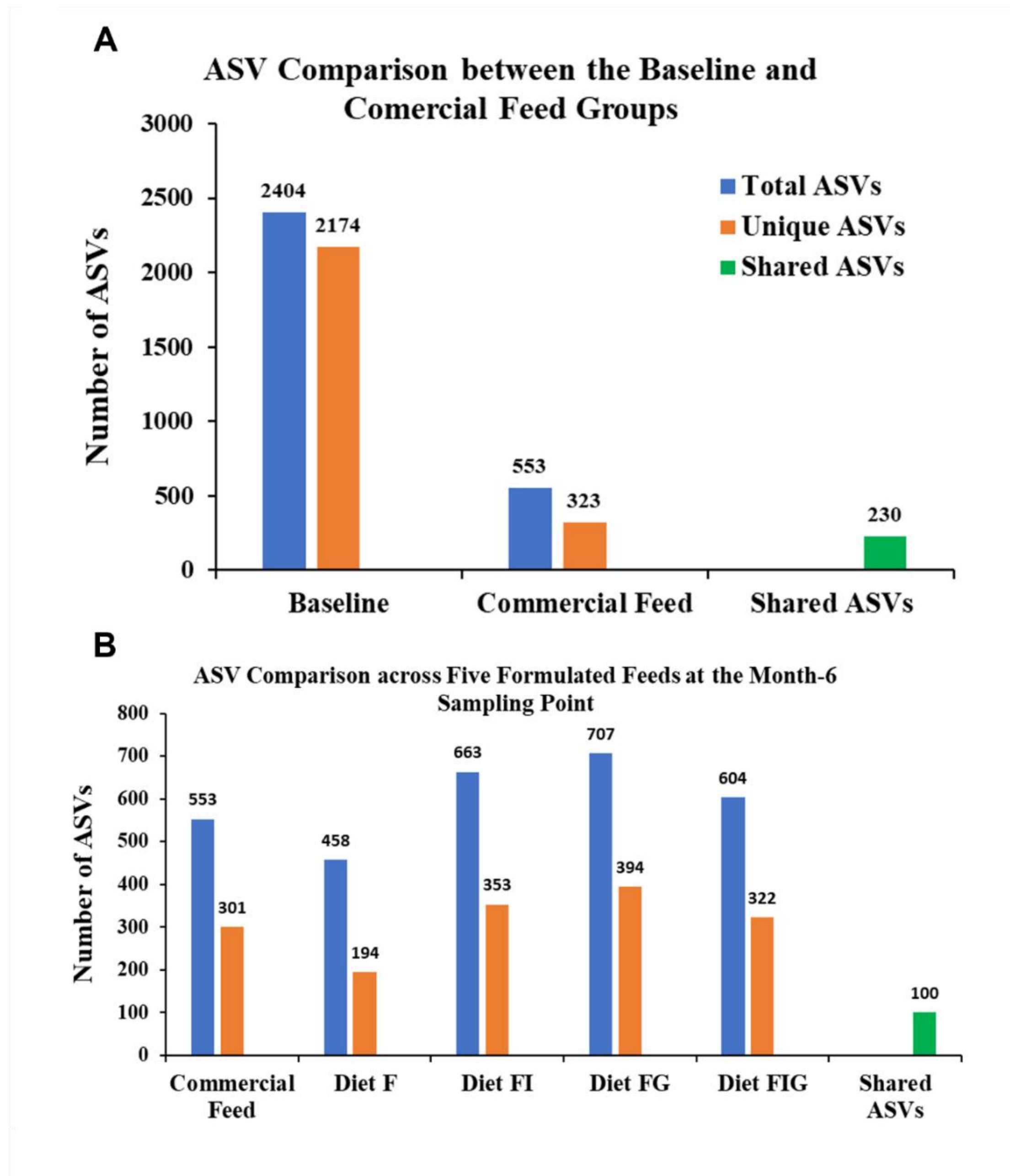


Figure 7.4 A) Comparisons of total, unique, and shared numbers of ASVs between the baseline and commercial feed groups and B) across all dietary treatments after the 165-days feeding trial. Abbreviations: CF, commercial feed; F, fishmeal only source of protein; FI, fishmeal + insect meal; FG, fishmeal + grape marc; FIG, fishmeal + insect meal + grape marc.

The taxonomic assignment returned 33 prokaryotic phyla of which 14 were shared by all diet groups (Appendix Table 7.A.2). Phylum Deinococcota was unique to Diet F, and Phyla Armatimonadota, Dadabacteria, Nanoarchaeota, and NB1-j were only unique to the baseline group. Proteobacteria, Bacteroidota, and Fusobacteriota were the top three bacterial phyla in all dietary treatments. The relative abundance of Firmicutes was high in baseline (before dietary treatment) and the relative abundance of Fusobacteria was high in diets FI and FG (Appendix Table 7.A.2 and Figure 7.5).

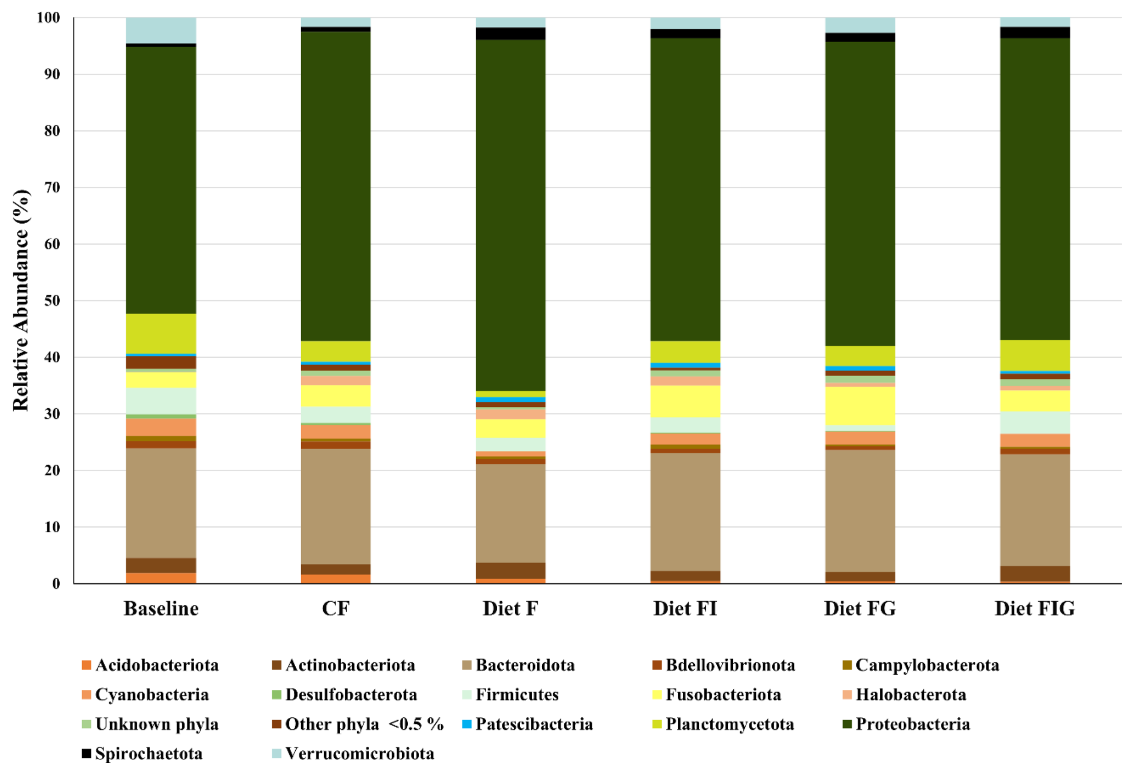


Figure 7.5 Relative abundance (%) of the most abundant prokaryotic phyla recovered from the farmed abalone gut content before and after dietary treatments. Abbreviations: CF, commercial feed; F, fishmeal only source of protein; FI, fishmeal + insect meal; FG, fishmeal + grape marc; FIG, fishmeal + insect meal + grape marc.

7.5.3 Histology

Histological analysis of the gills, foot, mantle, gonad, digestive tract, and kidneys revealed well-structured tissue architecture (Figure 7.6A). There were no discernible differences in the tissue health and quality from animals among dietary treatments. Several abalone had enlarged digestive gland and right kidney lumina as well as patches of autolysed tissue. This condition did not, however, correspond exclusively with any diet regime. Most of the abalone appeared to have food in the digestive system at the time of sampling (Figure 7.6B). There were no differences in the width of the stomach epithelium ($\chi^2(4) = 5.04$, $p = 0.28$)) and posterior intestine epithelium ($\chi^2(4) = 3.58$, $p = 0.47$) layers of the standardised section among the diet types (Table 7.3).

Table 7.3 Measurement of width of intestine and stomach epithelium of abalone among four experimental diets, commercial feed, and baseline.

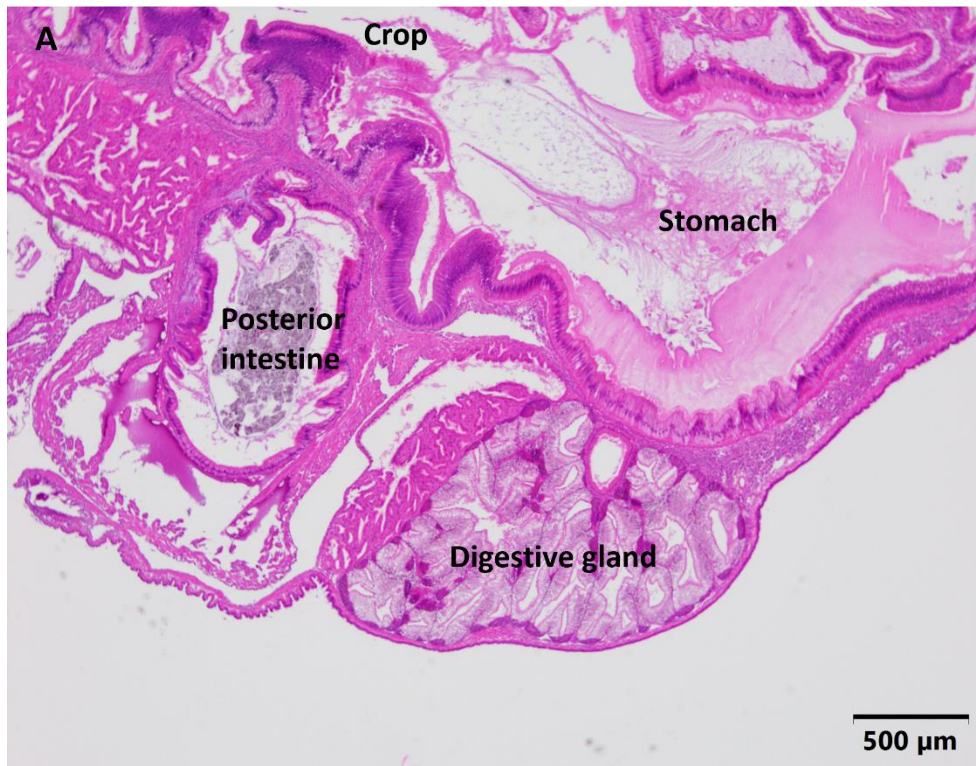
Diet	Average intestine epi thickness (μm)	Std dev	n	Average stomach epi thickness (μm)	Std dev	n
F	43.5	15.7	3	65.2	12.5	11
FI	45.5	12.4	5	68.2	11.7	11
FG	41.0	6.3	4	69.0	8.5	13
FIG	46.0	7.4	4	75.1	7.7	14
CF	40.2	7.5	5	70.7	9.0	10
Baseline	35.5	11.2	2	69.3	5.6	3

Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

The digestive gland tubules were observed to be well structured with healthy epithelial architecture. The baseline samples (before feeding trial) were compared with the samples collected at the end of the experiment to identify any variations in quality of the digestive gland. The baseline samples showed enlarged digestive tubule lumina and increased vacuolation of the epithelial layer, suggesting lower glycogen reserves and appearance of increased spacing of the interstitial spaces (Figure 7.7A and 7.7B, Table 7.4). Those abalone fed the commercial diet (after the feeding trial) appeared to have more

densely packed epithelial layers which produced a more eosinophilic staining appearance, likely due to increased levels of stored glycogen-like and fatty acid-like materials compared to the experimental diets and baseline sampling (before feeding trial). However, this has not been confirmed with other staining techniques, such as periodic acid-schiff (PAS) and PAS-D (with diastase) which specifically identifies glycogen-like substances (Figure 7.7C). Diets FG and FIG (Figure 7.7F, 7.7G and 7.7H) were associated with increased prevalence of vacuolation and depleted glycogen when compared to the other diets (Figure 7.7C, 7.7D and 7.7E). However, there was high variation among the diets as depicted in Figure 7.7G and 7.7H. Varying aspects and conditions of the digestive tissue architecture are described in Table 7.4.

