

**Investigation of the Pathophysiology
of the New Zealand Black-Footed Abalone
(*Haliotis iris*)**

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Abstract

This study was conducted to improve the knowledge regarding the immunological aspects of the New Zealand Blackfooted abalone (*H. iris*), to anticipate future occurrence of disease and to establish environmental-friendly therapeutic strategies in regards to the *H. iris* immune system.

An initial study to identify the immunological aspects were by conducting characterization upon haemocytes using a combination of classical and novel (flow cytometry with Sysmex XN-1000 and Muse® Cell analyser) techniques. Two types of haemocytes were identified in this study, including type I (monocyte-like) and type II (lymphocyte-like) cells. Monocyte-like cells showed higher phagocytic activity when encountering Zymosan A particles compared to lymphocyte-like cells.

The ability to successfully prepare and preserve haemocyte cells for microscopy and flow cytometry is critical for further investigation of the abalone immune system. Therefore, the study to apply different antiaggregants and handling protocols was conducted upon New Zealand black-footed abalone (*H. iris*) haemocytes. Results showed that Alsever's solution was an effective antiaggregant, whereas K₂EDTA Microtainer® tubes was similarly as effective as Alsever's solution. However, the influence of different mixing techniques of K₂EDTA Microtainer® tubes should be noticed.

Observation of the immune capacity in juvenile New Zealand Black-footed abalone (*H. iris*) was assessed by conducting an experiment with probiotic enriched diet. Two groups of abalone were fed over a four-month period with different diets: one control and the other a probiotic diet enriched with *Exiguobacterium* JHEb1), 7×10^5 (*Vibrio* JH1) and 4.10×10^8 (*Enterococcus* JHLDc) CFU g⁻¹ feed. Results showed that in comparison to control abalone (fed with the same diet but no probiotics), the probiotic fed abalone had a significantly higher ($p < 0.05$) total haemocyte count (1.9×10^6 cells), higher viable cell

counts (90.77%), higher reactive oxygen species (ROS) positive haemocyte cells (12.03%) and a higher amount of non-apoptotic cells (87.96%). Further, foot tissue samples were obtained for metabolomics GC-MS analysis. Six free amino acids (lysine, proline, asparagine, glutamine and serine), two fatty acid (adrenic acid and palmitoleic acid), three organic compounds (lactic acid, succinic acid and oxalic acid) were detected in a higher amount in the foot muscle tissue of the probiotic-fed abalone. In addition, probiotic-fed animals showed improved growth and survival compared to control fed abalone.

The immune response of the probiotic-fed abalone to an intramuscular *Vibrio splendidus* challenge was also observed. Each abalone in this experiment were challenged by 10^9 cells mL^{-1} of pathogenic bacteria. Probiotic-fed abalone had a significantly higher survival rate post challenge compared to non-probiotic fed abalone. It was observed that the infected probiotic-fed abalone had higher THCs, slightly lower proportions of haemocytes undergoing early apoptosis, and lower percentages of ROS-producing haemocytes compared to infected control-fed abalone. These results indicate that the probiotic diet enhances immune capacity by stimulating haematopoietic processes, with a simultaneous low-level upregulation of ROS production, as a priming mechanism of the antibacterial defence system. In addition, metabolite profiles of muscle tissues generated via GC-MS provided suggestions of a perturbed ROS-regulatory system in infected abalone through changes in key metabolites associated with glutathione biosynthesis. This study suggests probiotics as an immunostimulant strategy since it potentially induce a protective effect against bacterial diseases leading to enhanced production and sustainability of the growing New Zealand abalone industry.

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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”

Signed : Roffi Grandiosa

30 October 2019

Authorship Agreement Form

Chapter 2

Grandiosa, R., Mérien, F., Pillay, K., Alfaro, A., 2015. Innovative Application of Classic and Newer Techniques for the Characterization of Haemocytes in the New Zealand black-footed abalone (*Haliotis iris*). *Fish and Shellfish Immunology*. doi:10.1016/j.fsi.2015.11.039

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Grandiosa, R., Bouwman, M., Young, T., Mérien, F. and Alfaro, A. 2018. Effect of antiaggregants on the in vitro viability, cell count and stability of abalone (*Haliotis iris*) haemocytes. *Fish and Shellfish Immunology*. doi:10.1016/j.fsi.2018.04.038

Chapter 4

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Chapter 2

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A.C. Alfaro	Sampling	10	6	
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Thao V. N.	Sampling	20	4	
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CHAPTER 1

Introduction and Literature Review

1.1. Introduction

1.1.1. Shellfish Culture in New Zealand

The demand of food produced by aquaculture was expected to increase since the global population is continuously growing and expected to reach over 9.7 billion by 2050 (FAO, 2016). In addition rising income, urbanization, the growth of the middle class and the improvement in international trade has also played important roles in the increase of seafood demand (FAO, 2016; Alfaro *et al.*, 2014). Aquaculture production in 2014 reached an amount of 73.8 million tonnes, with an estimate of 16.1 million tonnes of molluscs (US\$19 billion value) produced. Thus, the opportunity to further develop shellfish aquaculture will continue to increase since aquaculture products from New Zealand are well accepted by the world market.

New Zealand has the reputation of highly sustainable production practices with strict quality assurance especially for shellfish aquaculture. Evidently, the aquaculture sector has been recognized by the New Zealand government as a high potential income earning industry through the New Zealand aquaculture strategy and action plan (Ministry for Primary Industries, 2015). Therefore, the government has committed to develop the industry to annual sales of NZ 1\$ billion by 2025.

The shellfish aquaculture in New Zealand itself have developed rapidly from being an experimental activity to become small-scale operations and further developing into an industrial scale gaining interest from major corporations. The production of two molluscan species through aquaculture, namely *Perna canaliculus* and *Crassostrea gigas*, highly contributes to the national economy in terms of earnings from export of these species. The Greenshell™ Mussels (*Perna canaliculus*) makes the largest contribution

of the aquaculture sector. The total production of mussels (live, fresh and processed) at 2016 reached 33,334.3 tonnes with the total value of NZ \$311.12 million (Seafood New Zealand, 2016). The reproduction of mussels in New Zealand were mainly conducted by Spat Production and Technology Ltd (SPATnz), based in Nelson, New Zealand (Young *et al.*, 2016). Although the mussel aquaculture industry is currently thriving, challenges are still present such as variability in larval growth and settlement rates and sudden mortality from the larval batch (Smith *et al.*, 2016).

The oyster (*C. gigas*) aquaculture is the second largest molluscan aquaculture industry in New Zealand which takes part as an important socio-economic activity in New Zealand. The scale of production in 2016 of 591.1 tonnes was relatively smaller compared to the production of Greenshell™ Mussels, with the export revenue of NZ \$ 7.4 million (Seafood New Zealand, 2016). Production of oyster is also currently facing challenges, mainly from the presence of pathogens for example OsHV-1 virus, bacterial pathogens such as *Vibrio* sp (Keeling *et al.*, 2014) and parasites such as *Bonamia ostreae* (Hutching, 2017).

The aquaculture of other potential molluscan species have also been identified during the development of aquaculture ventures in New Zealand. The New Zealand Black-footed abalone (*H. iris*) for example have been developed into a larger commercial scale and its products have been recognized by the world market. The aquaculture of potential species such as the New Zealand Geoduck Clam (*Panopea zelandica*) was also developed since Geoducks represent one of the most lucrative products in the seafood market (Le *et al.*, 2017). However, the aquaculture research and development of these species is still needed to develop a viable industry since it is still in its infancy.

1.1.2. Abalone (*Haliotis iris*)

There are three abalone species in New Zealand, including the yellow-foot abalone (*Haliotis australis*), the white-foot abalone (*Haliotis virginea*) and the black-footed

abalone (*Haliotis iris*) (Poore, 1972). The black-footed abalone (*H. iris*), is the largest abalone species in New Zealand, reaching a maximum of 195 mm in shell length (Dutton and Tong, 1981). Due to its larger size, the endemic New Zealand black-footed abalone or locally known in Māori language as pāua is an important food source for the indigenous people, who have harvested this species for at least 800 years (Smith, 2011).

H. iris is a shallow-water species that lives in both sheltered and exposed shores in sublittoral and subtidal areas of both main islands of New Zealand (North and South Island), Chatham Islands, Stewart Islands and the Snares Islands (Sainsbury, 1982). It is habitually most abundant at less than 5 m water depth, but its distribution may extend to 20 m in depth (Sainsbury, 1982, Schiel *et al.*, 1997). In the wild, the behaviour of the juvenile and adult *H. iris* is sedentary; with the presence of food and habitat disturbance that may influence their movement (Poore, 1972; Sainsbury 1982). However, pāua are often found growing in aggregations or large clusters where an abundance of natural diets (seaweed), preferably the Giant kelp (*Macrocystis pyrifera*) occur (Poore, 1972).

Like other abalone species, *H. iris* has a convex shell with a short oblique spire with five to seven raised holes along the shell margin (Figure 1.1.). These holes are used for the process of respiration, sanitation and reproduction. The shell lip is continuous and is produced beyond the body whorl.



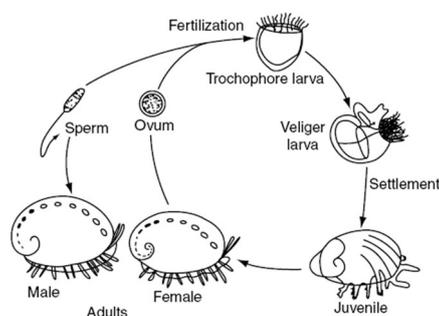
Source: Moana.co.nz

Figure 1.1. The New Zealand Black-footed Abalone (*H. iris*)

A ruffle of tissue (called the epipodium) lines the edge of the wide, muscular foot which the animal uses to crawl over hard substrates. The foot muscle is black in colour

with tubercles along the edges. The *H. iris* is also known to precipitate calcite and aragonite in their shells (Gray and Smith, 2004). Therefore, the composition and thickness of the mineral layers of the shells affect the color appearance. The inside of Pāua shells possess a pearly multi iridescent colour of Prussian blue and green predominating, but also with reflections of purple, orange and a little red. Thus, the shells are also valued as an important component in the jewellery and souvenir business in New Zealand (Gray and Smith, 2004).

Adult abalone have no discernible sexual dimorphism, except for the colour of the gonad, which is green for females and beige for males (Wilson and Schiel, 1995). Abalone are dioecious broadcast spawners with relatively high fecundity (7 million eggs) of females of about 140 mm (Wilson and Schiel, 1995, Webber, 1977; Poore, 1973). There are a variety of factors that regulate the spawning and gametogenesis of abalone, including sea-water temperature (Poore, 1973), physical disturbances, food supply, and genetic and hormonal factors (Webber and Giese 1969; Tutschulte and Connell 1988; Shepherd *et al.* 1985). Fertilisation of gametes occurs in the water column (Figure 1.2.), and is followed by a planktonic larval stage, which lasts less than 10 days (Tong *et al.* 1992; McShane, 1992). Thus, the successful establishment of abalone in the wild is influenced by many factors, such as the dispersal during the planktonic phase, and the ability of the organism to make an effective transition from larval to adult stage (McShane, 1995).



Source: Olin and McBride (2000)

Figure 1.2. The abalone reproduction cycle

There are many important biological processes that take place during metamorphosis, which is the transition from larval settlement to post-larval grazing (Tung and Alfaro, 2011 a). Metamorphosis in abalone has been shown to be mediated by chemical signals (e.g. GABA) produced by biofilms and coralline algae occurring in the environment (Wieczorek and Todd 1997; Roberts *et al.* 2007). Indeed, biofilms cover nearly every living and inert surface and play an important role in the lives of marine invertebrates and the food webs they are a part of (Pawlik, 1992).

From a genetic distribution standpoint, the abalone are present within at least four phylogeographic breaks occurring across the Chatham rise, in the western Cook Strait region, along the southeast coast of the South Island, and at East Cape in the North Island, with low to moderate levels of genetic differentiation (Will *et al.*, 2011). The distribution of abalone throughout New Zealand waters is thought to be closely linked to the effect of high energy currents that promote dispersal and transport of larval abalone (Stephens *et al.*, 2006). There are six major offshore currents (Laing and Chiswell, 2003), however dispersion and retention of larvae may also be affected with upwelling, eddies, river plumes (Sponaugle *et al.* 2002; Schiel 2004).

1.1.3. *Abalone Production and Market*

H. iris is highly valued by both non-commercial and commercial sectors since it has a cultural significance. This species has been commercially harvested for shell since the 1940s and for meat since the 1960s (Schiel, 1997), although recreational and illegal harvests still occur. The New Zealand's abalone fishery largely supplies an international market (Hooker and Creese, 1995; Cook and Gordon, 2010) where the commercial value of New Zealand abalone currently is valued from the exported products of fresh, frozen and processed product. In 2016, New Zealand exported \$37.5 million worth of abalone with a weight of 763.5 tonnes from processed products, 37 tonnes from frozen products and 29.1 tonnes from fresh products (Seafood New Zealand, 2016). The top destination

countries for New Zealand abalone exports in 2016 were Singapore, Hong Kong, People Republic of China, Malaysia and Australia (Seafood New Zealand, 2016). However, the export of abalone is limited by the total allowable commercial catch (TACC) each year. In 2010, the TACC for abalone was 1,058,499 kg, while the reported catch was 1,010,618 kg (Ministry for Primary Industries, 2015).

Currently, the production of abalone from the commercial fishery sector in New Zealand is managed by the Pāua Industry Council Ltd. This organization is an overarching service agency for five regional commercial stakeholder groups representing commercial Pāua fishery interests. Each regional representative group (PāuaMAC - derived from the Quota Management System designation for Pāua, and Management Area Council) draws its membership and mandate from fishing and non-fishing quota owners, ACE holders, permit holders, processors and exporters from within seven designated management areas. The Pāua industry is designed as a "bottom up" driven organisational structure, with decision making primarily resting with individual quota owners and dive crews. The primary aims of these groups are firstly to work to ensure the health of the New Zealand Pāua fisheries and secondly to act as advocate for its members interests (<http://www.Pāua.org.nz>).

1.1.4. Abalone Aquaculture in New Zealand

With increasing pressure being placed upon natural stocks of *H. iris*, aquaculture has been developed as an alternative source of shellfish for export purposes (Stuart & Brown, 1994). Aquaculture was sought as the best solution to provide a source for a sustainable production of abalone. The history of abalone aquaculture in New Zealand could be traced back to the late 1970's, when there was a considerable interest in spawning, culture and rearing of *H. iris* based on hands-on workshops in Pāua culture (Schiel, 1997). In the early phase of cultivation, abalone were also experimentally propagated for restocking wild populations (Tong, 1983). Optimization of abalone

aquaculture was developed by adapting techniques from other *Haliotis* species (Hahn, 1989 a, b) or developing innovative techniques from research in various fields of pāua culture.

The aquaculture of *H. iris* has contributed greatly to the New Zealand economy with an annual production of 80 tonnes in 2010 (Gordon and Cook, 2013) and increase to 100 tonnes in 2011, gaining a value of \$5 million (Forest and Bird, 2017). However, currently only one large scale commercial abalone farm exist in New Zealand, which is Moana New Zealand Abalone and was previously known as OceanZ Blue, located in Ruakaka Northern New Zealand. The farm has a capacity to produce up to 120 metric tons, or 2 million individual shells using a precise breeding regimen. The animals are produced through the full life cycle culture from spawning to spat, then to market size as a cocktail size abalone (of approximately 75mm length) which takes three and a half years to reach market size (Kearns, 2016). Moana New Zealand Abalone is only the fourth abalone producer globally to have recognition by the Aquaculture Stewardship Council, an independent non-profit organisation which sets a standard for sustainable aquaculture (Aquaculture Stewardship Council, 2017).

New Zealand has many regulations around development of the aquaculture industry. Thus, commercial cultivation requires compliance with local, regional and national laws, such as the Resource Management Act (1991) which requires impact assessments for commercial development of land and use of natural resources (New Zealand Legislation, 2017). Land-based aquaculture systems in New Zealand must fulfil technical compliance, such as water intake and water discharge (effects of the discharge and on-going monitoring may be required). Other permissions are required to trade aquaculture products and harvest resources from the wild, such as natural seaweeds if needed. Such procedures have limited the development of aquaculture abalone farms. A

census in 1996 showed that there were a number of abalone farms across the country (Schiel, 1997), however it is not known whether they are still operating at present.

1.1.5. Diseases in Abalone

Abalone diseases and outbreaks have occurred for a variety of abalone species around the world where it increases significantly with the expansion of the abalone aquaculture. Although infectious diseases rarely threaten the existence of a species in the wild (Smith *et al.* 2006), there were cases of infectious diseases causing extinction on local extent (Ben-Horin *et al.* 2013). In these cases, pathogens maintain high incidence and the ability to spread efficiently even as the susceptible host population declines, either through frequency-dependent transmission or the presence of a reservoir host species (de Castro and Bolker 2005). The main microbial diseases affecting marine cultured bivalves have been revised on the basis of the etiologic agents, pathogenesis and pathogenicity. Several recent bivalve-interaction models have been studied, including *Pecten* larvae-*Vibrio pectinica*, brown ring disease, juvenile oyster disease, Pacific oyster nocardiosis and summer mortalities of oysters. In addition, the taxonomy and phylogeny of new potential bivalve pathogens and their virulence factors have been established.

The ambiguity often exists when trying to establish whether a bacterial infection is a primary or secondary cause of disease. Therefore approaches have been developed to correlate the phenotype and genotype of potential pathogens. Evaluation of virulence mechanisms should be conducted on novel diagnostic tools to characterize the genes implicated in pathogenesis (Paillard *et al.*, 2004). Since the 1950's, various pathogens which have been attributed to a range of pathogens including prokaryotic bacteria, eukaryotic parasites and viral organisms, have been identified as causing clinical signs of disease and mass mortalities of abalone in the wild and cultured environments (Table 1.1.)

Table 1.1. Pathogens that affect abalone in wild and cultured environments

Author	Abalone Species	Pathogens
Millemann, 1951	<i>Haliotis</i>	Parasite - <i>Echinocephalus pseudouncinatus</i>
Elston and Lockwood, 1983	<i>Haliotis rufescens</i>	Bacteria - <i>Vibrio</i>
Bower, 1987	<i>Haliotis</i>	Parasite - <i>Labyrinthuloides haliotidis</i>
Lafferty <i>et al.</i> , 1993	<i>Haliotis cracherodii</i>	Bacteria
Goggin and Lester, 1995	<i>Haliotis</i>	Parasite – <i>Perkinsus</i>
Gardner <i>et al.</i> , 1995	<i>Haliotis cracherodii</i>	Parasite – <i>Prokaryotes</i>
Nishimori <i>et al.</i> , 1998	<i>Haliotis diversicolor</i>	Bacteria - <i>Vibrio carchariae</i>
Shields <i>et al.</i> , 1998	<i>Haliotis</i>	Parasite – Sabellid worms
Anguiano-Beltrán <i>et al.</i> , 1998	<i>Haliotis rufescens</i>	Bacteria – <i>Vibrio</i>
Nakatsugawa <i>et al.</i> , 1998	<i>Haliotis discus</i>	Virus
Karpov <i>et al.</i> , 2000	<i>Haliotis spp.</i>	Bacteria
Liu <i>et al.</i> , 2000	<i>Haliotis diversicolor</i>	Bacteria – <i>Vibrio parahaemolyticus</i>
Moore <i>et al.</i> , 2000	<i>Haliotis rufescens</i>	Parasite – Rickettsiales
Friedman <i>et al.</i> , 2000	<i>Haliotis spp.</i>	Parasite – <i>Candidatus xenohaliotis</i>
Lee <i>et al.</i> , 2001	<i>Haliotis diversicolor</i>	Bacteria – <i>Vibrio</i>
Nicolas <i>et al.</i> , 2002	<i>Haliotis tuberculata</i>	Bacteria – <i>Vibrio carchariae</i>
Raimondi <i>et al.</i> , 2002	<i>Haliotis cracherodii</i>	Bacteria –
Lleonart <i>et al.</i> , 2003	<i>Haliotis spp.</i>	Parasite – Spionid worm
Reece <i>et al.</i> , 2003	<i>Haliotis iris</i>	Parasite – Haplosporidian
Lee <i>et al.</i> , 2003	<i>Haliotis spp.</i>	Bacteria – <i>Vibrio parahaemolyticus</i>
Friedman <i>et al.</i> , 2003	<i>Haliotis spp.</i>	Bacteria
Chang <i>et al.</i> , 2005	<i>Haliotis diversicolor</i>	Virus – Herpes
Handlinger <i>et al.</i> , 2005	<i>Haliotis rubra</i>	Bacteria – <i>Vibrio harveyi</i> , <i>Vibrio splendidus</i>
Lester and Hayward <i>et al.</i> , 2005	<i>Haliotis</i>	Parasite – <i>Perkinsus</i>
Huchette <i>et al.</i> , 2006	<i>Haliotis tuberculata</i>	Parasite – Shell disease
Balseiro <i>et al.</i> , 2006	<i>Haliotis tuberculata</i>	Parasite – <i>Xenohaliotis</i> ; <i>Haplosporidium</i>
Cai <i>et al.</i> , 2006 a	<i>Haliotis diversicolor</i>	Bacteria – <i>Vibrio parahaemolyticus</i>
Cai <i>et al.</i> , 2006 b	<i>Haliotis diversicolor</i>	Parasite – <i>Shewanella</i>
Cai <i>et al.</i> , 2006 c	<i>Haliotis diversicolor</i>	Bacteria – <i>Vibrio alginolyticus</i>
Simon <i>et al.</i> , 2006	<i>Haliotis midae</i>	Parasite – Polychaetes
Hooper <i>et al.</i> , 2007	<i>H. laevigata</i> ; <i>H. rubra</i>	Virus – Ganglioneuritis
Cai <i>et al.</i> , 2007	<i>Haliotis diversicolor</i>	Bacteria – <i>Vibrio parahaemolyticus</i>
Sawabe <i>et al.</i> , 2007	<i>Haliotis discus</i>	Bacteria – <i>Vibrio harveyi</i>
Tan <i>et al.</i> , 2008	<i>Haliotis spp.</i>	Virus – Herpes-like
Cai <i>et al.</i> , 2008	<i>Haliotis diversicolor</i>	Bacteria – <i>Klebsiella oxytoca</i>
Travers <i>et al.</i> , 2008 b	<i>Haliotis tuberculata</i>	Bacteria – <i>Vibrio harveyi</i>
Moore <i>et al.</i> , 2009	<i>H. fulgens</i> ; <i>H. rufescens</i>	Virus – Withering syndrome
Rogers-Bennett <i>et al.</i> , 2010	<i>Haliotis</i>	Bacteria
Corbeil <i>et al.</i> , 2010	<i>H. laevigata</i>	Virus – Herpes like
Kamaishi <i>et al.</i> , 2010	<i>H. gigantea</i>	Parasite – <i>Francisella</i> sp.
Neuman <i>et al.</i> , 2010	<i>Haliotis cracherodii</i>	Bacteria
Corbeil <i>et al.</i> , 2012	<i>Haliotis cracherodii</i>	Virus - Ganglioneuritis
Ben-Horin <i>et al.</i> , 2013	<i>Haliotis</i>	Bacteria
Jiang <i>et al.</i> , 2013	<i>Haliotis discus hannai</i>	Bacteria - <i>Vibrio</i>
Crane <i>et al.</i> , 2013	<i>Haliotis</i>	Virus - Ganglioneuritis
Crosson <i>et al.</i> , 2014	<i>Haliotis</i>	Virus – Withering syndrome
Corbeil <i>et al.</i> , 2016	<i>Haliotis iris</i>	Virus - Ganglioneuritis
Dubief <i>et al.</i> , 2017	<i>Haliotis tuberculata</i>	Bacteria – <i>Vibrio</i>
Corbeil <i>et al.</i> , 2017	<i>H. laevigata</i>	Virus – Herpes

Unfortunately, there are only a few studies that have focused on immune responses of *H. iris* to diseases, and it is clear that this is a major gap in our knowledge and a hindrance to further advances in cultivation of this species.

1.1.5.1. Bacterial Pathogen in Abalone

Pathogenic bacteria induces infection through entry of pathogen and establishment and multiplication, as a result causing damage to host cells and tissues (Donnenberg, 2000). In the *Haliotis* genus, the most common disease in abalone species is the withering foot disease which is a syndrome for a variety of abalone disease causing severe atrophy of foot muscle due to the infections of the gastrointestinal tissue and makes the abalone easy to remove from its substrate (Haaker *et al.*, 1992; Gardner *et al.*, 1995; Lafferty and Kuris, 1993). Several pathogenic bacterial strains were responsible for the withering disease. Among the species were the *Candidatus xenohaliotis californiensis* (Balseiro *et al.*, 2006), *Vibrio parahaemolyticus* (Liu *et al.*, 2000), *Vibrio carchariae* (Nishimori *et al.*, 1998) and Withering Ryndrome Rickettsiales-like prokaryote (WS-RLP) (Friedman *et al.*, 2000). Infections signs may vary among species, however the suspected pathogen *Vibrio carchariae* caused white spots on the foot muscle, necrotic degeneration of muscle fibers and lesions (Nishimori *et al.*, 1998).

Bacterial pathogen could also affect the larval and post-larval stage of abalone and may lead to cases of mass mortality. Previously, mass mortality of larval abalone in cultured *H. diversicolor supertexta* in China was caused by *Klebsiella oxytoca* (Cai *et al.*, 2006 a; Cai *et al.*, 2008) while the *Francisella* sp. bacterium caused mass mortality of *H. gigantea* (Kamaishi *et al.*, 2010). However, the event of mass mortality may be related to specific environmental conditions such as the effect of temperature, oxygen supersaturation or other factors at the time of the event which increased the morbidity of the disease.

Opportunistic bacterial pathogens are also threats in abalone culture and normally found in moribund abalone. Commonly occurring, if not ubiquitous bacterial pathogens may affect cultured organism if physico-chemical environmental parameters are not

favourable (Elston and Lockwood, 1983). There are a number of bacterial pathogens falling to this category such as *V. aginolyticus* which affects *H. rufescens* (Elston and Lockwood, 1983), *Vibrio harveyi*, *V. splendidus* I and *Flavobacterium*-like species which affects *H. rubra*, *H. laevigata* and their hybrids causing clinical signs include loss of foot pigmentation, localized swelling (Handler, 2005).

Currently there has been little information provided regarding diseases of cultured Pāua (*H. iris*) in New Zealand. However, studies have provided information of pathogens that are able to cause mortality. Among the pathogens are the prokaryotic bacteria infections where *Flexibacter/Cytophaga*-like rods, and short *Vibrio*-like rod shaped bacteria were found to cause erosion of the mantle epithelium (Diggles *et al.*, 2002).

1.1.5.2. Eukaryotic Parasites

A diverse range of eukaryotic parasites (8 – 1800 µm), such as nematodes (platyhelminthes), protozoa, annelids, haplosporidia and perkinsozoan, have been found to affect abalone. These parasites often are species-specific and may have a wide variety of infection modes. For example, spionid polychaetes tend to bore in the shell to reach the mantle tissue of *H. midae* (Simon *et al.*, 2006). Other annelids may infect the foot tissue causing clinical signs of blistering (Millemann, 1951). The protozoan *Labyrinthuloides haliotidis* produces infective flagellated zoospores which can be seen in the mantle tissue, the muscle and the nervous tissues of *H. rufescens* (Bower, 1987).

Perkinsus is an important eukaryotic parasite on abalone, and is able to cause mass mortalities (Goggin and Lester, 1995). The zoospores of this intracellular parasite enters the body of the abalone through the digestive organ. Once developed, infections are often visible as brown nodules (0.5 to 8 mm in diameter) on the upper part of the foot, the mantle or as internal pustules (Goggin and Lester, 1995). The experiment conducted by Lester and Hayward, (2005), suggests infections was caused by abscesses

that rupture and release zoospores. However, abalone are not necessarily killed directly by *Perkinsus*, since it could maintain levels of low infection for several months even at temperature above 19°C (Lester and Hayward, 2005). However field observations found that *Perkinsus olseni* is highly pathogenic (Park and Choi, 2001).

Other parasite which should be noted are haplosporidians, which normally parasitize numerous invertebrate marine hosts causing heavy mortalities, and are the cause of significant economic losses in bivalve mollusks aquaculture around the world (Perkins, 1990; Hallett *et al.*, 2001; Hine and Thorne, 2002; Bower *et al.*, 1994; Burreson and Ford, 2004; Cranfield *et al.*, 2005). Haplosporidian parasites are less likely to be found in Haliotids (Balseiro *et al.*, 2006), however this parasite has been detected in a number of Haliotids, including the *H. iris* from New Zealand (Diggles *et al.*, 2002; Reece and Stokes, 2003).

Observations of haemocytes conducted by Diggles *et al.* (2002) on *H. iris* infected with a novel haplosporidian parasite observed that relatively few or no host haemocytes were visible in heavily infected Pāua compared to uninfected animals. The following research by Diggles and Oliver (2005), also observed haemocytes from abalone suffering with inflammatory lesions. The syndrome was characterised by focal areas of degraded muscle with prominent voids containing large numbers of haemocytes.

1.1.5.3. *Viral Pathogens*

Diseases caused by viral pathogens can also affect abalone. The notable herpes-like virus which causes abalone viral ganglioneuritis (AVG) has been documented to cause mass mortalities of farmed abalone in Victoria, South Australia (Hooper *et al.*, 2007 a). Dead abalone were observed to have no significant gross lesions. However histological examination showed lesions on the nerves innervating the labial apparatus, primarily the cerebral and buccal ganglia, cerebral commissure and peripheral nerve (Hooper *et al.*, 2007). Haemocyte infiltration were also observed in affected nerves.

However, the AVG virus surprisingly seems to be species-specific and appears to not affect pāua (*H. iris*), based on experimental challenges (Corbeil *et al.*, 2017). Other virus pathogens have been reported in abalone, such as the virus causing the crack-shell disease of *H. hannai* and *H. diversicolor* (Zhengli and Handlinger, 2004) and virus causing amyotrophia in *H. discus* (Nakatsugawa *et al.*, 1999). In general, a significant route of infection in aquatic organisms occur is when virus is shed from infected animals and affect nearby populations (Wolf, 1988; Chou *et al.*, 1998; Schikorski *et al.*, 2011). However further studies are needed to identify their mode of transmission, mechanism of infection and the immunological responses of the host to various pathogens under different environmental conditions.

1.1.6. Abalone Immune System

Abalone depend on innate immunity and they do not possess an adaptive immune system to fight infectious non-self agents, such as pathogenic microorganisms (Elvitigala *et al.*, 2013). Invertebrates in general show pathogen recognition processes through molecular mechanisms and effectors similar to those employed by vertebrates in eliminating pathogens and parasites (Hoffman *et al.*, 1999; Medzhitov & Janeway, 2000). In order to gain in-depth knowledge of this topic, most studies have used the observation of haemocytes as a technique to investigate molluscan immunity.

The immune system of abalone is a system of biological structures and processes that protects them against disease and other stresses. It consists of a cellular immune system and humoral response (Hooper *et al.*, 2006). Blood cells or haemocytes are the primary cells which circulate within the open circulatory system of gastropods (Bayne, 1973). Further, the haemocytes possess functions similar to macrophages in vertebrates (Loker, 2010; Emilia, 2011) where haemocytes are actively involved in immune defence with movement based on protein-mediated recognition of non-self molecules (Nikapitiya *et al.*, 2008; Wang *et al.*, 2008). The characterization of haemocytes have been conducted

in the species of *H. diversicolor* (Chen, 1996), *H. asinina* (Sahaphong *et al.*, 2001), *H. rufescens* and *H. cracherodii* (Martello and Tjeerdema, 2001; Martelo *et al.*, 2000), *H. tuberculata* (Travers *et al.*, 2008 a), *H. discus discus* (Donaghy *et al.*, 2010) and *H. laevigata* (Dang *et al.*, 2011). The characterization was applied using cytochemical staining and observation of phagocytosis activity followed by direct observation of cells under the microscope. However, the classic staining techniques applied may not produce reliable results since it depends on post-collection handling and/or variable staining approaches. In addition, flowcytometry instruments has been used to measure total cell populations and discriminate abalone haemocyte subtypes, based on cell size and internal complexity measured through specific flow cytometric parameters (Travers *et al.*, 2008 a; Donaghy *et al.*, 2010).

The characterization of different type of haemocytes from various resulted different terminology of haemocytes. Interestingly, granular features have been rarely observed in the haemolymph of abalone and even gastropod counterparts (Travers *et al.*, 2008 a; Hooper *et al.*, 2007; Chang *et al.*, 2005; Nakatsugawa *et al.*, 1999; Zhengli and Handler, 2004; Otsu and Sasaki, 1997). The presence of granulocytes in the haemolymph was only reported in *H. asinina* (Sahaphong *et al.*, 2001). It was suggested that the usual hyalinocyte and granulocyte classification might be unsuitable for haemocytes of marine gastropods (Martello and Tjeerdema, 2001). However, the classification of abalone haemocyte cells is also problematic since researchers have used different names and descriptions for the various cells observed (Travers *et al.*, 2008).

The New Zealand abalone (*H. iris*) haemocytes have previously been observed upon abalone infected with haplosporidian parasite, however no characterization study was conducted (Diggles *et al.*, 2002). The attempt characterize haemocytes in *H. iris* resulted identification of three types of cells, which were stem cells (Type I), hyalinocytes (Type II) and fibrocytes (Type III) ((Nollens *et al.*, 2004). However, the description of

fibrocyte cells was not clear and could not be found in our initial study. It is possible that this may have been the result of misidentification. Further studies of the different cell types in *H. iris* will be required using more accurate and effective means to describe and quantify haemocyte cells.

The abalone immune system functions through a complex signalling system which is initiated if antigens are found by two major signal transduction pathways, the NF- κ B and MAPkinases. The series of signalling systems eventually stimulate the synthesis of immune effectors (De Zoysa *et al.*, 2010 a, 2010 b; Jiang and Wu, 2007). Elimination of antigens in invertebrate are then conducted through internalization and phagocytosis of antigens (Bayne, 1990; Travers *et al.*, 2008 b), then enzymatic elimination occurs by means of acid phosphatase activity and reactive oxygen species (ROS), such as hydrogen peroxide produced by the haemocyte (Tiscar and Mosca, 2004). However, ROS production must be available in a certain amounts to avoid toxic effects. Therefore, ROS could be suppressed by “ROS-scavengers”, such as superoxide dismutase – SOD (Ekanayake *et al.*, 2006; Kim *et al.*, 2007; Li *et al.*, 2010). Other effectors are also present in the haemolymph, which act as immune helpers, such as the ion holders and the phenoloxidase pathway that could assist in limiting pathogen proliferation in abalone (Cheng *et al.*, 2004 a; Travers *et al.*, 2008 b).