Enlarged tubule lumens, increases in interstitial spaces, vacuolated epithelia (Figure 7.7) as well as thinning epithelia and sloughing can indicate either a potential health condition or general individual variation. Autolysis and sloughing appearance are likely to be an artifact of fixation, and bacteria were not observed in association with autolysed tissue.



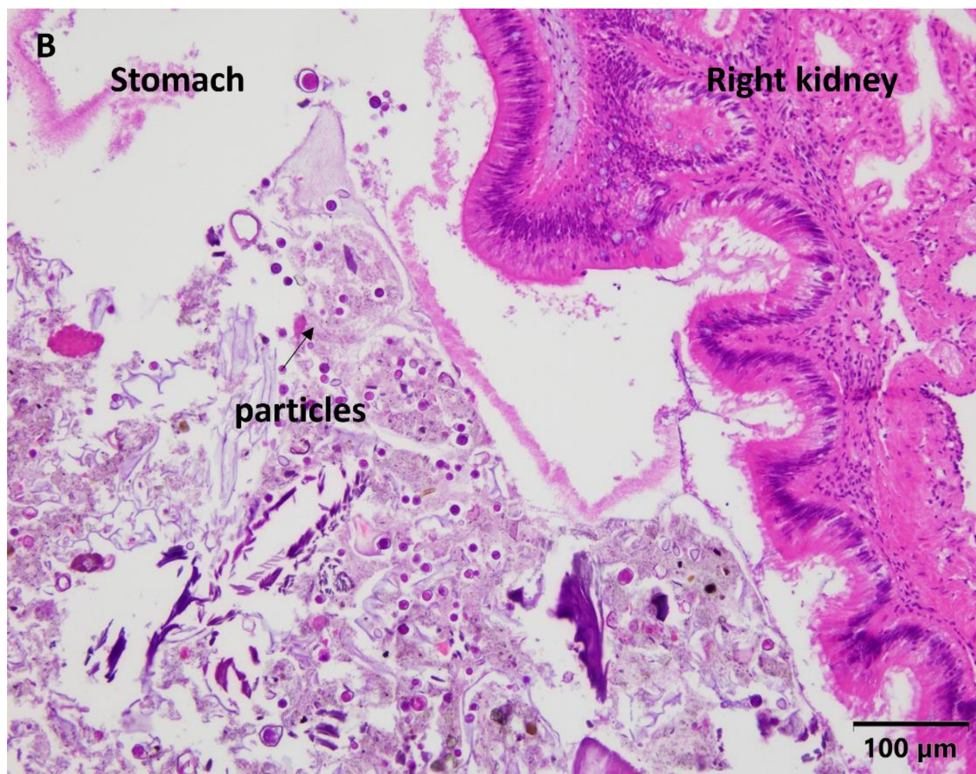


Figure 7.6 Gastrointestinal tract (stomach) regions showing examples of healthy individual *H. iris* from A) Diet F, and B) Example of particles observed in a couple of individuals fed using the encapsulated diets.

Table 7.4 Prevalence of digestive gland conditions among each of the dietary treatments. Data represents % prevalence and rounded to 1 decimal place.

		Diet				
	Baseline	F	FI	FG	FIG	CF
Good tubule architecture	55.6	28.6	20.0	46.7	53.3	33.3
Sloughing into lumen	0.0	0.0	20.0	0.0	0.0	26.7
Increased interstitial space	22.2	7.1	6.7	20.0	0.0	0.0
Enlarged lumina space	11.1	28.6	20.0	13.3	6.7	13.3
Enlarged lumina space and vacuolated	0.0	7.1	0.0	0.0	0.0	0.0
Large patches of autolysis in otherwise healthy tissues	11.1	21.4	20.0	0.0	0.0	26.7
Thinning epithelia	0.0	0.0	6.7	0.0	0.0	0.0
Vacuolated epithelia	0.0	0.0	0.0	20.0	40.0	0.0
No DG tissue available	0.0	7.1	6.7	0.0	0.0	0.0

Abbreviations: Dg = digestive gland. Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

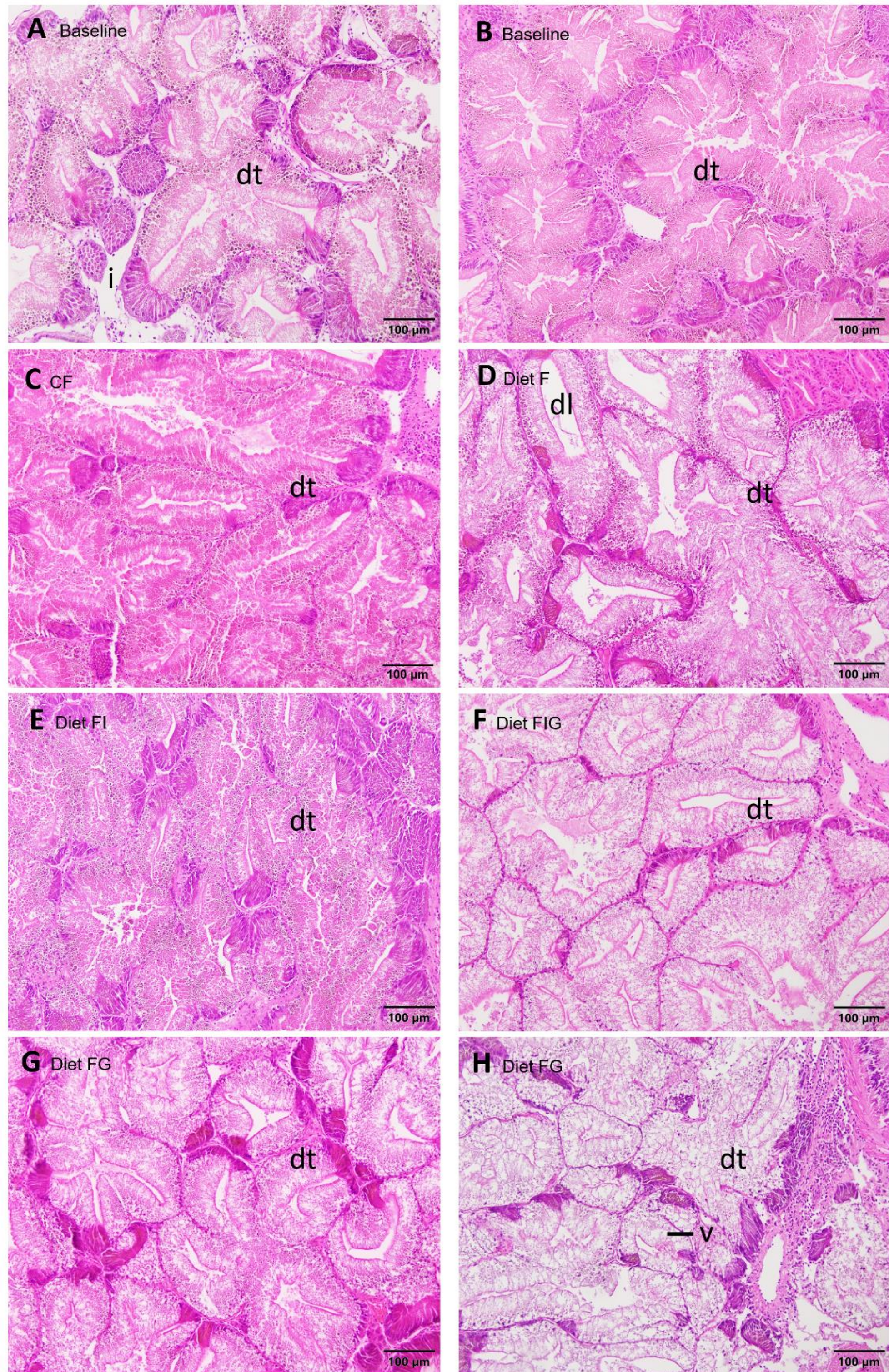


Figure 7.7 Histological images (Hematoxylin and eosin) of digestive gland tissue from *H. iris* fed with experimental diets and a commercial feed (CF) A) Baseline prior to diet treatment individual 1, B) Baseline prior to diet treatment individual 2, C) commercial feed, D) diet F, E) diet FI, F) diet FIG, G) diet FG individual one and H) diet FG individual 2 as an example of the variation within the treatment. Interstitial space (i), digestive tubule (dt), digestive lumen (dl), vacuole (v). Scale 100µm.

7.5.4 Metabolite profiles

Metabolite identification yielded 86 annotated compounds from spectra of abalone muscle tissues. The multivariate data analysis via PLS-DA score plot revealed some clear separations among diets throughout the feeding trial (Figure 7.8A). Overall, samples from diet FIG were clearly distinct from samples from the commercial feed group. However, there was no good separation between diet F, FG, and FI. The first two components contributed 27.1% of the total variation. The PLS-DA model cross validation *via* LOOCV showed accuracy (two components) of 0.74, R^2 of 0.91 and Q^2 of 0.71, indicating a good prediction model. Furthermore, PLS-DA analysis also identified 15 metabolites with VIP scores greater than 1 which are important classifiers (Appendix Table 7.A.3).

The univariate data analysis *via* one-way ANOVA identified 49 metabolite that were significantly different by the diet ($p < 0.05$) and none of them by the tank component (Appendix Table 7.A.4). The greatest difference was observed between diet FIG and commercial feed, with 19 significantly different compounds (increased and decreased) such as myristic acid, linoleic acid, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), aspartic acid, 2-phosphoenolpyruvic acid and α-linolenic acid (ALA). Among the experimental diets, major differences were observed between diet F and FIG, with 28 significantly different compounds. Other significant differences were observed between diet F and FG with compounds such as in myristic acid, DPA, palmitoleic acid, aspartic acid, cystathionine, ALA, linolenic acid (LA), lactic acid and lysine. A heatmap of these metabolites was generated to visualize the detail differences (Figure 7.8B), which divided the metabolites into 3 main clusters (A, B and C). Metabolites in cluster A are mostly amino acids, while most of the fatty acids are in cluster B. Cluster C is a mix of fatty acids, amino acids, and organic acids.

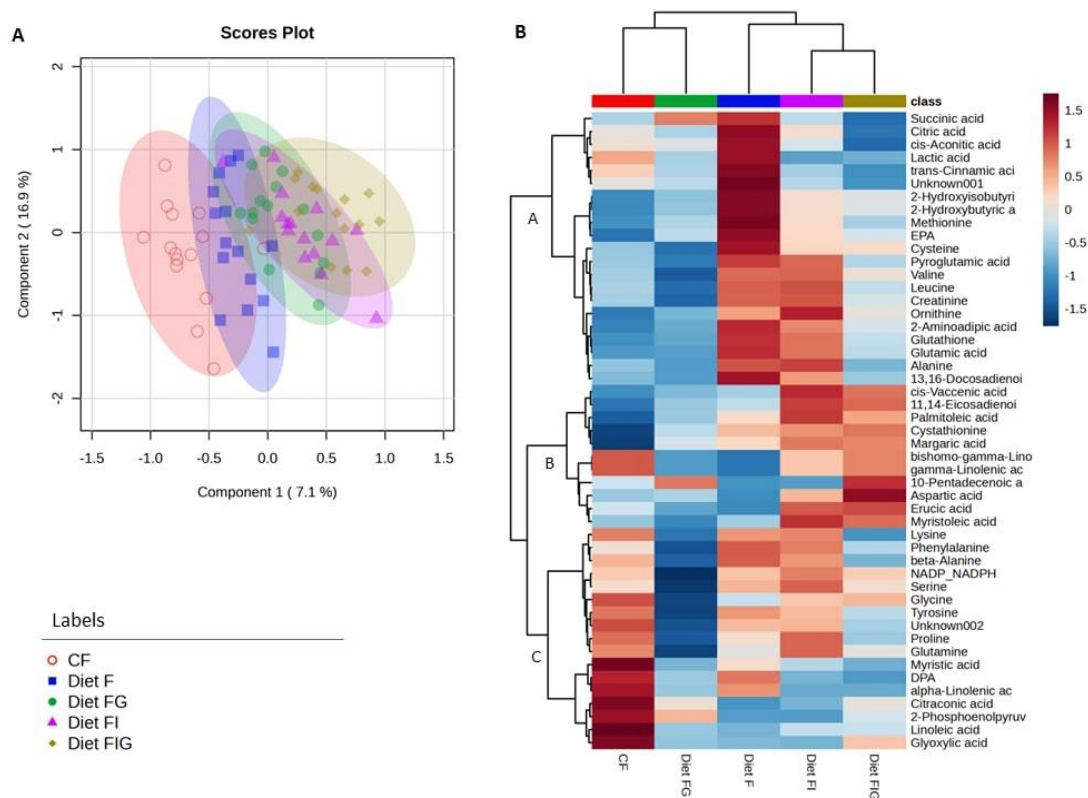


Figure 7.8 Effects of different diets on metabolite profiles of abalone. A) PLS-DA score plot. B) Heatmap of 49 metabolites significantly different among treatments (One-way ANOVA, $p < 0.05$). Cluster A contains mostly organic acids and fatty acids, cluster B mostly fatty acids and cluster C mostly amino acids. Abbreviations: CF: commercial feed, F: fishmeal only source of protein, FI: fishmeal + insect meal, FG: fishmeal + grape marc, FIG: fishmeal + insect meal + grape marc.