It is now well established from a variety of studies that the immune system of abalone especially haemocytes is also affected by physico-chemical changes in the aquatic environment. For example, *in vivo* exposure of the red abalone (*H. rufescens*) and the black abalone (*H. cracherodii*) upon pentachlorophenol (PCP) combined with salinity stress resulted reduction of chemotactic ability in both species (Martello *et al.*, 2000). The red abalone (*H. rufescens*) also had a reduction in phagocytic ability where as the black abalone (*H. cracherodii*) was relatively resilient upon salinity stress alone since it was known that abalone demonstrate greater phagocytic ability than red abalone both in the

presence and absence of PCP (Martello *et al.*, 2000). During a salinity stress experiment upon *H. diversicolor supertexta* conducted by Cheng *et al.* (2004 a), it was concluded that the abalone transferred from 30‰ to 20, 25 and 35‰ had reduced immune ability observed from the immune parameters of THC (total haemocyte count), phenoloxidase activity, respiratory burst, phagocytic activity. In a separate experiment designed by Cheng *et al.* (2004 b) to measure immune function upon water temperature, it was concluded that transfer of abalone from 28°C to 32°C reduced their innate immunity and resistance observed from the total haemocyte count, phenoloxidase activity, respiratory burst, and phagocytic activity. The study on *H. diversicolor* investigated the effects of different benzo(a)pyrene (B(a)P) concentration of 0.01, 0.02, 0.04 and 0.08 mg L⁻¹ for 7 days (Gopalakrishnan *et al.*, 2011). It was concluded that B(a)P was found to decrease significantly the total number of circulating haemocytes. Further, intracellular superoxide anion generation and phenoloxidase significantly increased on exposure to B(a)P, whereas phagocytic activity was decreased significantly at higher concentration (Gopalakrishnan *et al.*, 2011). In all of these cases, it is well-established that abalone will try to maintain homeostasis when exposed to external factors which cause stress. Parameters such as total haemocyte count (THC), phenoloxidase activity, respiratory burst activity (release of superoxide anion), antibacterial activity, NRR time and phagocytic activity have been known to be altered during stress condition (Moore *et al.*, 2000; Cheng *et al.*, 2004 a; Hooper *et al.*, 2014). Evidently, stress also alters the disease resistance ability and survival in abalone (Malham *et al.*, 2003; Elston and Lockwood, 1983; Hooper *et al.*, 2007). The status of the cellular defence related parameters usually reflect the health and physiological condition of the animal with respect to the condition of the aquatic environment (Chu, 2000). Thus, poor environmental conditions, such as elevated temperatures may lead to the disease outbreaks upon abalone in the natural in the aquaculture environment (Handlering *et al.*, 2005; Travers *et al.*, 2008 b).

1.1.7. Development of a Toolbox to Observe Abalone Immune Parameters

A considerable amount of literature regarding marine invertebrate immunology is available, and these contain various methods to observe the immune system of abalone and its response to biological or environmental stressors. Some of these techniques and approaches are discussed below.

1.1.7.1. Flow Cytometry Cell Analysis

Relatively little is known about the *H. iris* haemocytes and their defence mechanisms (Nollens *et al.*, 2004; Diggles *et al.*, 2002). In addition, the existing knowledge of haemocytes vary regarding the name, designation and function of different haemocyte cells from different species of the *Haliotis* genus (Sahaphong *et al.*, 2001, Travers 2008 a; Chen *et al.*, 1996; Martello and Tjeerdema, 2001; Martelo *et al.*, 2000; Travers *et al.*, 2008; Donaghy *et al.*, 2010 and Dang *et al.*, 2011). Thus, there is a need to resolve and optimise flow cytometry techniques for abalone haemocyte analyses.

Currently, flow cytometry is frequently used in shellfish immunology research to provide high-dimensional quantitative measurements of fluorescence and light scatter properties of a large number of individual cells within a sample. Rapid, accurate and direct quantification can be conducted to obtain information regarding the morphology and function of a large number of individual cells, hence leading to a better understanding of the immune system (Allam *et al.*, 2002; Goedken and De Guise, 2003; Donaghy *et al.*, 2009).

In the field of medical laboratory, flow cytometry is used to observe blood cells with sophisticated devices capable of observing various blood parameters related to the state of health (Green and Wachsmann-Hogiu, 2015). One of these advanced technologies is the Sysmex XN – 1000 automated analyser. In the field of biomedical research, this instrument is capable to provide complete counts of human White Blood Cells (WBC) and Nucleated Red Blood Cell (NRBC). The blood cells are analyzed by flow cytometry-

based optical measurement which could generate scattergrams based on detection of fluorescent cell signal according to cell volume, structure and fluorescence from blood cells that have been binded to a fluorochrome (Becker *et al.*, 2016). To date, no study has used this innovative tool for haemocyte of abalone.

In addition to the Sysmex XN – 1000 automated analyser, there is also a portable cell analyser, namely the Muse® Cell Analyser, which has advantages such as detecting cell health paramaters using specific kits provided by the manufacturer. The Muse® Cell Analyser packs 3-parameter analysis into a compact, easy-to-use benchtop device, making flow cytometry easily accessible. It is equipped with a user-friendly touchscreen interface, intuitive cell analysis software and optimized assays that work to simplify the research. The advantages of the Muse® Cell Analyzer is it is available to provide quantitative data at the single cell level with simple, effortless operation, rapid setup and analysis. It also has a compact size and basically is an affordable flow cytometry technology capable to perform a variety of optimized Muse® assays measuring, such as viability, apoptosis, autophagy, oxidative stress and cell signalling. The Muse® instrument is also equipped with the software for data acquisition. The micro capillary flow cell is engineered for acquisition of both suspension and adherent cells 2-60 microns in diameter. Prior to this study the Muse® Cell Analyser had never been used for marine invertebrates, and this technology promises to revolutionize the investigation of molluscan haemocytes, with endless applications.

1.1.7.2. *Metabolomics*

A biotechnological approach that has recently become available to study the pathophysiology of abalone is the study of metabolomics which observe chemical fingerprints or metabolites that are related to a specific cellular processes (Bennett, 2005). Cellular metabolites are the most sensitive to environmental changes thus information

retrieved from a metabolomics analysis could also provide information regarding the phenotype of the organism living in specific conditions (Patti *et al.* 2012). Recently, this innovative approach has been developed and applied over the past few years on molluscan species in New Zealand and has become a powerful tool to monitor molluscan health (Alfaro and Young, 2016; Young *et al.*, 2016).

Previous studies have utilized metabolomics to provide a rapid, inexpensive and multibiomarker analysis upon the effects of biological stressors upon the *Haliotis* genus. Metabolomics have revealed interesting findings in abalone such as gender, time and tissue specific metabolic responses in some studies (Lu *et al.*, 2016; Lu *et al.*, 2017). One of the first studies in the abalone was the discovery-based approach was utilized to investigate the withering syndrome on the red abalone (*H. rufescens*) along the Pacific coast of the United States using Nuclear Magnetic Resonance (NMR) metabolomics. The authors found that this method was able to distinguish biochemical profiles and obtain metabolic biomarker associated with the withering syndrome (Viant *et al.*, 2003). Using the same method and species, the effect of food availability, temperature, and bacterial infection was observed on the metabolic status of red abalone, and it was found that the glucose-to-homarine ratio was a potential marker for differentiating Withering Syndrome Rickettsiales-like prokaryote (WS-RLP) infection (Rosenblum *et al.*, 2005). Rosenblum *et al.* (2006) also observed the sequential metabolic changes that occur during pre-clinical WS, and demonstrated the application of metabolic phenotyping for understanding environmental effects on host-pathogen-drug interactions. Metabolomics analysis has also been shown to be a robust tool in other abalone research, such as the effect of toxins (Zhou *et al.*, 2015) and thermal and hypoxic environmental effects (Lu *et al.*, 2016; Tripp-Valdez *et al.*, 2017). The metabolic perspective of growth in abalone has been reviewed by Venter *et al.* (2016). The most recent research on abalone that applied metabolomics is the observation of metabolic response in *H. diversicolor* upon *Vibrio parahaemolyticus*

infection where the ^1H NMR spectroscopy together with pattern recognition methods was used (Lu *et al.*, 2017). Metabolic responses of infection was more pronounced in female abalones which is there is an indication of the accumulation of branched-chain amino acids and the depletion of organic compounds in the infected gills and hepatopancreas of female abalones (Lu *et al.*, 2017). Based on major metabolic functions, bacterial infections were able to cause disturbance in energy metabolism, nucleotide metabolism, osmotic balance oxidative stress, immune stress and neurotoxic effect in different tissues with various pathways (Lu *et al.*, 2017).

Development of advance metabolomics strategies in aquaculture samples to enhance sample processing and data interpretation have increased the possibility to obtain a new perspective regarding the health/immune status of a specific sample (Young and Alfaro, 2016). The application of these methods is advantageous for biomonitoring, particularly if the analysis is in conjunction with gene and protein expression profiling. This approach had never been applied to New Zealand black-footed abalone, and has shown to be a highly informative technique which will no doubt have huge application in this research are in the future.

1.1.8. Probiotic as an Application to Enhance the Immune System

Probiotics originated from the Greek word Pro and Bios, which mean for life (Gismondo, *et al.* 1999). Probiotics in aquaculture are referred to a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989). Aquatic animals are quite different from land animals for which the probiotics concept was developed. Thus, a consequence of the specificity of aquatic microbiota is that the most efficient probiotics for aquaculture may be different from those of terrestrial species (Gatesoupe, 1999).

Research on the application of probiotics to enhance the immunity of organisms have been carried out on various aquaculture species. Bacterial probiotic strains, either mono-species or multi-species have been shown to elevate phagocytic, lysozyme, complement, respiratory burst activity, as well as expression of various cytokines in fish (Nayak, 2010). Furthermore, mono-bacterial association studies (with non-probiotic bacterial strains) in gnotobiotic fish also indicate the up-regulation of various immune related genes (Nayak, 2010).

The use of probiotic supplement in the *Haliotis* genus has been previously conducted on several species with significant results in improving disease resistance and growth (Macey & Coyne, 2005; Iehata *et al.*, 2010; Jiang *et al.*, 2013). The supplementation of probiotics on feeds for *H. midae* have shown to serve as an immunoprophylactic control against pathogenic *Vibrio anguillarum* (Macey and Coyne, 2005). Successful research was also conducted by Doeschate and Coyne (2008), who found that the incorporation of probiotic *Pseudoalteromonas* sp. strain C4 in kelp diets improve growth rates of *H. midae*. Recent research done by Jiang *et al.* (2013) showed that the application of probiotics could enhance immunity in abalone (*H. discus*), specifically having the effect of increasing haemocyte numbers, respiratory burst activity, serum lysozyme activity and total protein levels after one week of probiotic administration. That research was one of the first to explore immune modulatory effects on abalone due to dietary effects of two promising probiotics *S. colwelliana* and *S. olleyana*. The bacterial species used was previously tested on in vitro characteristics, such as being non-virulent for abalone, ability to inhibit *Vibrio harveyi*, *Vibrio splendidus* and the ability to promote growth and decrease mortality of juvenile abalone *H. discus hannai* in vivo (Jiang *et al.*, 2013).

In addition, research on the effect of dietary probiotics on immunity of *H. rufescens* was conducted by Silva-Aciaries *et al.* (2013). The research group examined the

transcriptomic and cellular response to both bacterial pathogens and probiotics. Specific genes were analyzed through transcriptomic research using Real-Time PCR, and then compared to immunological responses in the abalone. The results showed that probiotics could increase haemocyte counts and phagocytic counts in that species. Further, specific genes, such as Macrophage Expressed Protein (MEP) and Caspase 8 in abalone fed with probiotics were differently expressed. Caspase 8 is known to be an initiator of the caspase cascade involved in apoptosis as a process for maintaining cellular and tissue homeostasis (Hildeman *et al.*, 2007). In the animals with pathogenic bacteria challenge and fed with probiotics, the expression of the caspase 8 gene increased over the entire experimental period, while the MEP gene expression increased significantly followed by a drastic decrease over time. In several studies with *H. diversicolor*, caspase 8 gene expression has been shown to increase after a bacterial challenge causing the death, mainly of haemocytes (Wang *et al.*, 2008a, 2008b; Huang *et al.*, 2010). In groups exposed to *Vibrio*, application of probiotics has been shown to cause a decrease in caspase 8 expression in the *H. rufescens* after 96 h, compared to animals from groups not fed probiotics, suggesting a decrease in apoptotic processes (Silva-Aciares *et al.*, 2013). The MEP gene, also known as perforin-like gene, has been reported to play a role in pink and red abalone in the process of elimination and death of bacteria (Mah *et al.*, 2004). A decrease of MEP gene expression was thought to correspond to an increase of pathogenic bacteria infecting abalone tissues, and it showed that probiotics could protect abalone (Mah *et al.*, 2004).

The use of probiotic bacteria in *H. iris* has also been studied by Hadi *et al.* (2014) although immunology parameters were not observed, however the survival rate of probiotic fed *H. iris* was significantly higher over a 60 days feeding trial. The main objective of the study was to evaluate the effect of two probiotic feeds containing two and three bacterial isolates upon the growth performance of New Zealand abalone (*H. iris*). The probiotic bacteria for the trial were isolated from the guts of a healthy cultured adult

abalone and probiotic candidates were selected based on the ability to hydrolyse nutrients (i.e. proteins, starch and alginate), produce acid and resist bile salts. Bacterial screening resulted the three bacterial species identified as *Exiguobacterium* JHEb, *Vibrio* JH1, and *Enterococcus* JHLDc using the 16S rRNA sequencing technique. The potential probiotic bacterial strains were utilized in a feeding trial to compare three treatments where the control treatments (unsupplemented feed) were compared to the 2-probiotic conglomerate treatment consisting of *Exiguobacterium* JHEb1 and *Vibrio* JH1 and the 3-probiotic conglomerate consisting of *Exiguobacterium* JHEb1, *Vibrio* JH1 and *Enterococcus* JHLDc. The probiotic feeding trial involved abalone juveniles (20–30 mm in maximum shell length). The study by Hadi *et al.* (2014) showed that both probiotic feeds significantly improved abalone growth compared to that of the unsupplemented feed. The 3-probiotic supplemented feed produced a significant shell length increase of 20.9%, a wet weight gain of 19.8% and a five-fold reduction in mortality compared to the controls. The 2-probiotic supplemented feed resulted in significant increases in shell length (15.4%) and reduced mortality (five- fold), but not in weight gain, compared to controls. The findings from that study offer a high opportunity to study the positive relation between probiotics, immunology and the possibility of probiotics to increase the disease resistance against selected bacterial pathogens.

1.2. Thesis Objective and Aims

This study was conducted based on the lack of information and knowledge gaps in regards to the *H. iris* immune system. The fundamental objective of was to develop a combination of classical and innovative methods to characterize the haemocyte as the main effector of the non-specific *H. iris* immune response. Further objectives were the observation of the immunological and physiological changes of the *H. iris* from an

experiment to compare abalone fed with and without probiotic-abalone. The final objective was to observe the effect of a bacterial pathogen to be used in a challenge to observe the immune response of the abalone (fed with and without probiotic-abalone) during pathogenic infection.

The general aims for this study are to:

- i. Identify and characterize the haemocyte cell types and observe phagocytic capability by implementing a combination of classic and newer innovative techniques for the study of haemocyte cells and their responses to a variety of pathogenic challenges and environmental conditions.
- ii. Develop methods to successfully prepare and preserve haemocyte cells for microscopy and flow cytometry investigation of abalone immune systems
- iii. Determine the effect of feeding with enriched with multi-strain probiotic on survival, growth, selected non-specific immune parameters and metabolite profile of the New Zealand black-footed abalone (*H. iris*).
- iv. Develop a bacterial challenge method upon the New Zealand black-footed abalone (*H. iris*).
- v. Determine the effect of feeding the probiotic enriched diet on disease resistance, survival, selected non-specific immune parameters and metabolite profile of the New Zealand black-footed abalone (*H. iris*).

1.2.1. Thesis Structure

There are a total of six chapters in this thesis. Chapters 1 and 6 are the general introduction and final discussion, respectively. The description from Chapter 2 through 5 are as follows:

Chapter 2: Innovative application of classic and newer techniques for the characterization of haemocytes in the New Zealand Black-footed abalone (*Haliotis iris*).

Overview: Identification using classical methods are conducted to characterize *H. iris* haemocytes and its importance in abalone immunological studies. Innovative applications of flow cytometry were also applied to observe abalone haemocyte cells. Chapter 2 has been published as full paper in the Fish and Shellfish Immunology scientific journal (Grandiosa *et al.*, 2016).

Chapter 3: Effect of antiaggregants on the *in vitro* viability, cell count and stability of abalone (*Haliotis iris*) haemocytes

Overview: This study involves improvement of techniques using antiaggregants to prepare haemocyte for further immunological studies in abalone. Chapter 3 has been published as full paper in the Fish and Shellfish Immunology scientific journal (Grandiosa *et al.*, 2018).

Chapter 4: Multi-strain probiotics enhances immune capacity and alters metabolic profiles in the New Zealand black footed abalone (*Haliotis iris*)

Overview: The study was conducted to observe the immune parameters of abalone fed with probiotic enhanced diet. In addition, the metabolic profile was also observed using innovative metabolomics studies. Chapter 4 has been published as full paper in the Fish and Shellfish Immunology scientific journal (Grandiosa *et al.*, 2018).

Chapter 5: Immune response of the probiotic fed New Zealand Black-footed abalone (*Haliotis iris*) under *Vibrio splendidus* challenge

Overview: This study develops a bacterial challenge with *Vibrio splendidus* upon abalone. Further, we observe the pathophysiological and immune response of probiotic fed New Zealand Black-footed abalone (*Haliotis iris*) under *Vibrio splendidus* challenge compared to control fed abalone. Chapter 5 is in preparation to be submitted to the Journal of Invertebrate Pathology.

1.2.2. Research Outputs

During my doctoral study at the Auckland University of Technology, I have participated in several conferences in New Zealand to present parts of my research. In relation to my study, I also have contributed as a co-author for a publication where I was involved in the development of the methods. The fruitful discussion developed in the Aquaculture Biotechnology Group at Auckland University of Technology have also linked developed methods in my study to be useful for other researchers in AUT or collaborating institutions conducting studies on the immunology of mussels (*Perna canaliculus*), geoducks (*Panopea zelandica*), scallops (*Pecten novaezelandiae*) and lobster (*Jasus edwardsii*) in New Zealand.

A list of these works are listed below.

Oral conference presentations:

- Grandiosa, R., Mérien, F., Alfaro, A.C. (2016). Investigation of the Pathophysiology of the New Zealand Black-footed Abalone (*Haliotis iris*). SASS Presentation, AUT University, Auckland.
- Grandiosa, R., Mérien, F., Pillay, K., Alfaro, A.C. (2016). Innovative application for the characterization of haemocytes In the New Zealand black-footed abalone (*Haliotis iris*). NZMSS Conference Presentation, Victoria University, Wellington, July.

- Grandiosa, R., Young, T., Nguyen V. T., Guitierrez, N., Mérien, F., Alfaro, A.C. (2017). Multi-strain probiotics enhances immune capacity and alters metabolic profiles in the New Zealand black footed abalone (*Haliotis iris*). NZMSS Conference. Canterbury University, Christchurch July.

Poster conference presentation:

- Grandiosa, R., Mérien, F., Alfaro, A.C. (2016) New Immunological Tools To Investigate Marine Invertebrate Haemocytes. NZ Aquaculture Conference Poster, Nelson, September.

Publications as co-author:

- Zha, H., Lewis, G., Alfaro, A., Wang, S., Dong, Y., Grandiosa, R., & Jeffs, A. (2017). Immune status of the spiny lobster *Jasus edwardsii* with tail fan necrosis. *Diseases of Aquatic Organisms*, 126 (3), 229-238.
- Nguyen, T. V., Alfaro, A. C., Merien, F., Young, T., & Grandiosa, R. (2018). Metabolic and immunological responses of male and female New Zealand Greenshell™ mussels (*Perna canaliculus*) infected with *Vibrio* sp. *Journal of Invertebrate Pathology*, 157, 80-89.

CHAPTER 2

Innovative application of classic and newer techniques for the characterization of haemocytes in the New Zealand Blackfooted abalone (*Haliotis iris*)

Note: This chapter has been published with the following citation:

Grandiosa, R., Mérien, F., Pillay, K., Alfaro, A., 2015. Innovative Application of Classic and Newer Techniques for the Characterization of Haemocytes in the New Zealand black-footed abalone (*Haliotis iris*). Fish & Shellfish Immunology. doi:10.1016/j.fsi.2015.11.039

Abstract

Haemocytes play an important role in innate immune responses within invertebrate organisms. However, identification and quantification of different types of haemocytes can be extremely challenging, and has led to numerous inconsistencies and misinterpretations within the literature. As a step to rectify this issue, we present a comprehensive and detailed approach to characterize haemocytes using a combination of classical (cytochemical and phagocytosis assays with optical microscopy) and novel (flow cytometry with Sysmex XN-1000 and Muse® Cell analyser) techniques. The Sysmex XN-1000 is an innovative fluorescent flow cytometric analyser that can effectively detect, identify and count haemocytes, while the Muse® Cell analyser provides accurate and rapid haemocyte cell counts and viability. To illustrate this approach, we present the first report on morphological and functional features of New Zealand black-footed abalone (*H. iris*) haemocyte cells. Two types of haemocytes were identified in this study, including type I (monocyte-like) and type II (lymphocyte-like) cells. Granular cells, which have been reported in other molluscan species, were not detected in *H. iris*. Cell types were categorized based on shape, size, internal structures and function. The lymphocyte-like haemocytes were the most abundant haemocytes in the haemolymph samples, and they had large nuclei and basic cytoplasm. Monocyte-like cells generally were larger cells compared to lymphocyte-like cells, and had low nucleus-cytoplasm ratios. Monocyte-like cells showed higher phagocytic activity when encountering Zymosan A particles compared to lymphocyte-like cells. The present study provides a comprehensive and accurate new approach to identify and quantify haemocyte cells for future comparative studies on the immune system of abalone and other molluscan species.

Keywords: *Haliotis iris*, haemocytes, cell morphology, cell characterization

2.1. Introduction

Numerous studies have been conducted to understand the mechanisms involved in the innate immune system of marine invertebrates, including mussels (Coles and Pipe, 1994; Wootton *et al.*, 2003, Costa *et al.*, 2009), oysters (Hégaret *et al.*, 2003; Lin *et al.*, 2014; da Silva *et al.*, 2008), clams (Matozzo and Bailo, 2015; Munari *et al.*, 2011; Prado-Alvarez *et al.*, 2012; Cima *et al.*, 2000), and abalone (Dang *et al.*, 2011; Travers *et al.*, 2008; Sahaphong *et al.*, 2001; Martello and Tjeerdema, 2001; Cheng, 1981; Vakalia and Benkendorff, 2005). In abalone, such studies have focused on cell-mediated responses (Travers *et al.*, 2008; Sahaphong *et al.*, 2001; Martello and Tjeerdema, 2001; Cheng, 1981), humoral responses (Cheng, 1981; Vakalia and Benkendorff, 2005), and stress responses (Baldwin *et al.*, 1992; Ryder *et al.*, 1994; Malham *et al.*, 2003). While these studies have provided a general characterization of the immune system of abalone, more in-depth investigations are needed to clearly elucidate how the different haemocyte cells function to eliminate pathogens and how the immune system can protect the animal in a pathogen-rich environment without adaptive immunity. As with other marine invertebrates, abalone mostly depend on innate immunity alone to defend against infections caused by pathogenic microorganisms (Elvitigala *et al.*, 2013). However, the issue of whether the abalone immune system is based solely on an innate immune system is challenged by the fact that recent observations of the invertebrate immune system provide the possibility of a degree of specificity and memory in their immune strategies (Rowley and Powell, 2007). Clearly, detailed investigations must be conducted to expand our understanding of the abalone immune system.

The New Zealand black-footed abalone (*H. iris*) is considered to be a high value species with a huge economic potential for aquaculture (Alfaro *et al.*, 2014). This species, also known as pāua, is endemic to New Zealand and is cultivated for the export market

(Hadi *et al.*, 2014). Historically, the culture of *H. iris* began for the purpose of restocking wild populations (Tong, 1983), but is now reared for commercial aquaculture (Tong *et al.*, 1992). Currently, the farm production is approximately 80 tons a year (Wright, 2011). The majority of this product is exported frozen or canned to overseas buyers, with live products being traded in small quantities in local markets (Wright, 2011). The New Zealand abalone industry has a sterling international reputation for providing a clean and healthy product with environmentally friendly and highly sustainable practices (Alfaro *et al.*, 2014).

A key aspect for the New Zealand abalone industry is to enhance the growth and health conditions of their stock. Efforts to increase growth rates have been targeted through selective breeding (Stuart and Brown, 1994), better design of culture systems (Preece and Mladenov, 1999), application of feed stimulants (Allen *et al.*, 2006), optimization of dietary protein and temperature for growth and health (Tung and Alfaro, 2011 a, b, c), and addition of probiotic supplements to their diets (Hadi *et al.*, 2014). However, little information is available regarding the health and potential pathogenic threats on *H. iris* cultured or wild populations.

Several recent studies investigating infectious diseases on abalone have reported the presence of bacterial, viral and parasitic agents. Notable events were mass mortality of postlarval abalone, *H. diversicolor supertexta* (L.) in South China in 2002 caused by pathogenic bacteria (*Vibrio parahaemolyticus*). Viral pathogens, such as the abalone virus ganglioneuritis caused mass mortality of cultured abalone in Australia in 2006 (Hooper *et al.*, 2007). Herpes-like virus infections causing neuropathy were reported in Taiwan (Chang *et al.*, 2005), while amyotrophy was observed in abalone in Japan (Nakatsugawa *et al.*, 1999). Other viral diseases causing significant mortality were also recorded in China in 1999 and 2004 (Zhengli and Handler, 2004; Otsu and Sasaki, 1997; Wang *et al.*, 1999; Wang *et al.*, 2004; Song *et al.*, 2000; Zhang *et al.*, 2001).

To date, there are no records of any outbreaks or reports of major disease threats on New Zealand abalone populations. However, preliminary assessment of health has been made based on available literature in order to identify potential disease threats that could affect the abalone industry (Webb, 2013). Despite the fact that knowledge of aquatic pathogens is constantly evolving and disease epidemiology is very complex in the marine environment, a list of pathogens that may significantly affect *H. iris* has been suggested (Webb, 2013). This list is based on pathogens and diseases identified as potential hazards for other abalone species overseas. The potential pathogen threats are *Haplosporidium* sp. and *Labyrinthuloides haliotidis* (protozoa); *Terebrasabella heterouncinata* (Annelid worm); shell mycosis (fungus); amyotrophy and viral ganglioneuritis (virus), *Francisella* sp., *Xenohalotis californiensis* and *Vibrio harveyi* (Webb *et al.*, 2013). Based on a first commissioned report, there are already measures in place (or being developed) to mitigate the introduction of aquatic pests and diseases into New Zealand (Webb *et al.*, 2013). However, these pathogens remain significant risks and substantial knowledge gaps exist. Additionally, the establishment and spread of new or existing pathogens, and the associated impacts on aquaculture, are virtually impossible to reliably predict (Castinel *et al.*, 2013). In order to evaluate the danger of these potential threats to *H. iris*, basic knowledge of pathogen biology, virulence and transmission conditions are required, as well as a clear understanding of the abalone immune system.

The main defense mechanism of molluscs against pathogens is by utilizing haemocytes which circulate within the open circulatory system (Browne *et al.*, 2008). 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 (Adema, 1991). Thus, these cells have a multifunctional purpose resembling that of macrophages in vertebrates (Loker, 2010; Emilia, 2011). The similarity of invertebrate innate immune response with the vertebrate

counterpart also extends to the mechanism of antibacterial peptides, the regulation of gene expressions and immune stimulators (Lehrer and Ganz, 1999; Salzet, 2001; Bulet *et al.*)

Previous studies have provided brief information of *H. iris* haemocyte characteristics (Nollens *et al.*, 2004) and also various other abalone species, such as *H. diversicolor* (Chen *et al.*, 1996), *H. asinina* (Sahaphong *et al.*, 2001), *H. rufescens* (Martello and Tjeerdema, 2001; Martello *et al.*, 2000), *H. cracherodii* (Martello and Tjeerdema, 2001; Martello *et al.*, 2000), *H. tuberculata* (Travers *et al.*, 2008), *H. discus discus* (Donaghy *et al.*, 2010) and *H. laevigata* (Dang *et al.*, 2011). These studies have used a range of techniques to classify and describe haemocytes, including direct observation of cells under the microscope (light, fluorescent and electron microscopy) before and after application of cytochemicals and phagocytosis assays (Dang *et al.*, 2011; Travers *et al.*, 2008, Sahaphong *et al.*, 2001; Martello and Tjeerdeema, 2001; Donaghy *et al.*, 2010). In addition, flow cytometry has been used to measure total cell populations and discriminate abalone haemocyte subtypes, based on cell size and internal complexity measured through specific flow cytometric parameters (Travers *et al.*, 2008; Donaghy *et al.*, 2010). However, these classic techniques have a great degree of subjectivity, since they often rely on direct observations and/or variable staining approaches. Another problem is the fact that these techniques can be time consuming and may produce unreliable results, depending on post-collection handling and preservation of the cells.

H. iris haemocytes were initially observed following infection with a haplosporidian parasite (Diggles *et al.*, 2002) and in abalone suffering inflammatory lesions (Nollens *et al.*, 2004). More characterization of haemocytes in *H. iris* identified three types of cells, including stem cells (Type I), hyalinocytes (Type II) and fibrocytes (Type III) (Nollens *et al.*, 2004). However, the description of fibrocyte cells was not clear, and may have been the result of misidentification. Further studies of these different cell types are required since observation was limited to simple microscopy methods. In addition, the terminology

used varies among studies and abalone species, leading to a range of confusing terms and inaccurate terminologies. For example, two types of *H. asinina* haemocytes were described as hyalinocytes and granulocytes (Sahaphong *et al.*, 2001), and blast-like cells were also described as haemocyte cells (Travers *et al.*, 2008). To avoid such reporting problems, we need more accurate and effective means to describe and quantify haemocyte cells.

Interestingly, granular features have been rarely observed in the haemolymph of abalone and even gastropod counterparts (Travers *et al.*, 2008; Preece and Mladenov *et al.*, 1999; Hooper *et al.*, 2007; Chang *et al.*, 2005; Nakatsugawa *et al.*, 1999, Zhengli and Handlinger, 2004; Otsu and Sasaki, 1997). The presence of granulocytes in the haemolymph was solely reported in *H. asinina*, but it was suggested that these granulocytes were morphologically different from the ones conventionally observed in bivalve molluscs (Sahaphong *et al.*, 2001). Later, it was suggested that the usual hyalinocyte and granulocyte classification might be unsuitable for haemocytes of marine gastropods (Martello and Tjeerdema, 2001).

Initial characterization of abalone haemocyte cells have been hampered by the fact that different researchers have used different names and descriptions for the various cells observed (Travers *et al.*, 2008). Here, we propose an innovative multi-technique approach to characterize *H. iris* haemocyte cells using a combination of traditional and novel techniques, including conventional microscopy, cytochemical staining, flow cytometry (Sysmex XN haematology analyser, Roche Diagnostics) and automated count and viability assays (Muse® rapid cell analyser, Millipore). The findings of this study form part of a larger project to investigate the immune system of *H. iris* under various biological and physicochemical stressors.

2.2. Material and Methods

2.2.1. *Abalone specimens*

H. iris specimens with a shell length of 55 ± 5 mm and wet weight of 22 ± 1 g were obtained from the abalone farm OceanNZ Blue Ltd (Bream Bay Aquaculture Park, Ruakaka, New Zealand). Immediately upon arrival to the Aquaculture Lab, Auckland University of Technology, Auckland, New Zealand, abalone were transferred to 20 L tanks. The water was continuously re-circulated, aerated and maintained at a temperature of $15\pm 1^\circ\text{C}$ and salinity of 35 ppt. Abalone were allowed to acclimatize in the tanks for 1 month prior to sampling. Animals were fed daily *ad libitum* with an abalone commercial diet (Abmax T20).

2.2.2. *Haemolymph collection*

A total of 20 healthy abalone were removed from the tanks and blotted with paper towels until dry. Haemolymph was taken immediately using a 25 gauge and 5/8" needle attached to a 3-ml sterile syringe (Terumo, Japan). The needle was gently inserted into the central anterior part of the foot muscle where the cephalic arterial sinus is located. As much as 700 μl of haemolymph was collected from each animal. A sterile antiaggregant solution (Modified Alsever's Solution; MAS) was mixed in equal volume with 500 μl of haemolymph from a single abalone in a 1.5 ml Eppendorf tube. Diluted haemolymph specimens were then gently mixed twice to obtain a homogeneous suspension of cells. MAS was prepared with 0.55 g/L citric acid, 8 g/L sodium citrate, 20.5 g/L D-glucose and 4.2 g/L sodium chloride (all from Sigma-Aldrich, USA) in deionized water and autoclaved at 121°C for 15 minutes (Grimes *et al.*, 2002).

2.2.3. Cell count and viability assay

2.2.3.1. Trypan Blue exclusion test

The cell density and viability of hemolymph samples in MAS were observed after 2 and 40 min from withdrawal at room temperature. The Trypan Blue test is a dye exclusion assay based on the principle that live cells possess intact cell membranes that exclude certain dyes (Strober *et al.*, 2001). Viable cells have a clear cytoplasm whereas non-viable cells have a blue cytoplasm. In this test, diluted haemolymph samples were incubated for 5 min in a 1:1 ratio with Trypan Blue 0.2% (Sigma-Aldrich, USA), placed inside a disposable Vetriplast counting chamber (S.A.S., Italy) and observed under a light microscope. The number of dead and live cells were recorded and means were calculated from triplicate abalone samples.

2.2.4. Muse Cell Analyser

Haemocyte cell counts and viability were also assessed with a Muse Cell Analyser (Merck Millipore, USA). The Muse® Cell analyser uses miniaturized fluorescence detection and microcapillary technology for rapid and accurate cell quantitative cell analysis compared to other manual methods. Based on differential permeability of two DNA-binding dyes, the Muse count and viability assay (Abacus, New Zealand) provides rapid and reliable determination of viability and total cell count. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying and dead cells. This proprietary combination of dyes enables the kit to distinguish viable and dead cells with extreme accuracy. Additionally, debris is excluded from the results, based on negative staining with the nuclear dye. Fresh haemolymph samples diluted 1:1 with MAS were incubated at room temperature without shaking for 2 min, 1 hr, 2 hr, 3.5 hr, 5 hr and 24 hr after withdrawal. Fifty μ l of each diluted haemolymph sample were then mixed with 450 μ l of Muse count and viability reagent and incubated for 5 minutes at room temperature as per manufacturer's instructions. Each cell suspension was thoroughly

mixed before acquiring samples for consistent and accurate results. The results obtained were cell counts per millilitre and the percentage of viability with high accuracy.

2.2.5. Flow cytometric identification of haemocytes using the Sysmex haematology XN-1000 modular system

Haemocytes were detected, identified and counted with a Sysmex XN-1000 (Sysmex Corporation, Kobe, Japan) multi-parameter automated haematology analyser based on the principal of fluorescent flow cytometry. A total of 200 μ l of haemolymph diluted 1:1 in MAS from each sample were injected in the analyser. Fluorescent labelling of cells takes place after perforation of the cell membrane caused by specific lysing reagents. After this, a polymethine dye enters the cell and binds to nucleic acid and bio-reactive proteins in the cytoplasmic organelles. Identification of cells is based on cellular structures measured with two types of scattered light (FSC and SFL). Data collected in dedicated channels (white cell nucleated channel WNR and white cell differential channel WDF) were analysed with the on-board XN Software, and differential counts were displayed in scattergrams. The analysis was repeated on 3 replicate abalone samples.

2.2.6. Morphological and cytochemical observations under light microscopy

2.2.6.1. Observation of fresh haemocytes

After withdrawal and dilution 1:1 in anti-clotting MAS, haemocytes from 3 abalone samples were observed under a light microscope without fixation to assess the morphological and physiological cellular features specific to different putative cell types without shrinkages due to fixation. A wet mount was prepared by placing 20 μ l from each diluted sample on separate glass slides and smeared evenly to create a thin homogeneous monolayer. The monolayers were then covered with a cover slip and observed under a Leica DM2000 microscope equipped with a Leica DF 290 camera (Leica Biosystems Pty

Ltd, Australia). Selected images were taken at 630x and 1000x magnification and processed with imaging software (Leica Application Suite).

2.2.6.2. *Cytochemical assay observations*

Haemolymph samples were collected and diluted 1:1 in anti-clotting MAS, as indicated above. Various staining methods were used to observe the haemocyte monolayers (HCM). Two different methods were used to prepare the HCMs, cell adhesion method and cytocentrifugation.