The enrichment analysis between diet FIG and commercial feed (the best separated diets based on a PLS-DA analysis) showed that 19 pathways were significantly enriched and impacted by the diets (Figure 7.9 and Appendix Table 7.A.5). Some of these pathways were increased in activity due to the inclusion of both IM and GM (fatty acid synthesis), whereas other pathways were decreased (linoleic acid metabolism, lysine degradation) with commercial feed. The majority of affected pathways were amino acid metabolism. Fatty acid biosynthesis was the most affected pathway while biotin metabolism and linoleic were the most enriched pathways.

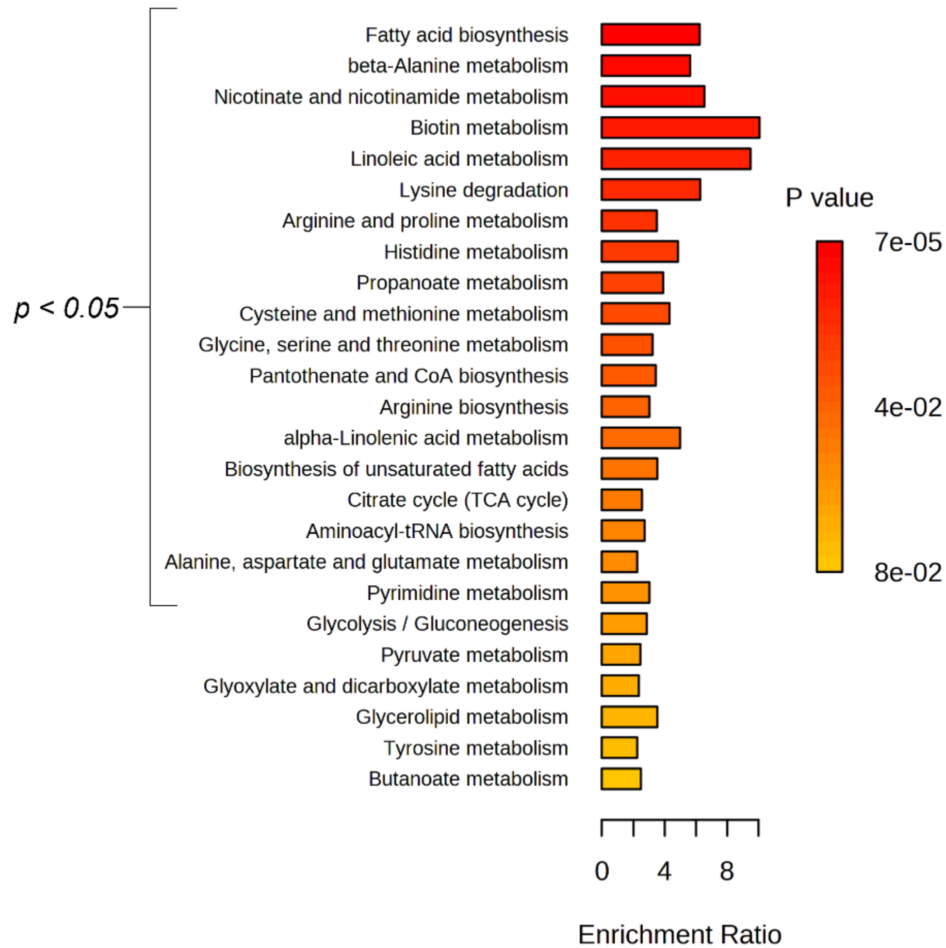


Figure 7.9 List of pathways (top 25) affected in abalone muscle fed on diet FIG and commercial feed.

7.6 DISCUSSION

The purpose of this study was to investigate the partial replacement of fishmeal with insect meal and inclusion of grape marc to promote better health in juvenile *H. iris* within farm conditions. Fishmeal is generally included in abalone feeds at 30-40% to promote faster growth (Bansemer et al., 2016). However, its use in aquafeeds has been questioned mainly due to sustainability issues. In this study, insect meal was included as a more sustainable protein substitute to achieve good nutritional profile, and grape marc was included as a carbohydrate source due to the antioxidant effect and the content of dietary fibre that can

be beneficial for the gut microflora. The inclusion of both ingredients would promote a more sustainable aquaculture in terms of reduced fishmeal use. In addition, it considers the animal's overall health, which integrates the intestinal microbiota interactions and gastrointestinal morphology. An integrated and comprehensive analyses of such factors can be used as a more effective assessment and indicator of fish/shellfish welfare.

The survival was maintained at 99% in all treatments, indicating good abalone overall health despite the ingredient inclusion. The inclusion of insect meal did not affect the protein levels, but affected the reducing sugars, dietary fibre, lipid, and ash content of the experimental diets. The inclusion of grape marc did not affect the protein level, but significantly affected the dietary fibre content, lipid, and ash content of the experimental diets, compared to the fishmeal/grape marc free diet (diet F). The inclusion of insect meal and grape marc did not affect abalone shell length and weight. However, the inclusion of grape marc significantly affected abalone shell width. The muscle yield, and the soft body/shell ratio (SB/S) were similar among abalone fed the experimental diets and the commercial feed, suggesting a similar growth regardless of the inclusion or diet.

The commercial feed was used as a positive control to compare against abalone fed with the experimental encapsulated diets. The limited knowledge of the ingredients on the commercial feed did not allow accurate performance comparison between commercial feed and experimental diets. Considering this, single experimental diets can be compared to one another based on the whole diet, not the ingredient differences. Commercial feeds usually contain highly palatable and digestible dietary ingredients, which include a variety of plant meals, fishmeal, cereal grains, feed enhancers, oilseeds, minerals, and vitamins, which are carefully formulated to optimise growth (Bansemer et al., 2016). For this reason, abalone fed with the commercial feed exhibited significantly superior biomass gain, SGR, and length increment compared to animals fed any of the experimental diets. The SGR (total weight) values of the experimental diets in this study coincide with the ones reported previously by Allen et al. (2006) and Bullon et al (2023b) with around 0.45% and 0.42%, respectively for *H. iris*.

In this study, abalone were fed commercial feed (Marifeed® S34) prior to the commencement of the study (since 6–8 months old). The commercial feed may have shaped digestion and palatability favouring commercial feed. The preference of the diet received after weaning over introduced diets has been documented in abalone (Hernández et al., 2009; Mulvaney et al., 2013). The introduction of experimental diets with alternative

ingredients may have had implications in growth promotion. Further studies need to consider the effect of the previous received diet on the feed intake of introduced diets.

7.6.1 Microbiome

In this study, the gut microbiome of abalone fed with four experimental diets and a commercial feed revealed four dominant bacterial phyla that comprised the majority: Bacteroidota, Fusobacteria, Proteobacteria and Firmicutes. The presence of these phyla coincides with what has been reported for *H. midae* (Erasmus et al., 1997), *H. discus hannai* (Tanaka et al., 2004), *Haliotis tuberculata* (Gobet et al., 2018), and *Haliotis diversicolor* (Zhao et al., 2012).

In this study, results showed that the inclusion of insect meal (diet FI) and grape marc (diet FG) did not significantly increase the diversity of microbial communities compared to the insect meal/grape marc free diet (diet F). One possible explanation for this finding is that the percentage of insect meal and grape marc used in this study may have not contributed to significant changes in the microbiome of abalone. A previous study by Terova et al. (2019) has documented that the percentage of insect meal inclusion determines the significance and degree of positive change in the microbiome of rainbow trout. Another possible explanation is that insect meal from *T. molitor* is highly acceptable by abalone. A previous study has documented that the inclusion of insect meal from the black soldier flies *H. illucens* at 50% replacement increased microbial diversity in rainbow trout and Atlantic salmon (*Salmo salar*) (Bruni et al., 2018; Weththasinghe et al., 2022). highlighting the role of insects in the normal diets of salmonid fish. However, a study by Terova et al. (2021) included insect meal from *T.molitor* at 100% replacement, without significant differences in the microbial diversity and richness in the gut of rainbow trout. These differences may be associated with nutritional profiles of the insect species themselves (Antonopoulou et al., 2019), and the fact that they possess their own microbiomes.

The inclusion of insect meal and grape marc produced an increased relative abundance of Fusobacterium and decreased abundance of Proteobacteria (Table 7.A.1). The high levels of indigestible fibre, such as chitin in insect meal and lignin in grape marc, may have contributed to these fluctuations as bacteria in Fusobacterium phyla have been associated with complex polysaccharide fermentation (Gobet et al., 2018) and the production of short chain fatty acids (SCFAs) (Levy et al., 2017; Parker-Graham et al., 2020). Microbial SCFAs are metabolic end products of microbial fermentation which enhance the renewal of gut epithelia (Pardesi et al., 2022; Vielma & Lall, 1997), regulate the diversity of

intestinal microbiota (Z. Li et al., 2019), and reduce intestinal inflammation in aquatic species (Liu et al., 2019). Chitin, the prebiotic oligosaccharide found in insect meals (Banerjee et al., 2021; Vogel et al., 2018) may have contributed to a more diverse bacterial microbiome in those abalone fed diets including insect meal, which were also found to be reduced in Proteobacteria, compared to diet F (insect meal/ grape marc free diet). In fact, chitin has been shown to have a significant effect on the reduction of pathogenic bacteria belonging to Proteobacteria, such as *Vibrio* (Rimoldi et al., 2021; Terova et al., 2019), *Shewanella, Aeromonas, Citrobacter*, and *Kluyvera* (Cai et al., 2006; Jiang et al., 2013; Rimoldi et al., 2019).

In this study, the inclusion of both insect meal and grape marc (diet FIG) promoted a relative increase in Firmicutes abundance compared to an insect meal/grape marc free diet (diet F). Previous studies have documented that dietary insect meals have promoted more abundance of Firmicutes in rainbow trout, mostly associated with beneficial lactic acid bacteria species, such as *Lactobacillus* and *Bacillus* (Rimoldi et al., 2021). Lactic acid bacteria increase the digestibility of indigestible products producing higher levels of short chain fatty acids in the digestive tract of aquatic animals (Burr et al., 2008; Soltani et al., 2019) and have antimicrobial effects (Newaj-Fyzul et al., 2007; Ringø et al., 2012). In addition, the polyphenols available in grape marc (anthocyanins, catechins, flavonols, and phenolic acids) have been shown to contribute to increase beneficial bacteria in rainbow trout, such as *Bifidobacterium* and *Lactobacillus* (Pulgar et al., 2021; Zorraquín et al., 2020). In previous studies, the increased presence of Firmicutes has been linked to higher levels of butyrate, which is the most preferred short chain fatty acid used by colonocytes, promoting growth in sea bream (Robles et al., 2013).

The results in this study showed general trends on microbial diversity across different dietary treatments with and without insect meal and grape marc. Further studies which include genus or species taxonomic classification are recommended for further association between beneficial bacterial species and alternative ingredients.

The results in this study showed that animals had significantly higher diversity of microbial communities before the feeding trial (2404 ASVs) compared to after the feeding trial (553 ASVs) in the commercial feed treatment. The relative abundance after the feeding trial showed a decreased abundance of Bacteroidota, Firmicutes, and Proteobacteria and varying levels of Fusobacteria compared to the baseline samples (before feeding trial). Although variations were present, the predominance of these phyla remained over time. In

this study, Proteobacteria has been identified as the predominant bacterial phylum, corroborating their importance in carbohydrate metabolism and digestibility of nutrients in *Haliotis* species (Choi et al., 2021; Gobet et al., 2018; Parker-Graham et al., 2020; Wang et al., 2021; Zhao et al., 2018). The increase in Proteobacteria has been associated with an attempt of abalone to improve feed efficiency (W. Yu et al., 2022) and the biosynthesis of amino acids (Xiong et al., 2022) to expedite abalone grow (Zhao et al., 2012). The differences found before and after the feeding trial are not conclusive and may be explained by additional factors that shape the intestinal microbiome in aquatic animals, such as seasons (Danckert et al., 2021; Gobet et al., 2018), developmental stage (Zhao et al., 2012), and the quality of the surrounding seawater (Medina-Félix et al., 2022). Further investigations are needed to reveal the contribution of these components on the microbiome of abalone.