The cell adhesion method was performed, as described above, for the observation of fresh haemocytes. An additional incubation step of 30 min at room temperature was added for the cells to adhere to the glass slides. The cells were fixed and stained with different staining protocols:

- a. *May-Grünwald Giemsa and Giemsa*: HCMs were fixed by immersion with methanol at room temperature for 1 min followed by air drying. For Giemsa staining, HCMs were stained for 3 min with a Giemsa solution (1:4 in deionized water). For May-Grünwald Giemsa (MGG), HCMs were stained by immersion with May-Grünwald (1:1 in deionized water) for 3 min, followed by a Giemsa staining (1:10 in deionized water) for 1 min. Glass slides were then gently washed in deionized water and left to dry.
- b. *Carbohydrate detection (Periodic Acid Schiff)*: Air dried HCMs were fixed with formalin-ethanol fixative solution for 1 minute. The fixative was prepared by mixing 5 ml of formaldehyde with 45 ml of 95% ethanol. Slides were rinsed for 1 min in slow running tap water, and then immersed with Periodic Acid Solution (Sigma-Aldrich, USA) for 5 minutes. Slides were rinsed and then immersed in Schiff's reagent (Sigma-Aldrich, USA) for 15 minutes. Slides were washed again in running tap water for 5 minutes, and counterstaining was performed with haematoxylin solution (Sigma-Aldrich, USA) for 90 seconds.

c. *Lipid detection (Sudan Black B staining)*: Air dried HCMs were fixed in chilled glutaraldehyde-acetone fixative (2-8°C) for 1 minute with gentle agitation. The fixative solution was prepared by adding 25 ml reagent grade acetone to 75 ml glutaraldehyde solution. Slides were stained in Sudan Black B (Sigma-Aldrich, USA) reagent for 5 minutes using intermittent agitation. Then, 70% ethanol was used to rinse the slides until no more dye washed out. Slides were then thoroughly rinsed in deionized water and counterstained in Gill's Haematoxylin (Sigma-Aldrich, USA) for 5 minutes. Slides were rinsed thoroughly in tap water and air dried. Finally, slides were mounted with DPX permanent mounting media (Sigma-Aldrich, USA) and coverslips.

The second method involved cytocentrifugation using the Cytotek centrifuge (Sakura Finetek, Japan). As much as 200 µl of haemolymph diluted 1:1 in anti-clotting MAS were cytocentrifuged at 2,500 rpm for 7 min onto glass slides placed in chamber holders. Then, slides were dried for 10 minutes and fixated with 95% ethanol for 1 minute. The cytocentrifuge method was used in combination with the Diff-Quik staining (Siemens, Germany). Slides were immersed in the Diff-Quik red dye for 1 minute, followed by the Diff-Quik blue dye for 1 minute. Slides were gently washed in deionized water and left to dry. Slides were then mounted and sealed with DPX mounting medium (Sigma-Aldrich, USA).

2.2.7. *Cell size measurements*

A minimum of 100 cells per haemolymph specimen were counted and classified into the different cell types based on cell size and morphology, and then the relative percentages were calculated. The cell and nucleus dimensions were measured and the value of the nucleus/cytoplasm (N/C) ratio was calculated to identify any differences in cell maturity within the haemocyte samples. This procedure was repeated four times with different abalone specimens. Cells obtained after cytocentrifugation and Diff-Quik

staining were also observed and measured. Digital pictures were obtained from a proprietary Leica software, and measurements were processed using “ImageJ 1.48v” software (Rasban, 1997) (Wayne Rasband, National Institute of Health). Statistical analysis of data were performed using unpaired Student’s t-tests. Data are presented as mean \pm SD. Differences were considered significant when $p < 0.05$.

2.2.8. Phagocytosis assay

In vitro phagocytosis activity assays were conducted using a modified method from Aladaileh *et al.* (2007). Zymosan (Sigma–Aldrich, Germany) was used as target cells for phagocytosis. One milligram of Zymosan was suspended in 10 ml of sterile filtered seawater (FSW). To measure phagocytic activity, haemolymph samples diluted 1:1 with MAS were used. A 50 μ l dilution of haemolymph was placed on a glass coverslip and the cells were allowed to adhere for 30 min at room temperature in a moist chamber. The coverslips were rinsed five times with FSW, overlaid with 50 μ l of Zymosan particles (1×10^6 cells/ml) and incubated in a moist chamber for 30 min at room temperature. After that step, phagocytosis was observed at different times over a 2 hour incubation period (15, 30, 45, 60, 90 and 120 min). Non-ingested Zymosan particles were removed by dipping each coverslip in FSW for 10 times. Haemocytes were stained by dipping coverslips in May Grunwald (1:1) for 1 min and Giemsa (1:10) for 1 min. The coverslips were carefully placed on glass slides and observed under a Leica DM 2000 microscope. Phagocytic activity was determined as the percentage of haemocytes that had ingested at least one yeast cell, and counting a minimum of 100 haemocytes on each coverslip. Means were calculated from triplicate abalone samples. Statistical analysis of data was performed with 1-way ANOVA followed by Duncan test for comparison.

2.3. Results

2.3.1. Morphology, cell count and viability assay

2.3.1.1. Unstained haemocytes under light microscopy and Trypan Blue test

Light microscopy of *H. iris* haemocytes revealed two types of cells. Type I cells are represented by large irregular agranular cells with vacuoles and low N/C ratios (Fig. 2.1. A). Type II cells are mostly represented by small round regular agranular cells with high N/C ratios (Fig. 2.1. B). Observations of these cells following haemolymph withdrawal indicated some haemocyte morphological changes, such as the development of thin pseudopodia and rapid attachment to glass slides. This was more obvious with type I cells (Fig. 2.1. A).

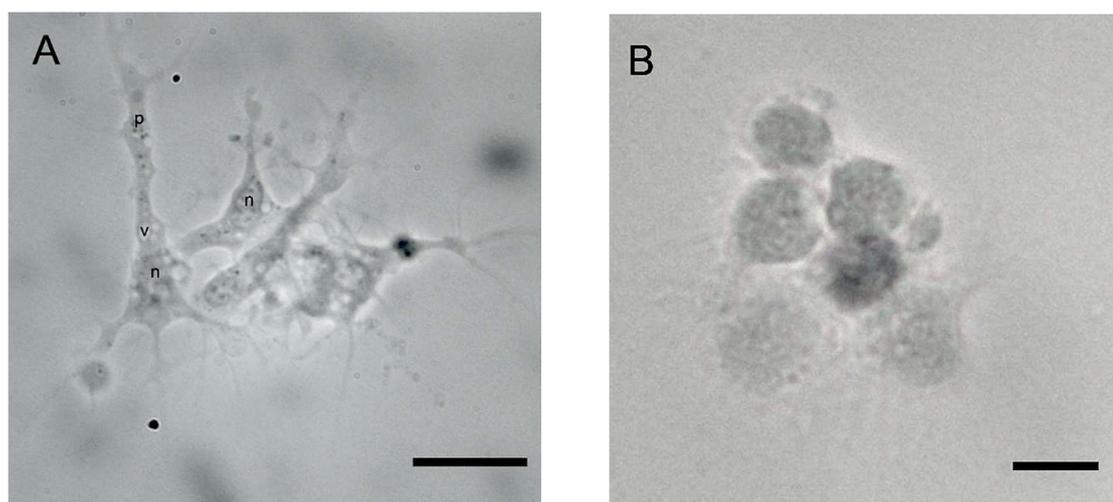


Figure 2.1. Light photomicrographs of fresh *H. iris* haemocytes attaching to glass slides, showing (A) cell nuclei (n), vacuoles (v) and extended pseudopods (p), and (B) cells aggregating. Scale bar is 10 μ m.

Amoeboid haemocyte cell locomotion was seen following attachment and aggregation. The number of cells involved in these aggregates increased over time, and formation of large cell clumps often resulted from collision of small cell clumps.

Cell viability was quantified through haemocyte cell counts using a Vetriplas haemocytometer after 2 and 40 min of haemolymph withdrawal and addition of Trypan

Blue solution. Cell densities were $1.12 \pm 1.50 \times 10^6$ cells/ml at 2 min and $1.06 \pm 0.39 \times 10^6$ cells/ml at 40 min (n=10 for each sampling period). Cell viability was almost unchanged over time with $98.1 \pm 1.4\%$ and $97.5 \pm 1.8\%$ of living cells after 2 and 40 min from the withdrawal respectively.

2.3.1.2. Muse cell analyser

Haemocyte counts and viability assays using the Muse® Cell Analyser (Merck Millipore) revealed that the average haemocyte cell density 10 min after haemolymph extraction ranged from 1.03×10^6 to 2.75×10^6 cells/ml (n=10) with a cell viability average of $98.1 \pm 1\%$ (Fig. 2.2.). These cells remained highly viable over time 1, 2, 3.5 and 5 hours after withdrawal with 97.2, 95.8, 94.6 and 93.6% viable cells, respectively. Significant cell mortality was observed 24 hours after hemolymph withdrawal with 84.1% viable cells (Fig. 2.3.).

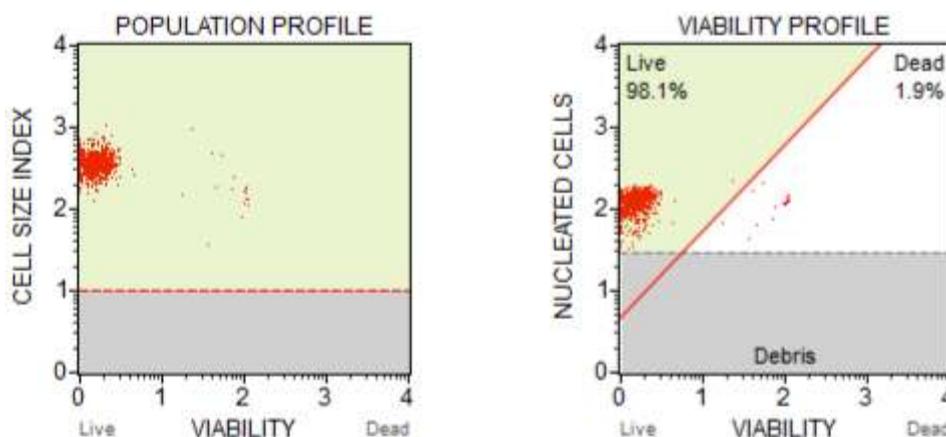


Figure 2.2. Scattergrams of haemocytes generated by the Muse Cell Analyser showing cell counts and viability of haemocytes after 10 min from withdrawal.

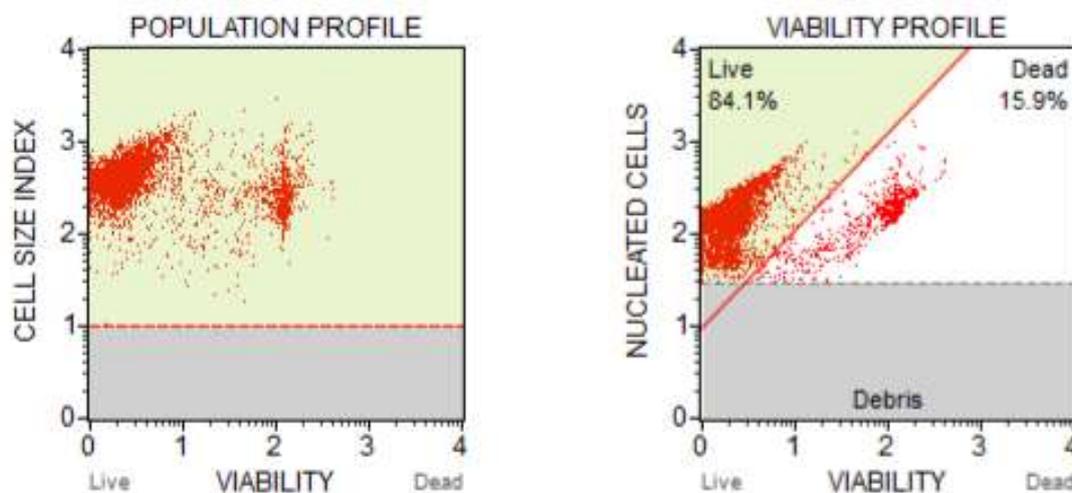


Figure 2.3. Scattergrams of haemocytes generated by the Muse® Cell Analyser showing cell counts and viability of haemocytes after 24 hrs from withdrawal.

2.3.2. Identification of haemocytes using the Sysmex XN-1000 analyser

Flow cytometric analysis of abalone haemocytes revealed two cell types based on Side-Fluorescent Light (SFL) and Forward-Scattered light (FSC) parameters. FSC is proportional to cell-surface area or size, while SFL gives information on DNA/RNA content of the cell (Diggles *et al.*, 2002; Briggs *et al.*, 2012). Dot plots of haemocyte cells analyzed with the XN-1000 showed two distinct populations of cells or clusters (Fig. 2.4.) included within the purple area (lymphocyte-like cells) and the green area (monocyte/macrophage-like cells). Based on the FSC and SFL parameters, cells in the purple area have a low internal complexity, while cells in the green area have a greater complexity.

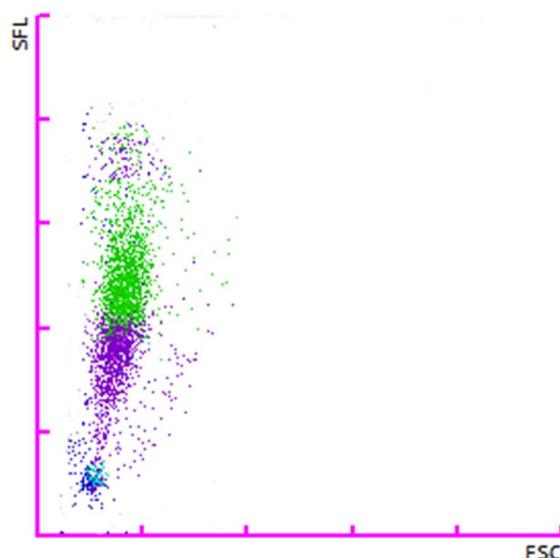


Figure 2.4. Scattergrams of haemocyte cells using the white blood cell differentiation (WDF) channel on the Sysmex XN-1000 haematology analyser showing SFL and FSC values. Automatically assigned lymphocyte-like cells and monocyte-like cells are shown in purple and green, respectively. Blue dots represent debris.

2.3.3. Cell size measurements after Diff-Quik staining

After cytocentrifugation and staining with Diff-Quik, haemocytes were observed showing mostly agranular cells within two distinct cell types based on morphology, size and N/C ratios based on statistical analysis of data that were performed using unpaired Student's t-tests (Table 2.1., Fig. 2.5.). Type I is represented by large irregularly shaped cells (20-23 μm) with a small nucleus (6.8-7.4 μm) and a low N/C ratio (0.76-0.86). Type II haemocyte cells are mainly round cells with a smaller size (11-20 μm), a larger nucleus (9.2-10.8 μm) and a higher N/C ratio (1.16-3.46).

Table 2.1. Measurements of cytocentrifuged haemocytes stained with a Diff-Quik kit. Values for Type I and Type II cells are mean \pm SD.

	Type I	Type II
Cell diameter (μm)	21.0 \pm 1.2	15.2 \pm 4.6
Nucleus diameter (μm)	7.1 \pm 0.3	10.0 \pm 0.8
Nucleus area (μm^2)	46.0 \pm 5.0	102.0 \pm 38.3
Cell area (μm^2)	306.7 \pm 60.0	157.4 \pm 75.5

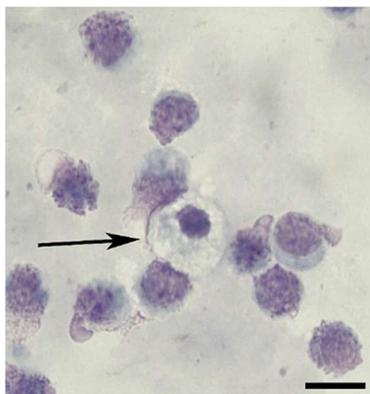
N/C ratio (μm^2) 0.81 ± 0.05 2.31 ± 1.15 

Figure 2.5. Micrograph of *H. iris* haemocytes after methanol fixation and Diff-Quik staining. Type I cell (black arrow) surrounded by Type II cells. Scale bar 10 μm .

2.3.4. Giemsa and May-Grünwald-Giemsa stainings

The two recognizable cell types were also observed after staining with Giemsa (Fig. 2.6. A) and May-Grünwald-Giemsa (Fig. 2.6. B). The majority of cells found in the haemolymph monolayer stained with Giemsa are type II cells or lymphocyte-like cells. The second population or type I cells is made of macrophage-like cells. These cytology results confirm our flow cytometry data reported above with the Sysmex XN analyser.

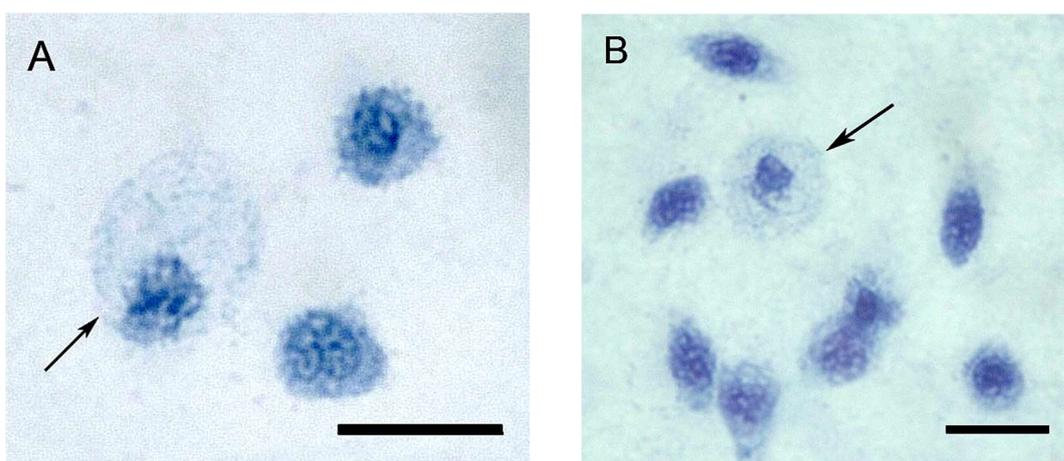


Figure 2.6. Micrographs of *H. iris* haemocytes with (A) Giemsa and (B) May-Grünwald-Giemsa staining. Type I cells (pointed with black arrow) were surrounded by Type II cells. Scale bar 10 μm .

2.3.5. Specific staining

Use of PAS staining system for staining the glycol-containing cellular components of *H.iris* haemocytes showed a strong red cytoplasmic stain with type II lymphocyte-like cells, revealing the presence of carbohydrates (Fig. 2.7.). Results show that the carbohydrate distribution differed among cell types. Type I monocyte-like cells showed no or poorly visible PAS staining. Usually performed on human blood and bone marrow films, the PAS technique stains lymphocytes and lymphoblasts. Our results obtained with *H. iris* haemocytes confirm the usefulness of PAS in the identification of lymphocyte-like cells only.

Use of Sudan Black B staining system for demonstration of phospholipids, neutral fats and sterols in *H. iris* haemocytes cytoplasm showed light blue-black staining (Fig. 2.8.) in type I cells (monocyte-like cells) and in type II cells (lymphocyte-like cells), with no granulation in either cell type. In human leukocytes, Sudan Black B stains cells that are differentiating along a monocytoid pathway (e.g., monocytes), while cells differentiating along a lymphoid pathway (e.g. lymphocytes) do not display do not stain. Our results confirm the usefulness of Sudan Black B in the identification of both monocyte-like and lymphocyte-like cells in *H. iris*.

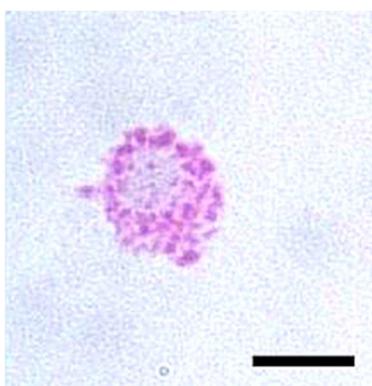


Figure 2.7. Periodic Acid Schiff staining showing glycogen deposits in cytoplasm of lymphocyte-like cells. Scale bar 10 μ m.

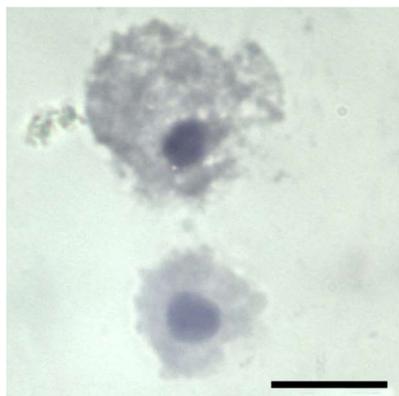


Figure 2.8. Sudan Black staining of Type I monocyte-like cell (top one) and Type II lymphocyte-like cell (bottom one). Scale bar 10 μm .

2.3.6. Phagocytosis assay

Phagocytic activity was used as an alternative method for corroboration of haemocyte types. The phagocytosis index in this research was determined as the percentage of cells that had engulfed at least one Zymosan particle (Fig. 2.9.). Both Type I and type II haemocyte cells showed phagocytic activity, but type I cells (macrophage-like cells) had a higher number of cells participating in phagocytosis (Fig. 2.9.). The phagocytosis percentage increased over time, indicating an increase of Zymosan particle uptake from less than 10% after 15 min to up to $51.2 \pm 3.9\%$ phagocytic cells after 90 min of incubation. After this point, the phagocytosis index did not change over time (plateau phase) (Fig 2.10.).

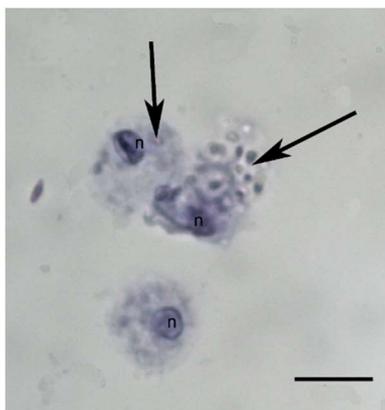


Figure 2.9. Light micrograph of haemocytes during the phagocytosis assay. Arrows point to Zymosan particles that were engulfed by haemocytes after 1 hr of incubation. (n) nuclei of haemocytes. Scale bar 10 μ m.

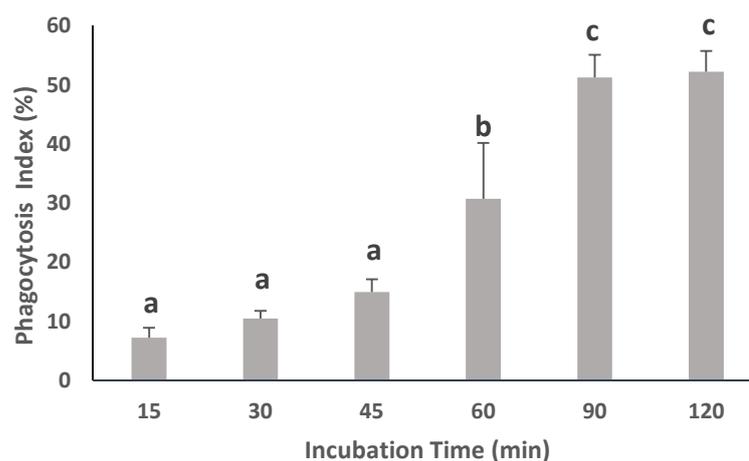


Figure 2.10. Mean \pm SD percent phagocytosis based on microscopy observation. Different letters above the bars indicate significant differences (Duncan post-hoc test, $p < 0.05$; $n = 4$).

2.4. Discussion

Few studies are available on the health and immunology of *H. iris*, and these include general descriptions of diseases (Diggle *et al.*, 2002), examination of infections by haplosporidian parasites (Diggle *et al.*, 2005), shell lesions (Nollens *et al.*, 2004), assessment of pathology threats (Webb, 2013) and a brief observation of haemocytes

(Nollens *et al.*, 2004). However, no detailed study of *H. iris* haemocytes has been available until now. Thus, the present study presents the first comprehensive investigation of *H. iris* haemocyte cells, and provides a basis for further studies on pathological, environmental and anthropogenic effects on the immune system of this species.

In the present study, haemocytes were classified based on morphological, biochemical and behavioural characteristics. Specifically, *in vitro* observations of *H. iris* haemocytes revealed specific behaviour, such as aggregation of cells, cell adhesion, development of pseudopods and phagocytosis. These behavioural characteristics were similar to those previously documented for *Haliotis* species (Travers *et al.*, 2008; Sahaphong *et al.*, 2001; Allen *et al.*, 2006; Tung and Alfaro, 2011 a) and bivalve haemocytes (Hine, 1999). Aggregation of haemocytes with formation of clumps is mainly attributed to homeostasis and wound healing (Sminia, 1981), while the extension of pseudopods by haemocytes and their adhesion capacity facilitate cellular migration and the immune defense (Letullier *et al.*, 2014).

Previous studies on other *Haliotis* species have used a variety of solutions to prevent haemocyte aggregation, such as MAS (Travers *et al.*, 2008), EDTA and caffeine (Chen *et al.*, 1996) and a solution of AASH; 2.5% NaCl and 1.5% EDTA in 0.1M phosphate buffer (Donaghy *et al.*, 2010). In the present study, a modified Alsever's solution (MAS) was used to avoid cell aggregation. MAS has been shown to be an effective anti-aggregant of haemocyte cells in other molluscs (Travers *et al.*, 2008; Bachère *et al.*, 2004; Carballal *et al.*, 1997), and was found to not compromise cell viability after 24 hrs in the present study.

Observation of *H. iris* haemocytes revealed two types in the haemolymph, in a variety of sizes, and were distinguished by their shape, size, internal structure and function, which are type I (monocyte-like) and type II (lymphocyte-like) cells. The terms used to name these cells are based on similarities of shape and function to monocyte and lymphocyte

white blood cells described in vertebrates (Abbas *et al.*, 2010). Similar terms were applied to haemocytes found in the snail *Oncomelania* sp. (Sasaki *et al.*, 2003) and the abalone *H. diversicolor* (Chen *et al.*, 1996). In addition, the small type II cells recognized in this study may be comparable to blast-like cells found in *H. tuberculata* (Travers *et al.*, 2008) and *H. discus discus* (Donaghy *et al.*, 2010).

Previous studies have classified *H. iris* haemocytes as stem cells (type I), hyalinocytes (type II) and fibrocytes (Type III) (Nollens *et al.*, 2004). However, fibrocytes were not encountered in the present study, and it is possible that the previously reported fibrocytes may have been hyalinocytes starting to spread their pseudopodia (Hooper *et al.*, 2007). Alternatively, the differences in these findings may have been due to variations in preparation of the haemolymph cell monolayers. Furthermore, the stem cells and hyalinocytes reported previously are likely to be lymphocytes (Type II), since all lymphocytes come from a common basic lymphocyte cell (blast-like/stem cell) before differentiating into a distinct lymphocyte type (Nollens *et al.*, 2004). Round/blast-like haemocyte cells are frequently considered to be of an earlier stage in haemocyte ontogeny (Loker, 2010).

An interesting result from this study is the fact that no granules were observed in the haemocytes. This result was similar to those found in previous studies with *H. tuberculata* (Travers *et al.*, 2008) and *H. discus discus* (Donaghy *et al.*, 2010), but different to studies on bivalves and several gastropods, which used the presence of granules for haemocyte characterization (Travers *et al.*, 2008; Cheng *et al.*, 1981; Allen *et al.*, 2006; Tung and Alfaro, 2011a). Granulocytes have been reported in *H. asinina* by transmission electron microscopy (Sahaphong *et al.*, 2001), featuring huge, rod-shaped, electron-dense structures that are completely different from previous descriptions in other molluscan species (Bacheré *et al.*, 2004; Carballal *et al.*, 2010). However, the term granulocyte in abalone was used for cells found in a mantle explant tissue culture of *H. varia*. These cells

had the ability to form pseudopodia and possess specific granules responsible for the nucleation of crystals during bio-mineralization (Suja and Dharmaraj *et al.*, 2005; Auzoux-Bordenave *et al.*, 2007). These inconsistencies in the literature highlight the need to develop more accurate techniques to characterize haemocyte cells, and to apply greater rigor when describing and naming cells.

The above mentioned problems with techniques and nomenclature are compounded by a general lack of knowledge regarding cell origin and hematopoietic tissue in abalone. As a first step to resolve these difficulties, this study provided confirmation that *H. iris* has two cell types recognized by flow cytometry using the XN-1000 haematology analyser (Sysmex). A clear advantage of this instrument is a new feature that uses color to effectively discriminate haemocyte populations. While this study is the first to use this instrument to examine invertebrate haemocytes (previously used for human blood cells), the results show great potential for future application in marine invertebrate haematology. The flow cytometry (FCM) results corroborated findings obtained with the XN-1000 haematology analyser, and mirrored the findings for *H. tuberculata* (Travers *et al.*, 2008) and *H. discus discus* (Donaghy *et al.*, 2010). These studies also reported small (blast-like) and large hyalinocytes (agranular haemocyte containing a central nucleus and little cytoplasm).

Haemocyte cells observed in this research displayed a wide range of cell diameters. This variation has recently been attributed to different maturation stages and functions of the same cell type (Rebelo *et al.*, 2013). Cells of different sizes were observed and attributed to both type I and type II cells.

Another important outcome of this research is the innovative use of the Muse® Cell Analyser as an accurate and quick method to count haemocyte cells compared to the manual microscopic method using the Trypan Blue exclusion assay, which is regarded as technically demanding and time consuming. The application of the automated Muse®

Cell Analyser to assess the viability of haemocyte cells was successfully applied in this research and should be extremely valuable in future studies of molluscan haemocytes.

The *H. iris* haemocyte counts obtained with the Muse®Cell Analyser ranged from 1.03×10^6 to 2.75×10^6 cells/ml. These values are similar to reported cell densities of haemocytes of *H. discus discus* (Donaghy *et al.*, 2010) and *H. laevigata* (Dang *et al.*, 2011). Variability in haemocyte counts may be attributed to seasonal differences (Travers *et al.*, 2008), environmental changes (Hooper *et al.*, 2007), pollution (Martello *et al.*, 2000) and pathogens (Nollens *et al.*, 2004). The evidence of transient drop in haemocyte counts (haemacytopenia) could be an indication of haemocyte apoptosis and haemolymph degradation (Matozzo and Marin, 2010). Haemocytes experiencing gradual deterioration were assumed to be removed by phagocytosis by other haemocytes (Hooper *et al.*, 2007). On the other hand, an increase in haemocyte counts is possible when abalone are exposed to temperatures higher than the preferred range (Cheng, 1981). The total number of haemocytes circulating in invertebrate haemolymph may be affected by the number of haemocytes migrating from inner tissues, such as the haematopoietic tissue, and infiltrating into connective tissues (Cochennec-Laureau *et al.*, 2003). In addition, complementary activity of storage and release of haemocytes within other organs is also possible (Matozzo and Marin, 2010).

Although *H. iris* haemocyte viability was never lower than 80% in this study, there was a decrease in viability after 40 min of incubation at room temperature, and a further decrease was observed after 24 hrs. This decrease in viability is attributed to ongoing metabolic activity, which would have produced reactive metabolites, and hence, would have negatively affected haemocyte cells through oxidative stress (Rebelo *et al.*, 2013). The MAS solution used to prevent cell aggregation appears to also have been instrumental in maintaining high counts of live haemocytes for longer periods. This is because the anti-aggregant solution contains components that are able to scavenge oxidative radicals, and

thus, provide a balanced extracellular micro-environment (Cochennec-Laureau *et al.*, 2003). However, further research is needed to conclusively determine the mechanistic action of the anti-aggregant solution on metabolic activity, membrane structure, and DNA integrity.

All lymphocyte-like cells encountered in *H. iris* were PAS positive, indicating that they contained carbohydrates. These findings are in agreement with studies in *H. tuberculata* (Travers *et al.*, 2008) and *H. asinine* (Sahaphong *et al.*, 2001), which found glycogen deposits in the cytoplasm. The carbohydrates found in haemocytes probably indicate the role of haemocytes in transport of carbohydrates, as has been reported in other invertebrates, such as *C. aestuarii* (Matozzo and Marin, 2010). Indeed, carbohydrates have been found to be abundant on haemocyte surfaces, and they have been shown to be important for pathogen recognition (Cochennec-Laureau *et al.*, 2003). However, monocyte-like cells did not stain positive with PAS, which indicates that these cells do not function as carbohydrate transport/storage agents.

In this study, Sudan Black B staining demonstrated that all haemocyte types of *H. iris* contained lipids, although deposits were not found in the cytoplasm. These lipids are likely to function as energy storage locales, as has been reported for the mangrove oyster *Crassostrea rhizophorae* (Johansson *et al.*, 2000). Furthermore, the fatty acid composition of haemocyte polar lipids in the oyster *Crassostrea gigas* and the clam *Ruditapes philippinarum* have been shown to be highly affected by the diet of the animal (Horak and Knapp, 1997). Phagocytosis activity is the front line of cellular defence in molluscs, and the role of haemocytes in immune response relies on the capacity of haemocytes to engulf and degrade foreign material through phagocytosis (Hooper *et al.*, 2007). Zymosan A contains particles prepared from yeast cell walls made of protein-carbohydrate complexes that are commonly used in phagocytosis experiments. The findings of this study indicate that while both lymphocyte-like and monocyte-like cells

showed phagocytosis activity with Zymosan A, monocyte-like cells began phagocytosis activity earlier and with greater success. Indeed, the results showed that most monocyte-like cells have the capacity to engulf 3 or more Zymosan particles and that phagocytosis rates increased over time, achieving a maximum rate of 56% after 120 min of incubation. However, haemocyte responsiveness during the *ex vivo* experiments may have been affected by the changes in the haemocyte solution and also the rapid morphological changes of the haemocytes after the collection of haemolymph (Barth *et al.*, 2005). Further evidence of the capacity of haemocytes to be involved in phagocytosis is the fact that they have been shown to secrete hydrolytic enzymes, such as lysosome enzymes in *H. tuberculata* haemocyte (Pichon *et al.*, 2013). Lysosome enzymes are involved in the intracellular degradation of engulfed foreign material (Donaghy *et al.*, 2010), and have been shown to break down various types of biochemicals, mostly proteins (Pichon *et al.*, 2013).

While phagocytic performance was successfully observed in this experiment, further research will be needed to confirm that the anti-aggregant does not affect this process. In some molluscs, phagocytosis has been shown to be affected by the use of MAS solution, and it remains unclear what the effects may be on haemocyte function (Cooper, 2000). Granulocytes have been shown to be the main phagocytic cells in bivalves (Hégaret *et al.*, 2003), and the lack of granulocytes in the haemolymph of *H. iris* provides an interesting divergence from previous findings, and demonstrates that even without granulocytes, phagocytosis is able to take place in abalone (Travers *et al.*, 2008).

In summary, the successful implementation of a combination of classic and newer techniques to observe, identify and quantify haemocytes resulted in the characterization of two distinctive types of haemocytes (lymphocyte-like cells and monocyte-like cells) in *H. iris*. We also illustrated the use of innovative tools (flow cytometry with Sysmex XN-1000 and Muse® Cell analyser) as accurate and efficient tools for future studies of

haemocyte cells and their responses to a variety of pathogenic challenges and environmental conditions.

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CHAPTER 3

Effect of antiaggregants on the *in vitro* viability, cell count and stability of abalone (*Haliotis iris*) haemocytes

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Abstract

The ability to successfully prepare and preserve haemocyte cells for microscopy and flow cytometry is critical for the investigation of animal immune systems. In this study, we observed the total cell count, in vitro viability and stability of New Zealand black-footed abalone (*H. iris*) haemocytes with different antiaggregants and handling protocols. Haemocyte stability was evaluated by direct observation of haemocytes under the microscope and calculating the aggregation index. Haemocyte counts and viability were measured via flow cytometry and tested for the effect of different antiaggregants (Alsever's solution at three concentrations, and specialised blood collection tubes containing lithium heparin and K₂EDTA) at different temperatures and storage times. Results showed that Alsever's solution is an effective antiaggregant at haemolymph:antiaggregant dilution ratios of 1:1, 1:2 and 1:3. Lithium heparin was ineffective as an antiaggregant, whereas K₂EDTA was similarly as effective as Alsever's solution. The influence of different mixing techniques (vortex, pipetting and flipping) were subsequently tested using the K₂EDTA Microtainer® tubes, revealing that proper mixing should be performed immediately. High cell viability can be achieved by mixing samples by either 10 sec of vortexing (1000 rpm), 10 times pipetting or 20 times flipping. The in vitro storage of abalone haemocytes in AS and K₂EDTA as antiaggregants at ambient room temperature was highly effective for up to 24 hrs (75–85% viability; 0.05–0.15 aggregation index) and is recommended for haemocyte studies in *H. iris*.