7.6.2 Histology

In this study, no inflammatory and/or degenerative changes were observed in any of the dietary treatments. The presence of antinutritional factors in insect meals, such as phytate, tannins, and polyphenols and lignin in grape marc may not have caused any signs of intestinal disease at the percentage included in the experimental diets. Antinutritional factors (ANF) are substances inherent to some ingredients that interfere with food utilisation, causing intestinal inflammation and affecting the health of animals (Francis et al., 2001) and constitute one of the main obstacles for inclusion of alternative ingredients in aquafeeds. Insect meals have been reported to have low levels of antinutritional factors (ANFs) compared to plant-based proteins, such as tannins (affect protein utilisation and digestion), oxalate, and phytate (which affect mineral utilisation and digestion) (Francis et al., 2001; Idowu et al., 2019). Therefore, inclusion of insect meals up to 75% in aquafeeds has demonstrated no gastrointestinal inflammatory effects for clownfish (*Amphiprion ocellaris*) and Atlantic salmon (Lock et al., 2016; Vargas-Abúndez et al., 2019). Conversely, grape marc is physically or chemically treated to remove potential antinutrients, such as lignin, which interfere with nutrient digestion (Moate et al., 2014) and reduce feed intake (Amyot et al., 2018). In this study, the inclusion of grape marc did not cause significant changes of the gastrointestinal epithelia and digestive gland. Therefore, it is presumed that percentages of 30% of grape marc can be included in abalone diets without further intestinal compromise.

In this study, insect meal replaced fishmeal to a maximum of 10%, thus maintaining fishmeal as the primary protein source in all experimental diets. This proportion of fishmeal

may have provided enough nutritional requirements to maintain a healthy gut condition. In diets where grape marc was included, only corn meal was completely replaced, but the main protein components (insect meal, fishmeal, or both) were maintained. Therefore, although insect meal and grape marc are foreign ingredients in the abalone diet, the levels included in our study may have been low enough to cause no detectable inflammation or disrupted intestinal morphology. Abalone fed on diets with grape marc may have adapted to this ingredient without much difficulty, considering that there was a proportion of digestible carbohydrates in the whole formulation. Based on to the above-mentioned studies, there is evidence to support that the final mixture of ingredients and nutrients, regardless of the antinutritional factors may provide a balanced and positive aquafeed nutritional profile for growth.

A critical factor in the development of aquafeeds is the level of inclusion of alternative ingredients. For instance, studies have shown that when soybean meal is included in high levels (more than 50% of the protein component), signs of enteritis can appear in the distal intestine of the rainbow trout (Kumar et al., 2021). Conversely, when a mixture of plant-based protein replaces a maximum of 30% fishmeal, there are no signs of intestinal inflammation in the European sea bass (*Dicentrarchus labrax*) (Bonvini et al., 2018). Compared to plant-based meals, insect meals seem to be more suitable to aquatic nutrition due to the presence of essential fatty acids (Belforti et al., 2015) and immunomodulatory factors, such as chitin (Ringø et al., 2012) which contribute to reducing proinflammatory substances in the intestinal tract of aquatic species (Kumar et al., 2021; Y. Li et al., 2019).

Another possible explanation for the absence of gastrointestinal morphology differences among diets may be that abalone adapt easily to nutritional sources. Abalone are highly adaptable species which, due to their limited capacity to move geographically, need to adapt quickly to available sources of food to survive (Agrawal, 2001; Schaefer et al., 2013). Since the digestive gland (site of nutrient absorption and storage), and the epithelial layer (provides insights into potential nutrient deficiencies) did not present any significant changes, it can be concluded that the digestive tract of abalone responded effectively to the introduction of insect meal and grape marc.

The stomach and the crop were targeted in histological observations due to their involvement in feed digestion. Typically, macroalgae are partially digested in the crop before moving into the stomach and spiral caecum. Although the stomach has half the capacity of the crop it has the same peristaltic contraction to move food to the caecum and intestine.

The stomach was targeted due to its proximity to both the digestive gland and the intestine, its ability to move food into the caecum and as the main site of digestion (Campbell, 1965, Crofts, 1929, Kemp 2001). The intestine was selected to observe whether there was any fibrous material making it through the whole gut tract and whether this was causing any damage. Additional sampling as the abalone developed (i.e., 1 year old) would have been beneficial to observe any future changes from the dietary alterations. Although these epithelial layer measurements can be subjective, they are worth recording to build a knowledge base for future research. Further studies that evaluate gradual increments of insect meal and grape marc on gastrointestinal morphology are recommended.

7.6.3 Metabolite profiles

The GC-MS based metabolomics approach revealed significant differences in metabolic profiles of abalone (adductor muscle) fed on experimental diets and a commercial feed. The dietary effects resulted in significant 49 metabolites and the enrichment analysis identified 19 pathways that were significantly impacted between diet FIG compared to commercial feed. This reflects the tissue-specific response of abalone to new dietary ingredients (Grandiosa et al., 2018; Venter et al., 2019; W. Yu et al., 2022). The up and down regulation of metabolites provide metabolic signatures of the relevance of fatty acid and amino acid products and metabolism in abalone growth and gut health.

In the present study, it was observed that the metabolite profile among the experimental diets varied significantly with the inclusion of insect meal and grape marc. The metabolite profiles of animals fed on insect meal (diet FI) showed significantly higher levels of palmitoleic acid and myristoleic acid compared to animals fed on the free insect meal/grape marc diet (F). The elevated levels of these compounds in abalone muscle may be associated with elevated levels of the fatty acid precursors, which are abundant in insect meals, such as palmitic acid and myristic acid (Belforti et al., 2015; Gasco et al., 2016; Sankian et al., 2018). The presence of myristoleic and palmitoleic acid in the abalone muscle fed on the insect meal diet (FI) may also be attributed to the abalone's ability to desaturate the precursors myristic and palmitic acid (Tocher, 2003), which are highly abundant in *T. molitor* species (Belforti et al., 2015; Dreassi et al., 2017).

The feed containing insect meal promoted elevated levels of proline, glutamic acid, aspartic acid, and erucic acid. The elevated levels of proline in diet FI support the fact that when dietary lipids are high (in diets with insect meal), proline regulates fatty acid oxidation (Venter et al., 2019). The high levels of aspartic acid and glutamic acid in diet FI may suggest

an increase in amino acid catabolism caused by insect meal inclusion, promoting the conversion from aspartate to glutamate, and glutamate to alpha-ketoglutarate (Fabrikov et al., 2020). As catabolism is increased, the utilisation of glucogenic amino acids (arginine, glutamate, glutamine, glycine, histidine, methionine, proline, and serine) to produce energy is possibly heightened and therefore found to be reduced in abalone muscle (Jarak et al., 2018; Venter et al., 2018). Reduced levels of these amino acids may suggest that abalone is using the low levels of digestible carbohydrates found in diets FI and FIG for energy metabolism. The utilization of glucogenic amino acids for energy may have contributed to a slow growth, as amino acids are ideally allocated for growth rather than energy fuel.

The insect meal/grape marc free diet (F) has shown to produce high levels of essential amino acids, such as methionine, valine, leucine, alanine, lysine, phenylalanine, intermediates of the tricarboxylic citric acid (TCA), such as succinic acid and citric acid and important fatty acids, such as eicosapentaenoic acid (C20:5n-3, EPA) and docosapentaenoic acid (DPA). Abalone fed on diet F showed elevated levels of these compounds, which may be due to the high levels usually present in fishmeal. However, the growth rates were similar to the other experimental diets that included insect meal and grape marc. This finding may corroborate that growth in abalone species is not limited to essential amino acid provision. As abalone is an herbivorous species, its metabolism is designed to mainly digest carbohydrates (Thongrod et al., 2003; Wang et al., 2009), moderate levels of protein and low levels of lipids (Lee et al., 2019). Abalone do not possess enzymes to metabolise fatty acids, and therefore an excess of dietary lipids might translate into slow growth (Lee et al., 2019).

The results showed that the accumulation of alpha-linolenic acid (C18:3n-3, ALA) and linoleic acid (C18:2n-6, LA) in adductor muscle of abalone fed commercial feed may have been linked with high levels of dietary digestible carbohydrates, resulting in animals with faster growth. As the lipid metabolism is closely involved with that of carbohydrates, an excess in carbohydrate consumption results in the formation of acetyl CoA which is the substrate for synthesizing fatty acids (Wang et al., 2009). The diets with substantially higher levels of digestible carbohydrates (F and CF) promoted abalone muscle with higher levels of docosapentaenoic acid (C22:5n-3, DPA), alpha-linolenic acid (C18:3n-3, ALA), gamma-linolenic acid (C18:3n-6, GLA), and linoleic acid (C18:2n-6, LA). Many of these fatty acids and their derived lipids, such as arachidonic acid (C20:4n-6, ARA), docosahexaenoic acid (C22:6n-3, DHA), and eicosapentaenoic acid (C20:5n-3, EPA) cannot be synthesised *de novo* by abalone and play a critical role in cell signalling, gene expression (Liu et al., 2023),

growth promotion, and overall health (Bautista-Teruel et al., 2011; Mai et al., 1996; Toledo-Agüero & Viana, 2009). Studies have shown that animals with higher growth upregulate fatty acid metabolism (Venter et al., 2022). However, further elucidation on the effect of specific dietary fatty acids on abalone is still limited.

7.7 CONCLUSIONS

The findings of this study indicate that the inclusion of insect meal and grape marc did not significantly affect the diversity of gut bacteria of abalone. In addition, the inclusion of insect meal and grape marc increased the relative abundance of gut bacteria possibly associated with the promotion of beneficial species in the gastrointestinal tract. The absence of signs of inflammation in abalone fed with both alternative ingredients suggest that abalone have a natural ability to utilise these substrates to maintain a healthy condition comparable to commercial feed. Moreover, the metabolite profile showed increased essential and non-essential fatty acid synthesis, which may be associated with higher growth rates attained with commercial feed. The inclusion of insect meal and grape marc did not decrease growth compared to diets without insect meal and grape marc reinforcing that these alternative ingredients can be included in abalone diets at 10% and 30% respectively, without affecting intestinal health and growth. These findings have significant implications for understanding the benefits of alternative ingredients in abalone diets, resulting in a more affordable final price and lower carbon footprint

7.8 APPENDIXES

Table 7.A.1 Number of Amplicon Sequence Variant (ASVs) recovered from the farmed abalone gut content before and after dietary treatments.

Phylum	Baseline	Diet F	Diet FI	Diet FG	Diet FIG	CF
Acidobacteriota	45	4	3	3	2	9
Actinobacteriota	64	13	12	12	17	10
Armatimonadota	3	0	0	0	0	0
Bacteroidota	466	80	138	152	119	113
Bdellovibrionota	31	4	5	5	6	7
Campylobacterota	22	2	5	2	2	3
Chloroflexi	8	0	0	1	0	0
Cyanobacteria	73	4	13	16	14	13
Dadabacteria	1	0	0	0	0	0
Deinococcota	0	1	0	0	0	0
Dependentiae	2	0	1	0	1	0
Desulfobacterota	19	0	1	1	0	2
Elusimicrobiota	2	0	0	0	1	0
Firmicutes	112	11	18	7	24	16
Fusobacteriota	66	15	37	48	22	21
Gemmatimonadota	2	1	0	0	0	0
Halobacterota	1	8	11	5	5	9
Hydrogenedentes	2	0	0	0	0	0
Myxococcota	10	0	1	2	0	0
NA	14	2	7	9	7	5
Nanoarchaeota	1	0	0	0	1	0
Nanohaloarchaeota	2	0	0	1	1	3
NB1-j	6	0	0	0	0	0
Nitrospinota	2	0	0	1	0	0
Nitrospirota	3	1	1	0	0	1

Phylum	Baseline	Diet F	Diet FI	Diet FG	Diet FIG	CF
Patescibacteria	10	4	6	6	3	3
Planctomycetota	170	5	25	25	33	20
Proteobacteria	1134	284	355	380	322	302
SAR324 clade (Marine group B)	5	0	0	1	1	0
Spirochaetota	14	10	11	11	12	5
Thermoplasmatota	1	0	0	0	0	2
Verrucomicrobiota	110	8	13	19	11	9
WPS-2	3	1	0	0	1	0
TOTAL ASVs	2404	458	663	707	604	553

Table 7.A.2 Relative abundance (%) recovered from the farmed abalone gut content before and after dietary treatments.

Phylum	Baseline	Diet F	Diet FI	Diet FG	Diet FIG	CF
Acidobacteriota	1.872	0.873	0.452	0.424	0.331	1.627
Actinobacteriota	2.662	2.838	1.810	1.697	2.815	1.808
Armatimonadota	0.125	0.000	0.000	0.000	0.000	0.000
Bacteroidota	19.384	17.467	20.814	21.499	19.702	20.434
Bdellovibrionota	1.290	0.873	0.754	0.707	0.993	1.266
Campylobacterota	0.915	0.437	0.754	0.283	0.331	0.542
Chloroflexi	0.333	0.000	0.000	0.141	0.000	0.000
Cyanobacteria	3.037	0.873	1.961	2.263	2.318	2.351
Dadabacteria	0.042	0.000	0.000	0.000	0.000	0.000
Deinococcota	0.000	0.218	0.000	0.000	0.000	0.000
Dependentiae	0.083	0.000	0.151	0.000	0.166	0.000
Desulfobacterota	0.790	0.000	0.151	0.141	0.000	0.362
Elusimicrobiota	0.083	0.000	0.000	0.000	0.166	0.000
Firmicutes	4.659	2.402	2.715	0.990	3.974	2.893
Fusobacteriota	2.745	3.275	5.581	6.789	3.642	3.797
Gemmatimonadota	0.083	0.218	0.000	0.000	0.000	0.000
Halobacterota	0.042	1.747	1.659	0.707	0.828	1.627
Hydrogenedentes	0.083	0.000	0.000	0.000	0.000	0.000
Myxococcota	0.416	0.000	0.151	0.283	0.000	0.000
NA	0.582	0.437	1.056	1.273	1.159	0.904
Nanoarchaeota	0.042	0.000	0.000	0.000	0.166	0.000
Nanohaloarchaeota	0.083	0.000	0.000	0.141	0.166	0.542
NB1-j	0.250	0.000	0.000	0.000	0.000	0.000
Nitrospinota	0.083	0.000	0.000	0.141	0.000	0.000
Nitrospirota	0.125	0.218	0.151	0.000	0.000	0.181

Phylum	Baseline	Diet F	Diet FI	Diet FG	Diet FIG	CF
Patescibacteria	0.416	0.873	0.905	0.849	0.497	0.542
Planctomycetota	7.072	1.092	3.771	3.536	5.464	3.617
Proteobacteria	47.171	62.009	53.544	53.748	53.311	54.611
SAR324 clade (Marine group B)	0.208	0.000	0.000	0.141	0.166	0.000
Spirochaetota	0.582	2.183	1.659	1.556	1.987	0.904
Thermoplasmatota	0.042	0.000	0.000	0.000	0.000	0.362
Verrucomicrobiota	4.576	1.747	1.961	2.687	1.821	1.627
WPS-2	0.125	0.218	0.000	0.000	0.166	0.000

Table 7.A.3 List of 30 metabolites with PLS-DA VIP scores greater than 1 (first component).

Compounds	Component 1	Component 2	Component 3	Component 4	Component 5
Myristic acid	3.045	2.989	2.910	2.822	2.795
DPA	2.167	2.125	2.077	2.014	1.997
Cystathionine	2.077	1.984	1.933	2.026	2.018
cis-Vaccenic acid	2.059	1.968	1.957	1.923	1.902
Palmitoleic acid	2.051	1.969	1.930	1.874	1.854
11,14-Eicosadienoic	1.822	1.752	1.731	1.681	1.664
Lactic acid	1.676	1.674	1.646	1.607	1.590
Margaric acid	1.630	1.563	1.561	1.529	1.513
Erucic acid	1.622	1.549	1.574	1.600	1.583
trans-Cinnamic acid	1.594	1.601	1.592	1.570	1.562
alpha-Linolenic acid	1.577	1.564	1.535	1.498	1.483
Aspartic acid	1.482	1.426	1.389	1.347	1.337
Citraconic acid	1.471	1.406	1.370	1.335	1.334
Linoleic acid	1.441	1.389	1.361	1.321	1.312
Stearic acid	1.428	1.386	1.382	1.364	1.349
Lysine	1.410	1.356	1.337	1.327	1.313
10-Pentadecenoic acid	1.401	1.353	1.318	1.281	1.271
Nicotinic acid	1.392	1.331	1.310	1.321	1.317
Succinic acid	1.359	1.315	1.283	1.246	1.233
cis-Aconitic acid	1.229	1.182	1.170	1.148	1.156
Unknown001	1.226	1.195	1.189	1.153	1.154
Proline	1.223	1.174	1.177	1.192	1.181
DHA	1.143	1.135	1.147	1.128	1.122
Adrenic acid	1.141	1.133	1.144	1.124	1.118
Dodecanoic acid	1.123	1.225	1.198	1.162	1.179

Compounds	Component 1	Component 2	Component 3	Component 4	Component 5
Citric acid	1.079	1.031	1.043	1.023	1.034
Unknown002	1.072	1.026	1.076	1.048	1.037
Myristoleic acid	1.070	1.060	1.041	1.016	1.006
Pyruvic acid	1.061	1.014	0.989	0.975	0.972
Ornithine	1.036	1.067	1.118	1.089	1.084

Table 7.A.4 List of metabolites identified as significantly different among the dietary treatments (One-way ANOVA, $p < 0.05$).

Compounds	F value	P value	FDR	Fisher's LSD
Myristic acid	26.986	<0.001	<0.001	CF - Diet F; CF - Diet FG; CF - Diet FI; CF - Diet FIG; Diet F - Diet FG; Diet F - Diet FIG
10-Pentadecenoic acid	16.499	<0.001	<0.001	CF - Diet F; Diet FG - CF; Diet FIG - CF; Diet FG - Diet F; Diet FIG - Diet F; Diet FG - Diet FI; Diet FIG - Diet FI
Proline	16.069	<0.001	<0.001	CF - Diet F; CF - Diet FG; CF - Diet FIG; Diet F - Diet FG; Diet FI - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG; Diet FI - Diet FIG
2-Hydroxyisobutyric acid	12.907	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet FIG - CF; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
bishomo-gamma-Linolenic acid	11.905	<0.001	<0.001	CF - Diet F; CF - Diet FG; Diet FI - Diet F; Diet FIG - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
gamma-Linolenic acid	11.905	<0.001	<0.001	CF - Diet F; CF - Diet FG; Diet FI - Diet F; Diet FIG - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
Citraconic acid	11.38	<0.001	<0.001	CF - Diet F; CF - Diet FG; CF - Diet FI; CF - Diet FIG; Diet FG - Diet F; Diet FIG - Diet F
2-Hydroxybutyric acid	11.375	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet FIG - CF; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Pyroglutamic acid	10.726	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG; Diet FI - Diet FIG
Linoleic acid	9.4395	<0.001	<0.001	CF - Diet F; CF - Diet FG; CF - Diet FI; CF - Diet FIG
Lysine	9.165	<0.001	<0.001	CF - Diet FG; CF - Diet FIG; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG; Diet FI - Diet FIG
Glutathione	9.093	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG; Diet FI - Diet FIG
Palmitoleic acid	8.9042	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet FIG - CF; Diet FI - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
Methionine	8.3057	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Leucine	8.2095	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG; Diet FIG - Diet FG; Diet FI - Diet FIG

Compounds	F value	P value	FDR	Fisher's LSD
Alanine	7.6194	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG; Diet FI - Diet FIG
Glutamic acid	7.4417	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG; Diet FI - Diet FIG
EPA	6.9658	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet FIG - CF; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Valine	6.8875	<0.001	<0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG
cis-Vaccenic acid	6.8659	<0.001	<0.001	Diet FI - CF; Diet FIG - CF; Diet FI - Diet F; Diet FIG - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
Creatinine	6.3238	<0.001	0.001	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG; Diet FI - Diet FIG
Succinic acid	5.9576	<0.001	0.001	Diet F - CF; Diet FG - CF; Diet F - Diet FI; Diet F - Diet FIG; Diet FG - Diet FIG
NADP_NADPH	5.8171	<0.001	0.002	CF - Diet FG; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG
Cysteine	5.6721	<0.001	0.002	Diet F - CF; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG; Diet FI - Diet FG; Diet FIG - Diet FG
DPA	5.6391	<0.001	0.002	CF - Diet FG; CF - Diet FI; CF - Diet FIG; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Erucic acid	5.4798	<0.001	0.002	Diet FIG - CF; Diet FI - Diet F; Diet FIG - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
2-Aminoadipic acid	5.4682	<0.001	0.002	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG
Aspartic acid	5.3038	<0.001	0.003	Diet FIG - CF; Diet FI - Diet F; Diet FIG - Diet F; Diet FIG - Diet FG
Cystathionine	5.2394	<0.001	0.003	Diet F - CF; Diet FG - CF; Diet FI - CF; Diet FIG - CF
trans-Cinnamic acid	5.1009	0.001	0.003	Diet F - CF; CF - Diet FIG; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Ornithine	5.0291	0.001	0.003	Diet F - CF; Diet FI - CF; Diet F - Diet FG; Diet FI - Diet FG; Diet FI - Diet FIG
Glycine	5.0285	0.001	0.003	CF - Diet F; CF - Diet FG; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG
Tyrosine	4.8995	0.002	0.004	CF - Diet FG; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG

Compounds	F value	P value	FDR	Fisher's LSD
Glutamine	4.45	0.003	0.007	CF - Diet FG; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG
11,14-Eicosadienoic	4.1971	0.004	0.010	Diet FI - CF; Diet FIG - CF; Diet FI - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
Margaric acid	3.8826	0.007	0.016	Diet F - CF; Diet FG - CF; Diet FI - CF; Diet FIG – CF
Serine	3.8737	0.007	0.016	CF - Diet FG; Diet F - Diet FG; Diet FI - Diet FG; Diet FIG - Diet FG
Lactic acid	3.8116	0.007	0.017	Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Phenylalanine	3.6903	0.009	0.019	CF - Diet FG; Diet F - Diet FG; Diet FI - Diet FG
2-Phosphoenolpyruvic acid	3.6471	0.009	0.020	CF - Diet F; CF - Diet FI; CF - Diet FIG
Unknown002	3.621	0.01	0.020	CF - Diet FG; CF - Diet FIG; Diet F - Diet FG; Diet FI - Diet FG
Citric acid	3.5822	0.01	0.021	Diet F - CF; Diet F - Diet FG; Diet F - Diet FIG
alpha-Linolenic acid	3.4358	0.01	0.025	CF - Diet FG; CF - Diet FI; CF - Diet FIG
Myristoleic acid	3.384	0.01	0.027	Diet FI - CF; Diet FI - Diet F; Diet FI - Diet FG; Diet FIG - Diet FG
13,16-Docosadienoic acid	3.2709	0.02	0.031	Diet F - CF; Diet F - Diet FG; Diet F - Diet FIG
Unknown001	3.1504	0.02	0.036	Diet F - CF; Diet F - Diet FG; Diet F - Diet FI; Diet F - Diet FIG
Glyoxylic acid	3.1142	0.02	0.037	CF - Diet F; CF - Diet FG; CF - Diet FI
beta-Alanine	2.941	0.03	0.046	CF - Diet FG; Diet F - Diet FG; Diet F - Diet FIG; Diet FI - Diet FG
cis-Aconitic acid	2.9404	0.03	0.046	Diet F - Diet FIG

Table 7.A.5 List of altered metabolic pathways enriched between diet FIG and commercial feed fed to *H. iris.*

Pathways	Total Compounds (in KEGG data)	Hits (in abalone samples)	Statistic Q	Expected Q	Raw p	Holm p	FDR
Fatty acid biosynthesis	47	4	21.489	3.448	<0.001	0.003	0.003
beta-Alanine metabolism	21	3	19.446	3.448	<0.001	0.017	0.006
Nicotinate and nicotinamide metabolism	15	2	22.64	3.448	0.001	0.024	0.006
Biotin metabolism	10	1	34.679	3.448	0.001	0.024	0.006
Linoleic acid metabolism	5	1	32.724	3.448	0.001	0.036	0.008
Lysine degradation	25	2	21.645	3.448	0.002	0.082	0.015
Arginine and proline metabolism	38	6	12.055	3.448	0.004	0.134	0.017
Histidine metabolism	16	3	16.753	3.448	0.004	0.138	0.017
Propanoate metabolism	23	3	13.494	3.448	0.004	0.139	0.017
Cysteine and methionine metabolism	33	4	14.89	3.448	0.004	0.139	0.017
Glycine, serine, and threonine metabolism	33	6	11.149	3.448	0.010	0.312	0.037
Pantothenate and CoA biosynthesis	19	4	11.857	3.448	0.011	0.335	0.038
Arginine biosynthesis	14	5	10.493	3.448	0.018	0.550	0.059
alpha-Linolenic acid metabolism	13	1	17.234	3.448	0.023	0.653	0.065
Biosynthesis of unsaturated fatty acids	36	7	12.223	3.448	0.023	0.654	0.065
Citrato cycle (TCA cycle)	20	6	8.8282	3.448	0.036	0.959	0.091

Pathways	Total Compounds (in KEGG data)	Hits (in abalone samples)	Statistic Q	Expected Q	Raw p	Holm p	FDR
Aminoacyl-tRNA biosynthesis	48	17	9.3649	3.448	0.037	0.959	0.091
Alanine, aspartate, and glutamate metabolism	28	9	7.7602	3.448	0.040	0.992	0.093
Pyrimidine metabolism	39	2	10.461	3.448	0.047	1.0	0.105

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Chapter

Discussion

8

8.1 THESIS BACKGROUND

The global fish consumption has increased from 9.96 Kg in 1961 to 20.5 Kg in 2018 (FAO, 2020), making fish production the fastest growing food production sector. As wild fisheries have remained stagnant over the last decades due to climate change (FAO, 2022), the aquaculture sector has increased its capacity to supply fish protein for a growing population. Although abalone contribute a small proportion of the aquaculture production, abalone are highly priced delicacies consumed manly in Asian countries due to the flavour and nutritional qualities of the meat. In New Zealand, the cultivation of the black-footed abalone (*Haliotis iris*) is very promising, contributing NZD 50 million to the seafood production sector in 2019 (Pāua Industry Council LTD, 2023). Most of the abalone production comes from wild fisheries, mainly from Chatham Island which contributes more than a quarter of the national wild harvest (Pāua Industry Council LTD, 2023). Abalone aquaculture is expected to grow as the New Zealand government plans to triplicate the revenues from exports by 2030 (New Zealand Government, 2012). However, this growth is hampered by the lack of sustainable feed supplies and feed alternatives that contribute to a more sustainable aquaculture.