Utilization of K₂EDTA Microtainer® tubes were advantageous since they are more cost effective compared to Alsever's solution, and samples can be prepared more efficiently.

Keywords: Abalone haemolymph, blood clumping and anticoagulants, Alsever's solution, K₂EDTA microtainer, Lithium heparin, Flow cytometry

3.1. Introduction

Most invertebrates have an open circulatory system, where blood (haemolymph) is collected from gills, pumped through the heart and released into haemocoel spaces where it directly bathes the internal body organs. Invertebrates therefore must rely on rapid non-inducible defence mechanisms to entrap and kill parasites, and immediate coagulation mechanisms to prevent blood loss after wounding. Haemocytes circulate readily in the haemolymph where they are the main components responsible for cellular immunity (Hooper *et al.*, 2006). Haemocytes are also involved in transport, excretion and digestion of nutrients (Beninger *et al.*, 2003), wound healing (Franchini and Ottaviani, 2000) and shell repair (Fleury *et al.*, 2008). Thus, haemocytes can be used to assess the immune status of an organism under a variety of physiological and environmental conditions or stressors.

Molluscs, such as abalone, are particularly susceptible to diseases and cultivation stresses that often go unnoticed until high mortalities are encountered (Prince, 2007). From an aquaculture perspective, identification and characterization of haemocytes can provide useful information regarding the health of stock within different cultivation settings, leading to improved management and production strategies.

Various laboratory methods have been published, including morphological observation of abalone haemocytes through various cytochemical staining techniques (differential staining, carbohydrate detection, acid phosphatase detection, lipid staining, lipid detection, lysosome staining and acid inclusion detection) (Travers *et al.*, 2008), measurements of intracellular superoxide anion (Malham *et al.*, 2003), quantitative cell counts (Malham *et al.*, 2003), haemocyte viability (Donaghy *et al.*, 2010; Grandiosa *et al.*), bacterial clearance (Cheng *et al.*, 2004), phagocytosis (Malham *et al.*, 2003; Macey and Coyne, 2005) and migration assays (Malham *et al.*, 2003). All of these methods require haemolymph samples to be taken from the animal before various other laboratory

analyses can be performed. However, it has been identified that the time and conditions of cell preparation are critical since cells begin to change (e.g., aggregation and lysis) as soon as they are extracted from the haemolymph circulatory system. To overcome this complication, effective techniques are required for the preservation and stability of haemocyte cells. Since such techniques can be highly species-specific (Hooper *et al.*, 2006), it is important to establish appropriate protocols for each species, especially for those commercially cultivated.

Some of the issues that affect the invertebrate haemocyte tests are cell clumping or cell aggregation (Travers *et al.*, 2008; Malham *et al.*, 2003; Donaghy *et al.*, 2010; Grandiosa *et al.*, 2016; Hinzmann *et al.*, 2013; Hégaret *et al.*, 2003; Cardinaud *et al.*, 2015, Sminia and van der Knaap, 1987). These events are influenced by the presence of divalent cations, such as calcium and magnesium (Chen and Bayne, 1995; Shozawa and Suto, 1990; Kenney *et al.*, 1972). Previous studies have demonstrated that the use of various solutions with metal cation chelators, such as ethylenediaminetetraacetic acid (EDTA) and citrate, are able prevent cell clumping in abalone haemocytes (Travers *et al.*, 2008a; Grandiosa *et al.*, 2016; Cardinaud *et al.*, 2015; Travers *et al.*, 2008b; Ding *et al.*, 2016; Poncet and Lebel, 2003; Lebel *et al.*, 1996).

An antiaggregant concentration is also known to affect the cellular structure, morphology and viability of haemocytes (Hinzmann *et al.*, 2013). Thus, the appropriate antiaggregant and concentration should be identified for each species in question to ensure optimal *in vitro* stability and cell viability. In addition, the optimal concentration of the widely used Alsever's Solution should be identified to ensure accurate measurements of cell count, stability and viability.

One of the antiaggregants worth studying is K₂EDTA, which is available as a sterile evacuated predetermined 500 µl microtainer tubes (BD Microtainer®). The microtainer tube® is advantageous for human blood collection (Hicks *et al.*, 1976) and has been used

widely in clinical laboratories to assist in complete blood cell count (CBC) and white blood cell (WBC) differential counts (Xu *et al.*, 2010). To our knowledge, use of Microtainer® tubes have been restricted to collection of vertebrate blood samples, but may provide advantageous for handling invertebrate haemocytes, since they provides a tube cap closure and a precise volume in the container that eliminates additional pre-analytical pipetting of antiaggregants.

This study also focused on sample preparation, such as mixing and temperature storage of haemocytes. Sample mixing has been identified as a potential source of error, when the complete interaction between the haemocytes and the antiaggregant is not achieved. In addition, temperature plays an important role in *in vitro* haemocyte preparation, since temperatures that exceed a certain stress threshold can enhance oxidative metabolism of haemocytes (Donaghy and Volety, 2011). Previous studies have shown that molluscan haemocytes stored at 10°C for 1 h produce higher levels of reactive oxygen species compared to haemocytes stored at 20°C and 30°C (Donaghy and Volety, 2011). Previous studies have also highlighted the importance of *in vitro* haemocyte storage in cold temperature before further analysis (Torreilles *et al.*, 1999; Cardinaud *et al.*, 2014). It appears that this cooling procedure prevents cell aggregation, but the effect on cell metabolism is unknown. To date, no studies have been conducted to clarify the association between temperature during storage and haemocyte viability in antiaggregant media.

Thus, the aim of this study is to establish effective handling and preservation techniques (i.e., antiaggregant type, time and temperature of storage, and mixing technique) to improve haemocyte cell viability and stability of the New Zealand black-footed abalone (*H. iris*) for immunological studies. We envisage that this information will be valuable for commercial cultivation of *H. iris*, which is considered a high-value endemic species for the New Zealand aquaculture industry (Alfaro *et al.*, 2014).

3.2. Material and Methods

3.2.1. Animals and haemolymph sampling

Specimens of *H. iris* (55.5 ± 5 mm mean shell length) were obtained from a commercial hatchery (OceaNZ Blue Ltd.; Bream Bay Aquaculture Park, Ruakaka, New Zealand) and transported to the AUT aquaculture facility (Auckland University of Technology, New Zealand). Animals were kept in 20-L recirculation tanks containing filtered (5 μ m) seawater (FSW) maintained at a temperature of $15 \pm 1^\circ\text{C}$ and salinity of 35 ppt. Abalone were acclimatized in the laboratory for 2 weeks and fed with a commercial abalone feed (Abmax T20, E.N. Hutchinson Ltd. Auckland). Haemolymph samples (1–2 mL) were collected from the pedal sinus region (vessel on the midline of the pedal sole; ca. 20mm from the anterior foot margin) with a 25 gauge and 5/8” needle attached to a 3-ml sterile syringe (Terumo, Japan, Terumo).

3.2.2. Experimental designs

Three separate experiments were conducted to assess: (i) the effect of Alsever’s Solution [AS] concentration on haemocyte count, viability and aggregation to identify the best dilution ratio/s for future use; (ii) the effectiveness of alternative antiaggregants (AS vs K_2EDTA vs Lithium heparin) at different temperatures and storage times to determine whether an improved method can be obtained compared to AS treatment; and (iii) based on the previous results, the effect of different mixing techniques (vortex, pipetting and flipping) with K_2EDTA Microtainers® to further optimise protocol development using pre-made blood collection tubes.

Total haemocyte counts and viabilities were determined by flow cytometry (MUSE® Cell Analyzer; Merck KGaA, Darmstadt, Germany) using the MUSE® Count and Viability Assay Kit (Merck) following the manufacturer’s protocol, where 20 μ l of haemolymph were collected and placed in micro-centrifuge tubes containing 380 μ l of assay reagent (Grandiosa *et al.*, 2016). Cell stability was provided by measuring the index

of aggregation, which was defined from two measurements: at 10 min (initial number) and at 30 min. The index was calculated according to a developed formula (Gebbinck *et al.*, 1993) by counting the number of particles (single cells plus clusters of more than four cells) in the specific area of the haemocytometer. The index describes full aggregation with the index of > 0.90 and no aggregation index of < 0.10 . The following formula was used:

$$\text{Aggregation index} = 1 - (\text{remaining } N / \text{initial } N).$$

General morphologies of Type II lymphocyte-like cells (most abundant haemocyte type in *H. iris* (Grandiosa *et al.*, 2016)) were assessed via microscopy (Leica DM 2000 microscope equipped with a Leica DF 290 camera [Leica Biosystems Pty Ltd, Australia]) to characterise presence/absence of clumping, unusual cell shrinkage or swelling, and membrane blebbing. The aggregation index and morphological features of haemocytes were observed by placing 50 μL of haemolymph sample on a haemocytometer. Images were taken from wet mounts which were prepared by placing haemolymph on glass slides and smeared evenly to create a thin homogeneous monolayer. The slides were then covered with coverslips and selected images were taken at 630x magnification.

3.2.2.1. *Effect of Alsever's antiaggregant concentration*

In order to evaluate the optimal AS concentration for *H. iris*, we tested different haemolymph:AS ratios (1:1, 1:2, and 1:3) and measured total cell count, viability and stability. Haemolymph extracted from three abalones were randomly assigned to the different dilutions in 1.5 mL centrifuge tubes with three replicates each. The Alsever's solution (Sigma-Aldrich; A3551) was composed of: NaCl (4.2 gL^{-1}), Citric Acid \cdot 3Na \cdot 2H $_2$ O (8.0 gL^{-1}), Citric Acid \cdot H $_2$ O (0.55 gL^{-1}) and D-Glucose (20.5 gL^{-1}). Diluted samples were vortexed (Scilogex MX-S Vortex Mixer) at 1000 rpm for 10

seconds (Grandiosa *et al.*, 2016), and then cell count, viability and stability were assessed after 10 min, 3 hrs, 6 hrs and 24 hrs post extraction.

3.2.2.2. *Effectiveness of alternative antiaggregants, temperature and storage time*

Three antiaggregant media types (K₂EDTA [BD Microtainer®], Lithium Heparin [BD Microtainer®] and AS at a 1:1 haemolymph:AS ratio) were tested for their comparative ability to preserve cell viability and stability at room temperature (RT; 21°C) and low temperature (LT; 4°C) during 24 hrs of storage (assessed after 10 min, and 3, 6, and 24 hrs). The three antiaggregants possess different active components. The commercial K₂EDTA (1 mg for 500 µL haemolymph) and Lithium Heparin (1 mg for 500 µL haemolymph) blood collection tubes contain spray dried substances inside (Lippi *et al.*, 2007), while AS, containing citrate, is readily available in a liquid solution and can be used at different dilution ratios.

Eight randomly selected healthy abalone were used to draw 1500 µL of haemolymph per animal. Once drawn, the haemolymph was placed into the respective treatment media, with three replicates per treatment, and immediately vortexed for 10 seconds. Treatments of K₂EDTA and Heparin were conducted by placing 500 µL of haemolymph inside each Microtainer® tube. The AS treatment was achieved by placing 500 µL of haemolymph in a 1:1 ratio with AS in micro-centrifuge tubes. Cell count, viability, stability and morphology were assessed in all samples as previously outlined at 10 min, and 3, 6 and 24 hrs.

3.2.2.3. *Effect of different mixing techniques with K₂EDTA Microtainer® tubes*

Three mixing procedures (vortexing, pipetting and inversion [flipping the tubes]) were tested independently with different times for their performance with haemolymph in K₂EDTA. Only K₂EDTA was tested in this experiment since the purpose was to identify if this easy-to-use blood collection tube could effectively replace AS. Vortexing was conducted at 1000 rpm for 10, 15 or 20 sec. Mixing with a pipette was conducted

10, 20 or 30 times, whereas inversion mixing was achieved by gently inverting the tubes 10, 20 or 30 times. Each treatment was replicated three times with haemolymph from eleven animals (randomly assigned to different treatments and replicates). In addition, three K₂EDTA tubes without mixing were assigned as negative controls. All procedures were conducted at RT (21°C), then cell count, viability and stability were assessed as previously outlined at 10 min, and 3, 6 and 24 hrs.

3.2.3. Statistical Analyses

Measurements of percent viability and cell count were analysed with Mixed Design (Split-Plot) ANOVAs with Tukey post-hoc tests, while the effect of time was analysed by a Bonferroni test. The cell count data were normalized using logarithmic transformation. In all statistical tests, differences were deemed significant with $p < 0.05$. Results are reported as means \pm standard deviations. All statistical analyses were performed using SPSS v22.0 (IBM Corporation, Armonk, New York).

3.3. Results

3.3.1. Effect of Alsever's antiaggregant concentration

The population and viability profiles of haemocytes treated with AS were generated from the MUSE® flow cytometer (Fig. 3.1. B and 3.1. C). The viability of haemocyte cells exposed to all AS concentrations did not differ significantly ($p > 0.05$) to each other over the 24 hr sampling period (Fig. 3.1. D; Table 3.1). There was an indication that a 1:1 (haemolymph:AS) ratio resulted in slightly higher cell viability ($75.8 \pm 6.14\%$) than the other dilutions (haemolymph:AS ratios of 1:2 and 1:3) after 24 hrs. Haemocytes treated with Alsever's solution did not remain in a fully non-aggregated state since a small number of haemocytes were observed to be aggregating after 30 minutes of mixing with Alsever's solution. Therefore, the aggregation index from the treatment of AS 1:1, AS 1:2 and AS 1:3, were 0.14, 0.07 and 0.04, respectively (Fig. 3.1. A).

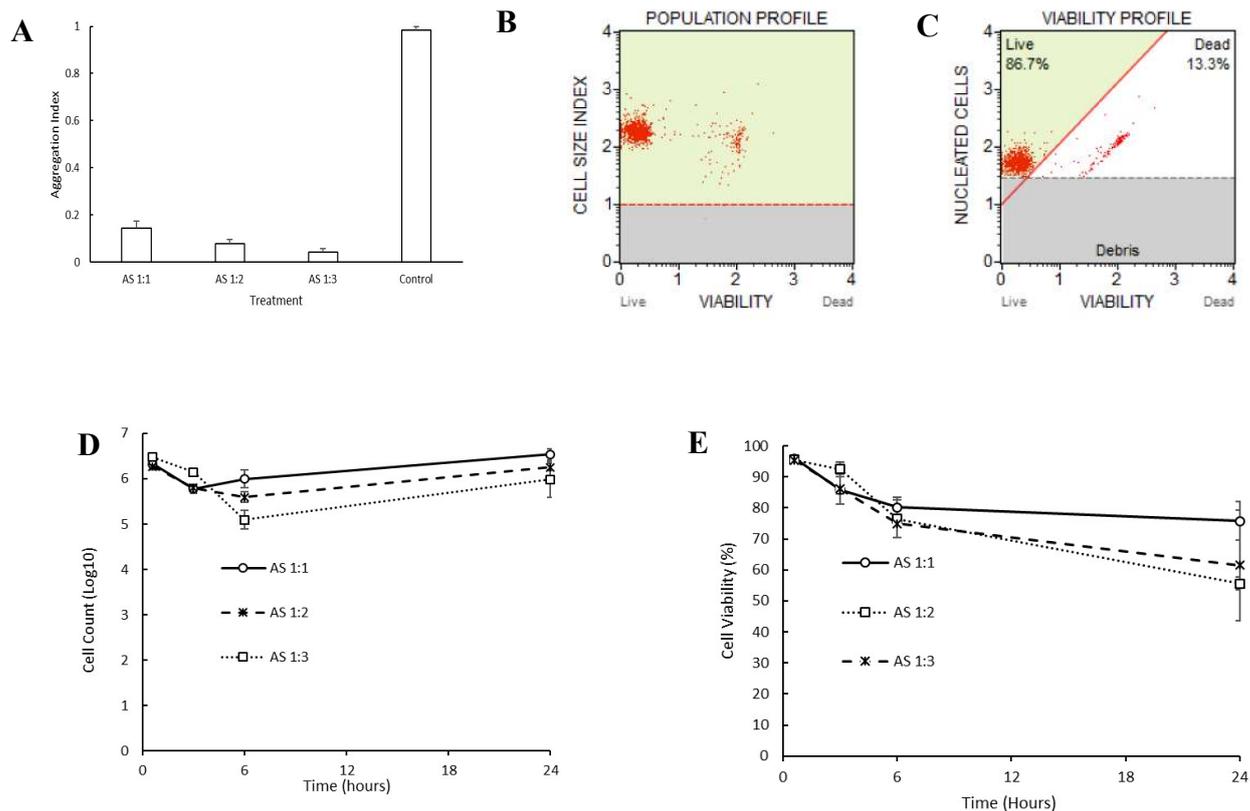


Figure 3.1. Effect of Alsever's solution concentration on (A) aggregation index after 30 min; (B) Population profile and (C) viability profile of haemocytes measured by the MUSE® Cell Analyzer; (D) cell count over time; (E) cell viability over time.

The average total haemocyte cell count (THC) from antiaggregant treatments at 10 minutes after withdrawal ranged from 2.19×10^6 to 3.02×10^6 cells.mL⁻¹ across samples. Initial measurements showed that the THC of treatments did not differ significantly ($p > 0.05$) (Table 3.1.). There were temporal variations in THC measurements and significant interactions with storage time in the THC analyses but no interaction was found between the treatments and storage time upon cell viability (Fig. 3.1. D; Table 3.1.).

3.3.2. Effectiveness of alternative antiaggregants, temperature and storage time

K₂EDTA and AS successfully prevented haemocyte clumping after 24 hrs incubation with low aggregation values (< 0.2) regardless of storage temperature (Fig. 3.2. A); Type

II cells were also of similar size and shape as previously characterised under ideal conditions (Grandiosa *et al.*, 2016) (Fig. 3.2. C and 3.2. D). Untreated haemocytes and those treated with lithium heparin resulted in fast cell aggregation regardless of temperature (Fig. 3.2. A and 3.2. B). THC and viability analyses of clumped haemocytes were not conducted since aggregates may yield inaccurate and inconsistent results due to differential staining in clumps and the possibility that aggregates may clog or result in coincident events hampering the flow cytometry assay.