One of the main reasonings behind the development of more sustainable feeds for abalone is the use of fishmeal. Fishmeal is used to feed aquatic species due to its high digestibility, excellent composition of essential amino acids, and long chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compared to other sources of protein. Almost 25–30% of the globally available fishmeal comes from Peru (Hardy, 2006), followed by Chile and Thailand, at around 10–15% and 6%, respectively (Green, 2016). Approximately 4–5 tonnes of whole fish are required to produce 1 tonne of dry fishmeal (Miles & Chapman, 2006). The aquaculture sector currently uses ¾ of the global fishmeal production (Shepherd & Jackson, 2013), and fishmeal is the main ingredient of compounded feeds for carnivorous and herbivorous species. The utilisation of fishmeal as a feed ingredient for aquaculture has been questioned due to the reliance on the availability of wild-caught, short-lived pelagic fish, and increasing price ranging from USD 452 per tonne in 2000 to USD 1596.54 per tonne in 2018, which makes aquaculture products more expensive (Jannathulla et al., 2019). Due to a stagnating fishery production and a raising aquaculture production, it is expected that the price of fishmeal increases by 29% in 2030 (The World Bank, 2013). Therefore, there is a need of sustainable and more socially responsible alternative ingredients to effectively replace fishmeal.

Among the most used alternative ingredients in aquafeeds are plant protein sources, insect meals, and fruit waste by-products. Plant protein sources have an acceptable nutritional profile, but their low digestibility, palatability, and presence of substances that reduce absorption (antinutrients) represent a concern in animal health (Gatlin III et al., 2007). Some plant meals produce intestinal inflammation (Djordjevic et al., 2021; Kumar et al., 2021) and poor growth compared to fish protein, due to lack of the essential amino acids methionine, lysine, and threonine (Gatlin III et al., 2007). Insect meals are a good source of nutrients which are considered more sustainable than fish protein and microalgae (Maiolo et al., 2020; Oonincx & De Boer, 2012). Insect meals can contain a protein proportion of 50–70%, depending on the insect species and stage of life. In addition, the amino acid profile of insect meals tends to be similar to fishmeal, thus requiring little nutritional enhancement (Barroso et al., 2014). Insects also have a low ecological impact due to reduced land required for rearing and low water use, high production efficiency with a high feed conversion ratio. In addition, insects have the potential to revalorise waste as they can feed on bio-waste. Insect meal use has recently been authorised for use as aquaculture feed in the European union (European Comission, 2017), and this trend is expected to be reflected in other areas of the world. Insect meals have been included in feeds for Atlantic salmon (Belghit et al., 2018), Nile tilapia (Fontes et al., 2019), European seabass (Basto et al., 2020) and rainbow trout (Rimoldi et al., 2019). However, to our knowledge no inclusion has yet been trialled for *Haliotis* species, and no scientific reports have evaluated the effect of insect meal on the overall health and nutrition profile of this species.

Another alternative ingredient included in animal feeds is grape marc. Grape marc disposal represents a problem due to the 73, 000 tonnes of grape marc produced annually in New Zealand (Ministry for Primary Industries, 2020) and the lack of approaches to dispose this waste sustainably. There have been many studies using grape marc or derivatives as potential prebiotics and antioxidants for ruminants and chickens resulting in improved meat quality (Bennato et al., 2020; Ianni et al., 2019; Turcu et al., 2020) and intestinal microbial diversity (Pulgar et al., 2021). In fishes, grape marc supplementation has been included in feeds for rainbow trout (Pulgar et al., 2021) and grass carp (Souza et al., 2019), showing improved antioxidant capacity and reduced lipid peroxidation in animals. Grape marc has only been included in feeds for abalone in the form of Acti-Meal®, steam distilled grape marc, for *Haliotis laevigata* promoting abalone growth when included up to 20% (Currie et al., 2019).

This research sought to investigate whether alternative ingredients can be used to formulate sustainable feeds that benefit the growth and general health of *H. iris*. In this study, the nutritional profile and chemical composition of *Halioris iris* in two aquaculture farms in New Zealand were characterized. The development and evaluation of sustainable feeds that included insect meal and grape marc on the growth and overall health of *H. iris* were then further investigated. Firstly, the approach of the feed design started with the characterization of the nutritional and metabolite profile of juvenile *H. iris* across different seasons over one year in a farm located in the North Island of New Zealand. Results from this study reported on the growth performance and metabolite profile fluctuations of juvenile abalone in four different seasons. Secondly, experimental diets were developed based on the protein, carbohydrate, and lipid levels that were similar to the commercial feed. Thirdly, the experimental diets were encapsulated to improve seawater stability and their chemical and physical characteristics were evaluated to determine performance during storage, handling, and abalone feed intake. Lastly, the experimental diets were trialled in an abalone farm in the South Island of New Zealand for 165 days. Growth performance, intestinal health (microbiome and histological studies), metabolic profile, flavour compounds, nutritional profile, digestibility, and shell colour variations were measured at the end of the feeding trial.

This study aims to evaluate the effects of the inclusion of insect meal and grape marc on somatic growth, gut health, meat quality, nutritional profile, shell colour, and metabolite profile of *H. iris*. Furthermore, this study provides a comprehensive understanding of nutrition, which considers the animal's growth, as well as the use of feeds which contribute to less environmental pollution and reduced feed waste. It is envisaged that the information from this study can contribute to further optimisation of feeds for further growth of the New Zealand aquaculture industry aligned with the national initiatives for sustainable development and waste management to be achieved by 2050. It is believed that the insights from this thesis open new avenues for a more circular economy where fewer synthetic products are utilised and is closer to Māori *Kaitiakitanga* values (guardianship for nature conservation).

8.2 DISCUSSION

"Change happens by listening and then starting a dialogue with the people who are doing something you don't believe is right."

— Jane Goodall

Every scientific advancement has involved more integration of different aspects that were neglected or dismissed before. In the field of fish nutrition, the focus has shifted from a narrow view of solely seeking animal growth to the wider view of understanding the multiple effects of nutrition. For instance, researchers now aim to understand how nutrition affects energy maintenance, development, reproduction, and animal health simultaneously (Wu, 2017). Although aquaculture practices theoretically acknowledge the holistic view of nutrition, there is still a large gap between understanding and producing formulated feeds that are beneficial beyond animal growth. The development of aquatic feeds has largely been focused on replacing unsustainable products like fishmeal with alternatives that produce similar animal growth, without paying sufficient attention to the animal's gut health, environmental pollution, and waste production. However, the understanding of the effect of nutrition on animal health, particularly the role of prebiotics, changed the *status quo* of animal nutrition in 1995 (Gibson & Roberfroid, 1995). This discovery highlighted the crucial role of nutrition in the overall health and welfare of animals, shifting the focus of animal feed development to promoting animal health and welfare and producing safe and high-quality animal products (FAO, 2023).

Aquaculture compounded feeds are designed to provide a standardized nutrition with reliable ingredients that can be manipulated to meet the different needs of the farms. Originally, formulated feeds were developed to replace seaweed due to the lack of seaweed availability for industrial purposes. Fishmeal was a key component in compounded feeds due to its the excellent nutrient characteristics, high palatability, and digestibility. However, the environmental concerns of unsustainable maintenance of fishmeal production for animal feeding, and the decline in wild-caught pelagic fishes, have questioned its use and deemed it unsustainable. Although the use of fishmeal in aquaculture feeds has significantly decreased in the last 20 years (Tacon & Metian, 2008), the questions remain about the future of compounded feeds in aquaculture, how they can contribute to a better understanding of the role of nutrition, and if replacing fishmeal is an effective approach to

generating a more sustainable aquaculture industry that is expected to grow by 25% in the next 30 years (FAO, 2022).

Fish nutrition research has successfully tested a variety of alternative sources to replace fishmeal, such as plant protein, insect protein, and other meals, which have been improved in their digestibility through sophisticated purification techniques. However, it remains to be determined if the success of aquafeeds is based on the use of one ingredient or a mixture of many. The prioritisation of amino acids over carbohydrates or lipids have been overestimated, with protein sources being the main focus of feed development in pursuit of a similar product to fishmeal. This narrow view overlooks other key components, such as fatty acids and carbohydrates, which are often considered feed fillers.

Currently, the fish nutrition research paradigm is mostly focused on the question: '*can we produce a dietary replacement that promotes similar fishmeal performance?'* rather than '*what can we use for aquaculture feed?'*. Although it seems more of a semantical difference, it unveils the intention behind the science. The first view unveils the goal of attaining something similar to fishmeal and the second view unveils open discovery to find possible solutions to the bigger problem of environmental sustainability. This thesis subscribes to the latter view (*what can we use for aquaculture feed?)*, which requires the integration of knowledge from fish nutritionists, marine policy makers and regulators, environmental scientists, food scientists, aquaculturists, farmers, and social scientists. The differentiation of these two views creates different directions in research questions, design, and funding. Rather than subscribing to a right or wrong view, these differences are displayed with the aim to promote further discussion on the most advantageous path for aquaculture feed production.

In the following sections of this discussion, the potential contributions of the inclusion of two alternative ingredients (insect meal and grape marc), and the delivery strategy (feed encapsulation), will be discussed. Based on these statements, potential advantages of customised compounded feeds are discussed in relation to the results in this thesis in terms of 1) overcoming limitations to achieve more efficient abalone growth, 2) exploring alternative features for commercialisation of abalone, 3) utilising food by-product wastes for species-specific formulated diets and 4) tailoring compounded feeds to meet specific nutritional requirements.

Beyond protein replacement: Overcoming limitations to achieve more efficient abalone growth.

If you can prove a statement one-hundred percent true, it does not describe the world.

-Bart Kosko, paraphrasing Einstein, *Fuzzy Thinking*

The findings in this thesis support the importance of non-protein components in overall abalone growth performance, particularly the role of fatty acids. Increasing levels of EPA (eicosapentaenoic acid) in the diet was found to promote more growth in abalone fed the commercial feed (CF) compared to those fed the experimental diets. While fatty acids are known to be important for energy use and membrane constitution, their role in animal growth is not yet fully understood. Although a direct comparison between the experimental diets and commercial feed is not possible due to the feed enhancers typically included in industrial feeds, commercial feed contained nearly three to four times more EPA than the experimental diets. The dietary level of EPA was reflected in abalone tissue, with significantly higher levels of EPA found in abalone fed the commercial feed compared to those fed the experimental diets, suggesting an essential role for EPA in abalone growth.

Eicosapentaenoic acid (EPA) plays a very important role in abalone nutrition and growth (Rao et al., 2022). EPA is a precursor to DHA (Wu et al., 2002; Wu et al., 2003), eicosanoids (Sargent et al., 2003), and other immune molecules that are responsible for disease resistance (Zuo et al., 2012). Both EPA and DHA (docosahexaenoic acid) seem to modulate feed efficiency response and weight gain of aquatic species (Xu et al., 2018). When dietary EPA is provided along with DHA, abalone can synthesize DHA from EPA, leading to improvements in growth performance and immune status (Xu et al., 2011). The DHA/EPA ratio has been used to determine growth efficacy or depression in various species. For example, higher amounts of DHA and higher DHA/EPA ratios resulted in growth depression rather than efficacy in juvenile golden pompano (Zhang et al., 2019) and Japanese seabass (Xu et al., 2016) with documented ratios of 1.69, 2.12 (Zhang et al., 2019), and 2,4 (Xu et al., 2016). In our study, all experimental diets resulted in a DHA/EPA ratio of approximately 3.0, while the commercial feed had a ratio of 0.9. This suggests that the DHA/EPA ratio in the commercial feed was closer to a moderate level compared to our experimental diets. In previous studies, a DHA/EPA ratio of 1.69 in abalone showed the best growth compared to lower ratios of 0.01–0.9 and a higher ratio of 8.8 (Toledo-Agüero & Viana, 2009). Currently, there are no studies on DHA/EPA requirements in abalone species.

Our results showed that the inclusion of insect meal and grape marc caused a significant variation in the fatty acid profile of diets and abalone tissue (Chapter 5). In terms of the diets, the inclusion of insect meal (IM) resulted in significant increments of myristic acid, palmitic acid, oleic acid, linoleaidic acic, α-linolenic acid (ALA), docosahexaenoic acid (DHA), and a reduction of arachidonic acid (ARA), and eicosapentaenoic acid (EPA) compared to the free-insect meal diet (diet F). This result corroborates the previous reports of Belforti et al. (2015) and Gasco et al. (2016) which documented that the inclusion of insect meal increases the concentration of omega-6 fatty acids compared to omega-3. Typically, long chain polyunsaturated fatty acids (PUFAs), such as EPA, DHA, and DPA are not present in *Tenebrio molitor*. However, oleic, linoleic, and palmitic acid are the most present (Gasco et al., 2016), and therefore are more likely to be transferred to the animal's tissue fed on insect meal. The inclusion of grape marc (GM) resulted in an increment of linoleadic acid, arachidic acid, eicosenoic acid, oleic acid, palmitic acid, heneicosanoic acid, and in a reduction of behenic acid compared to the free-grape marc diet (diet F). Grape marc is naturally rich in omega 6-fatty acids, such as linoleadic acid, which is the cis-isomer of linoleic acid, omega-9 oleic acid, and the saturated fatty acids stearic and palmitic acid (Gómez-Brandón et al., 2019).

Our study found that the inclusion of insect meal (IM) in the abalone's diet resulted in significantly higher levels of oleic acid, linoleadidic acid, α-linolenic acid (ALA), eicosadienoic acid, eicosatrienoic acid, and arachidonic acid (ARA) in the abalone compared to animals fed the free-grape marc/insect meal diet (diet F). This suggests a direct positive influence of the insect meal fatty acid profile on the abalone's fatty acid profile. However, the inclusion of grape marc (GM) resulted in abalone with significantly higher levels of linoleaidic acid, α-linolenic acid (ALA), eicosadienoic acid, and eicosatrienoic acid compared to animals fed diet F. The main difference observed between abalone fed diets with IM and GM was the increase in ARA in animals fed the IM diets. This is particularly interesting because diets with IM had the lowest levels of level of ARA compared to the free-insect meal diet or diets with grape marc. ARA is an essential omega-6 fatty acid that, along with docosahexaenoic acid (DHA), are the major fatty acids in higher invertebrates. ARA has a predominant role as an eicosanoid precursor, and its dietary nutritional requirements have not been fully understood. However, dietary ARA supplementation has been associated with better immune function and improved mechanisms to cope with environmental stress (Bell & Sargent, 2003; Koven et al., 2003). In our study, it is possible that the chitin present in *Tenebrio molitor* (Belforti et al., 2015) have had stimulated the production of arachidonic acid in abalone. Increased expression of phospholipase A2 in

response to doses of chitosan, a derivative of chitin, has resulted in increased serum arachidonic acid levels in piglets, improving immune function (Li et al., 2017).

In future investigations, it is recommended to evaluate different levels of synthetic DHA, ALA, ARA, and EPA in abalone diets. Synthetic sources are encouraged to be used to precisely relate dietary ingredient addition with nutritional results. The levels of inclusion of these fatty acids should be adjusted to maintain a constant level of carbohydrates to avoid excess lipid input, as abalone metabolism is not equipped to metabolise high levels of lipids (Lee et al., 2019) and they can synthesize lipids from carbohydrates (Wang et al., 2009). The lipid: carbohydrate ratio in abalone feeds should be determined, as abalone primary utilize carbohydrates for growth (Thongrod et al., 2003). In formulating these diets, the gross energy of the feeds from an adiabatic calorimeter should also be considered, as abalone feeding is based on energy requirements rather than nutrient requirements (Montano-Vargas et al., 2005). In this way, the DHA, ALA, ARA, and EPA requirements of abalone can be determined, and their roles in growth, immunity, and improved digestion efficiency can be elucidated through metabolomics, quantitative determination of essential fatty acids, and growth performance assessment.

Beyond growth: Exploring alternative features for commercialization of abalone.

Honest disagreement is often a good sign of progress.

Mahatma Gandhi

The inclusion of insect meal (diet FI), grape marc (diet FG), and a combination of both (diet FIG) resulted in abalone with healthy intestinal epithelia without signs of inflammation, as supported by the microbiome evaluation. Microbial diversity determined by Amplicon Sequence Variants (ASVs) suggested that the inclusion of both, insect meal and grape marc increased the microbial diversity, yet not in a significant manner. This suggest that the inclusion of 10% insect meal and 30% grape marc constitute a safe dose for abalone. Differences were mainly detected before and after the feeding trial, in those animals that remained in the commercial feed treatment (Chapter 7). This finding suggests a possible contribution of age and environmental factors to shape the abalone microbiome. The increased relative abundance of the phylum Fusobacterium and Firmicutes in abalone fed the experimental diets with insect meal and grape marc may suggest a possible beneficial effect of those ingredients. The fibre contained in the form of lignin and cellulose

in grape marc, and chitin in insect meal may have caused this increase, allowing fermentation of complex-carbohydrates in the intestinal tract of abalone. The products of this fermentation, such as short-chain fatty acids like butyrate or propanoate, have been associated with improved fish health (Aguilar-Toalá et al., 2018) and their pathways were enriched in abalone fed the diet that included both insect meal and grape marc (Chapter 7).

The inclusion of complex polysaccharides that benefit gut health without promoting growth has been documented in various studies. For instance, Sutriana et al. (2021) found no significant improvement in the growth of striped catfish fed with the prebiotic mannanoligosaccharide (MOS). However, the apparent protein digestibility, protease activity, and Fusobacterium and Firmicutes phylum abundance was increased. Similarly, Dimitroglou et al. (2010) showed that dietary MOS inclusion did not improve the growth or modified the gross histological morphology of gilthead sea bream. However, it did improve the microvilli density and microbial abundance and richness of beneficial bacterial species. In abalone, Meng et al. (2019) demonstrated that MOS fed at higher levels than the optimal dose did not improve survival, weight gain, or shell growth gain, but increased immune parameters that can potentially provide protection against pathogens.

The potential benefits of including insect meal (IM) and grape marc (GM) in immunity and disease resistance can be observed when abalone is subject to stress. To test this, future studies, should incorporate a bacterial challenge at the end of the feeding trial period to determine survival and recovery time. To reduce the effects of abalone adaptation to a new feed, a baseline feeding period for a couple of weeks is recommended to homogenize the feeding pattern of the animal cohort. Sampling can occur afterward, and the animal cohort can be further divided for dietary treatment. In this manner, biases are reduced, and populations can have the same baseline measurements (enzymes, microbiome, histological morphology, and metabolomics). Another recommendation is to quantitatively determine the presence of certain compounds that reflect improved gut health, such as short fatty acids (SCFA). If the dietary ingredient has a potential prebiotic effect, it should generate a change in gastrointestinal concentrations of propanoic, butyric, and acetic acid. As the metabolomic results showed (Chapter 7), the propanoate metabolism (propanoic acid) was the most enriched between the diet that included both insect meal and grape marc (diet FIG), and commercial feed (CF), suggesting a beneficial effect that is unseen. Therefore, further studies can include the quantitative determination of the three main SCFAs in a specific section of the digestive tract. Microbiome and enzyme activity studies of the gastrointestinal region dissected is highly recommended as enzymes and microbial populations are

allocated differently along the digestive tract. Growth performance evaluation and faecal collection for faecal microbiome and digestibility studies can provide valuable information to understand the dynamism between microbiome (gastrointestinal and faeces) – growth and nutrient metabolism.

The inclusion of insect meal and grape marc promoted a different profile of volatile compounds compared to the insect meal and grape marc free- diet. The diet that included grape marc (diet FG) and both IM/GM (diet FIG) promoted a better quality of meat that was less prone to lipid peroxidation compared to diet F (Chapter 6). The free-grape marc/insect meal diet (F) resulted in abalone with significant higher levels of peroxidation products, including ketones, aldehydes, and alcohols. The high presence of these products in animals fed this diet suggests that the high levels of fishmeal included in this diet may have been a key component in lipid oxidation. Lipid oxidation is the main process responsible for meat deterioration and has been associated with changes in colour, texture, nutritional value, and the generation of off-flavour compounds that reduce consumers acceptance (Amaral et al., 2018). As lipids are one of the most chemically unstable food components, they are prone to react with reactive species of oxygen (ROS) generated by metabolism, enzymes, metal ions, pH, and temperature. It is possible that the high levels of unsaturated fatty acids available in the fishmeal contributed to a high level of unsaturated fatty acids in the diet F, resulting in significant higher levels of arachidonic acid (ARA), erucic acid, and DHA compared to the other experimental diets (Chapter 4). Therefore, abalone fed on diet F showed significant higher levels of aldehydes (benzaldehyde, hexanal, heptanal), ketones (2,3 pentanedione, 2-butanone, 2-pentanone, 3-octanone), and alcohols (1-hexanol, 2-hexanol, 1-pentanol, 1-octen-3-ol) compared to the diets that included grape marc (FG and FIG). The low levels of peroxidation products in animals fed diets including grape marc corroborate the potential of grape marc to act as a natural antioxidant. The phenolic compounds and non-phenolic antioxidants (tocopherol and beta-carotene) found in grape marc are the responsible compounds for reducing lipid oxidation (Yu & Ahmedna, 2013) by reacting more actively with the hydroperoxyl radical (ROS) than unsaturated fatty acids (Waterhouse & Laurie, 2006).

The findings related to volatile compounds have important implications for developing more natural formulated diets where synthetic antioxidants can be fully replaced. This is particularly relevant for feed companies as nowadays, consumers have a preference for products with a smaller ecological imprint (Szendrő et al., 2020). In animal nutrition, synthetic antioxidants are used to extend the shelf life of aquatic feeds and fishmeal and

improve animal growth and health. The most used synthetic antioxidants are ethoxyquin (EQ), followed by butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Lundebye et al., 2010), as well as taurine (Tau), a sulphur-containing amino acid that improves fish growth performance (Salze & Davis, 2015). Currently, only the European Union has set regulations on the synthetic antioxidant dose limits for animal feed due to the pesticide use of ethoxyquin (World Health Organization, 2023). Taurine supplementation in animal feed is not listed by the Food and Drug Administration (FDA) and its use in some aquatic species has been associated with liver damage due to excess of oxidable amino acids (Hoseini et al., 2017).

As grape marc diets (diet FG and FIG) produced abalone meat with the lowest profile of peroxidation products, their inclusion in aquafeeds can improve grape marc valorisation and expand the alternatives for natural formulated feeds. This is an important point for future research, as grape marc can be included in finishing diets administered before abalone harvesting. Grape marc included as a natural antioxidant might extend the shelf-life of abalone meat, improve meat stability and preservation, and therefore longer periods of export/import can be attained. The phenolic compounds present in grape marc can extend the shelf-life of the opened package of abalone (Soro et al., 2021) by acting as an antimicrobial, reducing the enzymatic degradation of proteins in abalone (Chapter 5). In this direction, there is abundant room for further progress in terms of optimization of grape marc substrate. First, techniques to improve the prebiotic and antioxidant capacity of grape marc without excessively increasing the price. Second, the inclusion of grape marc chemically treated to reduce the potential antinutrients and improve acceptability. Third, the delivery of grape marc as a potential prebiotic along with beneficial bacteria in the form of *symbiotics*, as they have demonstrated superiority compared to single prebiotic and probiotic (Sutriana et al., 2021). Fourth, the determination of optimal dose of grape marc as a supplement to avoid growth retardation and promote gut health as shown in Chapter 7.

A critical aspect of exploring alternative ingredients is affordability. For this thesis, grape marc was donated by the Bragato Research Institute, an institute dedicated to wine research. If this ingredient was to be processed (optimised) for bioavailability or chemically treated to removed deterrents, then the final price will increase. Similarly with insect meal, which was purchased from a local market, where prices are generally elevated. If the production of aquafeeds scale up, a cheaper supply of insect meal must be sorted. In this study, the final price of the powder formula was around NZD 5.0 per Kg of feed, compared to the NZD 3.5 per Kg of commercial feed. The final price for encapsulated feed was around

NZD 50 per Kg, which price can be justified by the inclusion of particular bioactives aimed at promoting immunity traits, digestive health or boost growth.

Circular aquaculture production: Utilising food-by-product wastes for species-specific formulated diets.

Profit is what happens when you do everything else right

Yvon Chouinard – Owner of *Patagonia*

The current global focus on sustainable development and the current climate crisis has increased the importance of reducing waste. One solution is to re-use, recycle, compost, or recover energy from waste by-products (Giannakitsidou et al., 2020). Food and green waste account for over 50 percent of waste generated worldwide (Hoornweg & Bhada-Tata, 2012). According to the World Bank, New Zealand is among the top ten countries in the world in terms of waste generation, with almost 2 kg of waste per capita per day in 2016 (Kaza et al., 2018). In New Zealand, waste amounts to 327 tonnes per year, with fruit and vegetable waste accounting for 52,165 tonnes per year and seafood processing waste for 23,945 tonnes per year (Reynolds et al., 2016). As a result of this global concern about waste, there has been an emergence of opportunities to use plant and fish-by products in fish nutrition over the last decade. These products have a high nutritional profile and quality when sourced from reliable sources, and their low cost makes them the ideal for contributing to a more circular economy (Ministry for the Environment, 2023).

In this thesis, the replacement of 100% cornmeal with red dried grape marc was used without affecting growth and weight, while enriching the microbiome of *H. iris* compared to animals fed a grape marc free diet (diet F). The reutilisation of grape marc (GM) represents a steppingstone for New Zealand in attaining a circular economy by 2050 (Ministry for the Environment, 2023). There are many examples of waste by-products that have been used in fish nutrition in other countries. For example, in Brazil (the largest producer of banana and mango globally), banana meal has been used to replace cornmeal at 8% in feeds for the omnivorous freshwater fish Tambaqui without decreasing growth or weight gain (Felix e Silva et al., 2020). Also, mango meal has replaced cornmeal up to 100% for Nile tilapia without decreasing the specific growth rate (SGR) and weight gain (Melo et al., 2012). In Iran, lemon peel waste has been dehydrated and supplemented at 1.5% in diets for rainbow

trout without decreasing growth and weight gain, yet positively modulating the enzyme activity of superoxide dismutase and catalase (Chekani et al., 2021). These studies suggest that fruit by-products can be reutilised to provide antioxidants, antimicrobial compounds, and immunostimulants for aquatic animals if included at determined percentages (Dawood et al., 2022). However, the feasibility of their inclusion must be investigated in terms of the cost of feed production and animal digestibility.

Contrary to fruit by-products, insect meal is a product obtained from rearing insects, and although it has been cited as more sustainable than fishmeal, its use still rises concerns in terms of waste production and animal welfare. Through the findings of this thesis, it is observed that the inclusion of the insect meal from *Tenebrio molitor* does not reduce growth performance and may potentially act as an immunostimulant due to the presence of chitin. The next step, aligned with the global reduction of waste, would be to use fish waste in aquafeeds. Fish wastes are normally discarded and are usually described as putrescible due to the high level of lipids. The direct utilisation of fish waste in aquafeeds is still limited as fermentation must occur before inclusion to prevent bacterial growth and spoilage. Hence, two options for more circular aquafeeds are envisaged 1) to include fish waste in the insect rearing and 2) to ferment fish waste to obtain a protein replacement. The inclusion of fish waste in insect rearing has produced insects with enriched omega-3 fatty acids, elevating the total polyunsaturated fatty acids (PUFAs) from 4.3% to 14.8% (Barroso et al., 2019). The replacement of fishmeal at 100% with insects reared on fish waste has shown to increase the antioxidant capacity (catalase) and reduce intestinal inflammation in juvenile barramundi (Chaklader et al., 2021). The fermentation of fish waste and lemon peel during 120 h with *Saccharomyces cerevisiae* and *Lactobacillus reuteri* has shown to provide a product with a protein percentage of 48.5% and lipid of 15.6% adequate for fish nutrition (Tropea et al., 2021). These characteristics can be taken advantage of to produce more circular aquafeeds that are less dependent on synthetic compounds.

Optimizing Integrated Aquaculture: Tailoring compounded feeds to meet specific nutritional requirements.

All adventures, especially into new territory, are scary.

Sally Ride – American Astronaut and physicist

Integrated mariculture has traditionally been concentrated in China, Japan, and South Korea where fish net pens, shellfish, and seaweed are farmed in proximity in bays or lagoons. In contrast, modern integrated aquaculture has been slow to gain momentum in Western countries, only being revitalised at the end of the 20th century (Neori et al., 2004). Integrated multitrophic aquaculture systems (IMTA) have emerged as a promising approach to reducing nutrients release into the environment while enhancing overall productivity. In these systems, the nutrients from uneaten feed and excreted waste of fed species become food for other species. Typically, IMTA systems avoid the use of formulated feeds and instead cultivate seaweed for animal feeding (Capinpin Jr et al., 2020). Although IMTA systems remain interesting in terms of less water pollution, they require expensive initial operations and have not been implemented in big scale at least for abalone (FAO, 2022). Nevertheless, other integrated practices, such as the cultivation of IMTA-seaweed and the simultaneous provision with compounded feeds to aquatic animals, or the use of IMTA-seaweed to replace fishmeal have shown promising results in promoting growth and improving the nutritional profile of aquatic species (Shpigel et al., 2017). Scientific reports have highlighted the beneficial effects of enriched seaweed produced in IMTA when included in abalone diets, promoting good growth, and improving protein deposition compared to compounded feeds (Bansemer et al., 2016; Viera et al., 2011). Furthermore, integrating IMTA-seaweed with or within compounded feeds can reduce the need of wild-seaweed and reduce fishmeal inclusion, respectively. IMTA-seaweed typically contains a higher level of protein compared to non-enriched seaweed (Bansemer et al., 2016; Viera et al., 2011) and it often promotes better growth when provided as a mixture of seaweeds rather than as a single species.

Compounded feeds can be used in integrated aquaculture as a supplement or a vehicle to supply of specific nutrients while contributing to less environmental pollution. These nutrients can boost animal health by enhancing the immune status or improve meat quality by changing the colour or increasing the omega-3 fatty acid content. For example, in our experimental diets, the encapsulation method improved physical characteristics, such as seawater stability and reduced leaching of phosphorus (Chapter 4). The experimental diets showed improved seawater stability and reduced degradation after 24 and 48 h. In addition, similar values of water absorption in commercial feed (CF) and diet FIG were found, indicating that the ingredient mixture played a significant role in the stability of formulated diets. These findings suggest that the encapsulation method contributes more to the water stability of diets than the ingredient formulation.

It is hypothesized that the encapsulated delivery strategy creates a protective effect against leaching of nutrients such as phosphorus (P), nitrogen (N), and amino acid. The inclusion of both insect meal and grape marc significantly reduced the P levels and N levels in uneaten feed reducing the over-enrichment of P and N in waste waters. Our encapsulated feeds with insect meal and grape marc not only reduced the P and N levels, but also gradually absorbed P waste from the environment. The high stability and protective effect of the capsule is an advantage of hydrogels. Alginate hydrogels are considered non-toxic membranes, possess high levels of biocompatibility, and have a reduced impact in the environment (Aadil et al., 2016; Arafa et al., 2022; Campos et al., 2015).

The higher seawater stability of the encapsulated diets makes them an interesting option for adding feed supplements that require special care. For instance, thermolabile components which do not require heat-drying processes (Savic-Gajic et al., 2021), bacterial probiotic cells that require refrigeration or freezing for shelf-life extension (Hekmat & McMahon, 1992), or labile ingredients that are susceptible to lipid oxidation such as fish oil and fat-soluble vitamins (A,D,E,K) (Fu et al., 2007). Our results showed that abalone nitrogenous waste present in faeces and respiration products affect the levels of dissolved P and N in uneaten feeds. However, the inclusion of grape marc and insect meal promoted less phosphorous (P) and reduced nitrogen levels (N) in diets, before and after seawater immersion compared to diets without those supplements. Grape marc seems to provide the most protective effect in terms of leaching, showing the lowest levels of P even after 96 hours of seawater immersion (615.4–1192.9 ppm) compared to other experimental diets. The advantage of feed encapsulation not only results in more seawater stability, but also extends the time of available P, amino acids, and fatty acids inside the capsule until abalone is ready to eat, approximately 12 hours after feeding (Bei-ping et al., 2002).

8.3 LIMITATIONS

The first experimental section of this thesis (Chapter 3) supported the fact that nutritional changes, metabolites in particular amino acids are affected by seasons even when animals are fed the same diet for one year in a land-based aquaculture setting. This seasonal study hypothesized that the nutritional profile of abalone is not only the result of the dietary treatment, but it is also affected by natural fluctuations through seasons, such as water temperature. However, the one-year duration of the study limited comparisons among seasons from different years. Future studies will need to evaluate the nutritional and metabolomic profile for longer periods of time, obtaining samples in different seasons and years to have a better understanding of specific fluctuations, especially during stress-like events, such as heatwaves.

Another limitation in our study was the unavailability of the gross energy measurement for our experimental diets and commercial feed. Instead, a calculation based on expected calories was used as shown in the proximate composition information (Chapter 4). This is relevant because the optimum protein: energy (PE) ratio for abalone is close to 100 (mg protein: calories present), according to previous studies (Gómez-Montes et al., 2003). However, the P:E ratios of the experimental diets in this study ranged from 12,8–16,6 based on theoretical calculations, which may have resulted in abalone receiving less gross energy per gram of diet compared to the commercial feed. This may have increased the energy expense in feed intake, ingestion, and digestion, leading to more faecal biomass collected from the experimental diets than the commercial feed (Chapter 4).

Previous research has shown that insect meal is a more sustainable ingredient than fishmeal (Oonincx & De Boer, 2012). However, this must be assessed based on the small-scale production of insects in New Zealand. Life cycle assessments (LCA) should evaluate the environmental impact of formulated feeds, including insect meal and the grape marc supplementation, for the New Zealand farmed abalone. The results from these evaluations may promote the generation of a new legislation framework to regulate the use of fishmeal inclusion in feeds and incentivise optimised compounded feeds for specific purposes such as: immune protection for warmer-temperature seasons (by adding prebiotics), synthetic-free abalone meat (by adding non-synthetic dietary components in aquafeeds), and naturally improved abalone meat with longer preservation times.

All the experiments in this thesis used juvenile abalone, which require more energy for growth than adults. It is believed that as adult abalone require less energy for growth, the

experimental diets developed in this thesis can be of particular interest for farmers, not only reducing the use of fishmeal in aquafeeds but also reducing pollution in waste waters. Further studies on adult abalone population are recommended to evaluate the impact of insect meal, grape marc, and encapsulation feeds on their growth, digestibility, protein efficiency ratio, protein deposition ratio, and faecal deposition. As adult abalone produce more faecal biomass, this should be analysed for nitrogen, protein, lipid, and carbohydrate composition to understand the digestibility of compounded feeds under an alginate capsule. A microbiome analysis of faeces is also recommended to evaluate the microbial changes without sacrificing animals.

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Chapter

Conclusions 9

This thesis has successfully shown that insect meal and grape marc are adequate alternatives to be included in aquafeeds. Developed diets were carefully evaluated in terms of their physical characterisation and their potential for maximising productivity and delivery of nutrients in the farms. The encapsulated feeds were more stable in seawater compared to a commercial feed and are adequate for abalone feeding even in their frozen form. The frozen form delivery adds an advantage to the formulations as temperature-labile substances can be incorporated in the diets. In addition, encapsulated diets that included insect meal and grape marc showed reduce levels of nitrogen and phosphorus in the uneaten feed reducing their environmental pollution in seawater.

Within this thesis, the evaluation of the effect of nutrition went beyond the traditional aspects of growth performance. The developed diets were evaluated in their digestibility resulting in reduced digestibility when insect meal and grape marc were included. However, the growth and weight gain were not significantly reduced compared to the insect meal/grape marc free diet (diet F). The inclusion of insect meal and grape marc significantly affected the fatty acid profile of abalone meat, which suggest a critical role of fatty acids in growth. The inclusion of insect meal and grape marc produced modifications in the gut microbiome of abalone, suggesting a potential prebiotic effect of both ingredients that needs further elucidation. In addition, the inclusion of the alternative ingredients did not affect the gut-morphology of abalone showing healthy abalone gut tracts. The inclusion of insect meal and grape marc modified the flavour-volatile composition suggesting a modification of flavour according to the ingredients placed in the diets. In addition, the inclusion of insect meal and grape marc did not significantly modify the shell colour of abalone.

There is a need for future studies to evaluate all aspects affected by alternative dietary nutrients in animals. It is important to investigate the role of nutrients on gene expression (nutrigenomics), the gene variability on the nutritional effect (nutrigenetics), the role of nutrients on protein synthesis (nutritional proteomics), and lipid metabolism (nutritional lipidomics) with a large set of individual samples and different life stages. In addition, future research should integrate those tools with phenotypic characteristics, such as behaviour, fitness, and food intestinal absorption. This integration will allow the manipulation of nutrient delivery according to the needs of the species, leaving aside excessive use of synthetic ingredients to maximise productivity, and relying on natural nutritional programming to adapt to new ingredients that seem more sustainable. Overall, it is envisioned from this thesis that feed development and optimisation will include the

understating of other aspects of nutrition that provide a more comprehensive view of animal welfare, environmental impact, and affordability of diets.