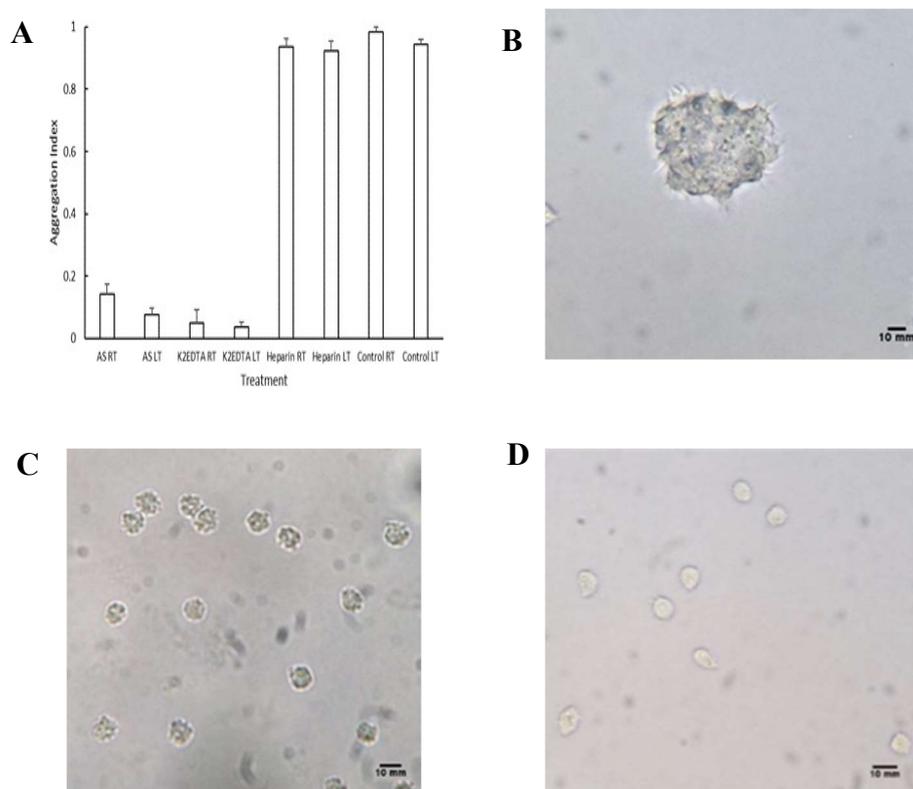


Figure 3.2. (A). Aggregation index of haemocytes treated with various antiaggregants at different temperatures (RT= Room temperature 21°C, LT = Low temperature of 10°C). Images of haemocytes (B) without the addition of antiaggregant shown as one big aggregation and haemocytes treated with (C) K₂EDTA and (D) Alsever's Solution.

The highest haemocyte cell viability was observed at room temperature (RT) treatments, where K₂EDTA and AS exposures resulted in a cell viability of 82.5% and 75.8%, respectively (about 10–20% decrease after 24 hrs exposure). The values were

significantly higher compared to the viability of haemocytes treated with K₂EDTA at low temperature, which resulted the lowest viability with an average of 35.2% viable cells after a 24 hour storage period (Fig 3.3.; Table 3.2.).

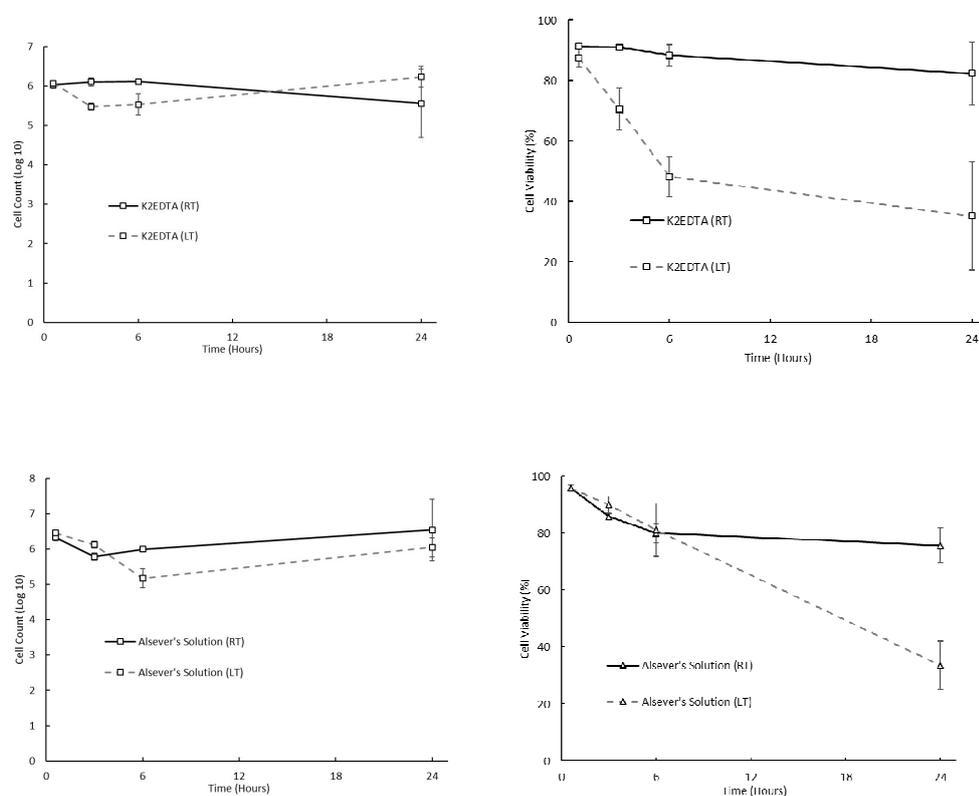


Figure 3.3. Total haemocyte cell count (log₁₀) and viability of haemocytes exposed to different antiaggregants (K₂EDTA, Alsever's solution [dilution ratio 1:1]) at different temperatures (RT = Room temperature [21°C], LT = Low temperature [4°C]) over a 24 hr storage period.

Differences among AS treatment effects at room temperature and low temperature were more pronounced after 24 hrs, with higher cell viability from AS at room temperature. Statistically significant differences for cell counts were found for within-subject effects which were attributed to temporal variations among observations (Fig. 3.3; Table 3.2). Thus, the range in THC across treatments and controls throughout the experiment were 3.04×10^5 to 5.26×10^6 cells.mL⁻¹.

3.3.3. Effect of different mixing techniques with K₂EDTA

Results from these experiments indicated that the use K₂EDTA microtainers is an effective method to prevent cell aggregation and enhance cell viability and stability after mixing compared to controls (no mixing), which resulted in almost 100% cell mortality after 24 hours (Fig. 3.4.; Table 3.3.). However, slight differences were observed in mixing technique (vortexing, pipetting and inverting) and times.

Microscopic observations of haemocyte cells were consistent with the above findings, where enhanced visualization of separate cells and normal cell features were associated with high viability. The opposite cell characteristics were found for controls, where small clumps consisting of 3-10 cells and abnormal cells were encountered more routinely.

Cell viability from all of the K₂EDTA vortex treatments were significantly higher than the control (Table 3.3.), However, a slight decrease in cell viability from the vortex treatment was observed after 24 hrs, with significant differences of about 16% between 10 and 15 seconds and a difference of about 21% between 10 and 20 seconds (Fig. 3.4., Table 3.3.). The THC analysis from the vortex mixing experiment showed that all treatments and controls had similar and constant values throughout the experiment, with significant differences only between 10 and 20 secs of vortexing. The mean range of THC for haemolymph samples across treatments and control ranged from 1.07×10^6 to 2.13×10^6 cells.mL⁻¹ throughout the experiment.

Results from the pipette mixing technique were not as conclusive as those with vortexing. Although all pipette mixing times resulted in significantly higher cell viability compared to controls (no mixing), there was great variability among mixing time and storage time effects (Fig. 3.4.; Table 3.4.). Among the mixing treatments, there was a general decrease in cell viability with increasing storage time. Regardless, the storage

time did not significantly affect the viability and cell count among the mixing treatments after 24 hrs of storage time.

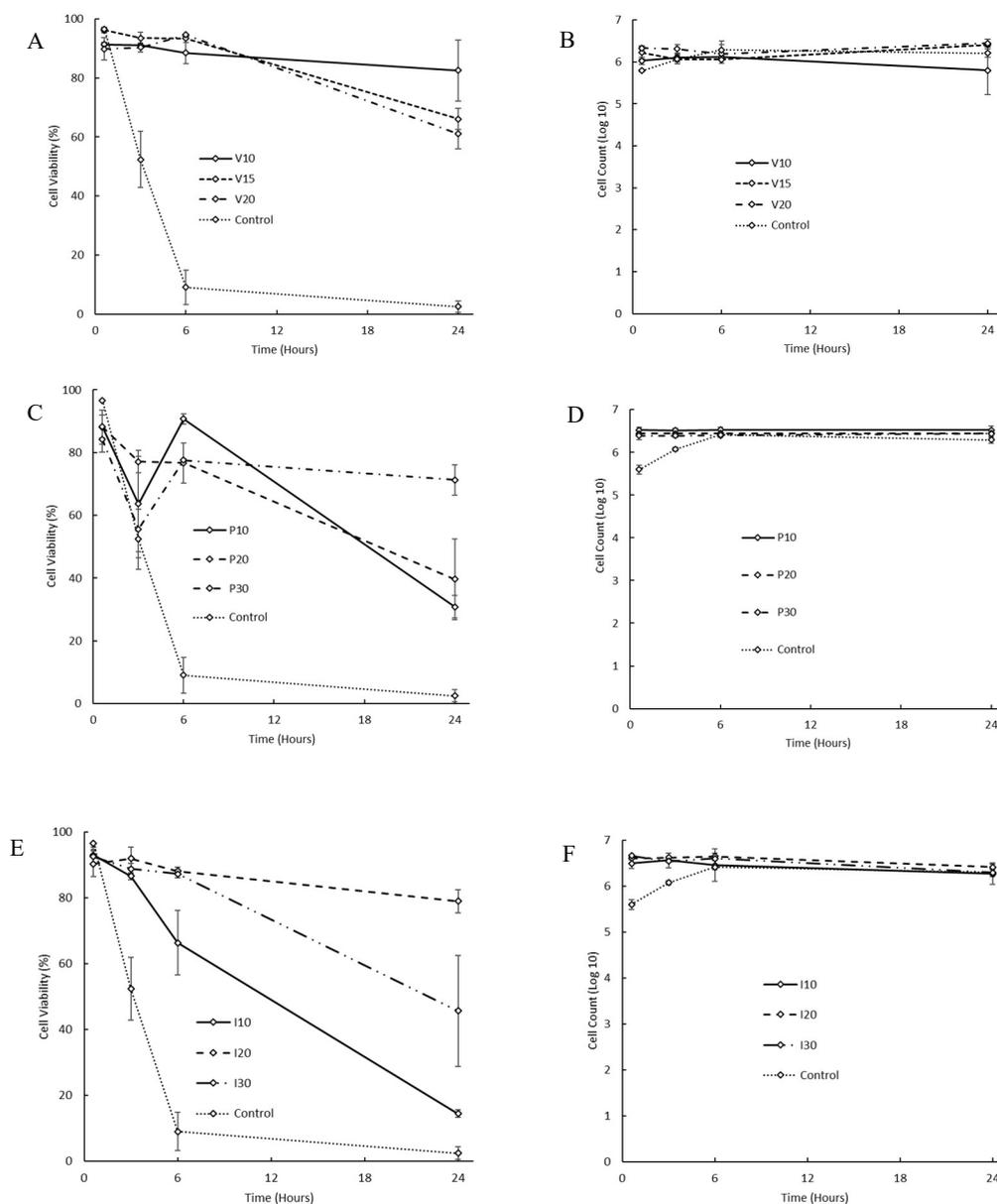


Figure 3.4. Total haemocyte cell count (log₁₀) and viability of haemocytes in K₂EDTA Microtainers® exposed to different mixing methods over a 24 hr storage period. (A and B) Effect of vortexing for 10, 15 and 20 secs (V10, V15, V20 respectively) compared to non-mixed control samples; (C and D) Effect of pipette mixing 10, 20 and 30 times (P10, P20, P30 respectively) compared to non-mixed control samples; (E and F) Effect of inversion flipping the Microtainer® tubes 10, 20 and 30 times (I10, I20, I30 respectively) compared to non-mixed control samples.

The THC measurements across treatments ranged from 1.96×10^6 to 2.61×10^6 cells.mL⁻¹, compared to the controls which ranged from 3.00×10^5 to 2.76×10^6 cells.mL⁻¹ throughout the experiment. Significant differences were observed between control and pipette treatments (Fig. 3.4.; Table 3.4.).

Results from the tube inversion technique also revealed significant differences in cell viability between inversion treatments and the control. However samples that were inverted 30 times showed higher viability compared to 10 and 20 times of inversion. (Fig. 3.4.; Table 3.5.). Thus, there were pronounced differences among inversion time treatments after 24 hrs of storage.

Table 3.1. Split-plot ANOVAs with Tukey post-hoc tests for cell viability and cell count (log₁₀) with different haemolymph:Alsever's solution ratios over time.

Source	df	MS	Cell Viability		MS	Cell Count		
			F	p - value		F	p - value	
<i>Between-subject</i>								
Dilution (D)	2	88.167	3.092	0.119	0.286	4.682	0.06	
Error	6	28.515			0.061			
<i>Within-subject</i>								
Time (T)	3	1670.656	42.402	0.001	1.573	117.008	0.001	
D x T	6	99.126	2.516	0.06	0.318	23.665	0.001	
Error	18	39.4			0.013			
<i>Significant Tukey HSD Tests</i>					<i>Significant Tukey HSD Tests</i>			
Dilution: -					Dilution: -			
Time : T1xT2, T1xT3, T1xT4, T2xT3, T2xT4					Time : T1xT2, T1xT3, T2xT3, T3xT4			

Table 3.2. Split-plot ANOVAs with Tukey post-hoc tests for cell viability and cell count (log₁₀) with different antiaggregants (K₂EDTA, Alsever's solution) at different temperatures (RT = Room temperature [21°C], LT = Low temperature [4°C]) over a 24 hr storage period.

Source	df	Cell Viability			Cell Count			
		MS	F	p - value	MS	F	p - value	
<i>Between-subject</i>								
Treatments	2	1851.08	27.03	.0001	Treatments	0.23	2.591	0.125
Error	6	68.48			Error	8	0.089	
<i>Within-subject</i>								
Time (T)	3	2869.9	69.87	0.001	Time (T)	0.63	9.42	0.001
Treatments x T	9	486.02	11.84	0.001	Treatments x T	0.42	6.21	0.001
Error	24	41.06			Error	0.07		
<i>Significant Tukey HSD Tests</i>				<i>Significant Tukey HSD Tests</i>				
K ₂ EDTA LT x AS LT								
K ₂ EDTA LT x AS RT								
K ₂ EDTA LT x K ₂ EDTA RT								
AS LT x K ₂ EDTA RT								
Time : T1xT2, T1xT3, T1xT4, T2xT3, T2xT4, T3xT4				Time : T1xT2, T1xT3, T2xT3				

Table 3.3. Split-plot ANOVAs with Tukey post-hoc tests for cell viability and cell count (log₁₀) of haemocytes in K₂EDTA Microtainers® and mixed via vortex for different times (10, 15, 20 sec; V10, V15, V20 respectively) compared to non-mixed controls (C) over a 24 hr storage period (measured at 10 min, and 3, 6, and 24 hrs).

Source	df	Cell Viability			Cell Count			
		MS	F	p - value	MS	F	p - value	
<i>Between-subject</i>								
Vortex (V)	3	6498.86	420.77	0.001	Vortex (V)	0.167	3.261	0.081
Error	8	15.45			Error	0.051		
<i>Within-subject</i>								
Time (T)	3	3535.89	170.45	0.001	Time (T)	0.017	0.332	0.802
V x T	9	1185.27	57.14	0.001	V x T	0.096	1.818	0.117
Error	24	20.74			Error	0.053		
<i>Significant Tukey HSD Tests</i>				<i>Significant Tukey HSD Tests</i>				
C x V10, C x V15, C x V20								
Time: all pairwise comparison Significant				Time: no significance				

Table 3.4. Split-plot ANOVAs with Tukey post-hoc tests for cell viability and cell count (\log_{10}) of haemocytes in K₂EDTA Microtainers® and mixed via pipette for different numbers of occurrences (10, 20, 30 times; P10, P20, P30 respectively) compared to non-mixed controls (C) over a 24 hr storage period (measured at 10 min, and 3, 6, and 24 hrs).

Source	df	Cell Viability			Cell Count			
		MS	F	<i>p</i> - value	MS	F	<i>p</i> - value	
<i>Between-subject</i>								
Pipette (P)	3	2759.67	47.93	0.001	Pipette (P)	0.424	27.606	0.001
Error	8	57.58			Error	0.015		
<i>Within-subject</i>								
Time (T)	3	5669.31	128.21	0.001	Time (T)	0.105	49.534	0.001
P x T	9	1390.47	31.45	0.001	P x T	0.095	44.430	0.001
Error	24	44.22			Error	0.002		
<i>Significant Tukey HSD Tests</i>					<i>Significant Tukey HSD Tests</i>			
C x P10					C x P10			
C x P20					C x P20			
C x P30					C x P30			

Table 3.5. Split-plot ANOVAs with Tukey post-hoc tests for cell viability and cell count (\log_{10}) of haemocytes in K₂EDTA Microtainers® and mixed via inversion treatments for different numbers of inversion (10, 20, 30 times; I10, I20, I30 respectively) compared to non-mixed controls (C) over a 24 hr storage period (measured at 10 min, and 3, 6, and 24 hrs).

Source	df	Cell Viability			Cell Count			
		MS	F	<i>p</i> - value	MS	F	<i>p</i> - value	
<i>Between-subject</i>								
Pipette (P)	3	5092.06	328.38	0.001	Pipette (P)	0.558	12.159	0.002
Error	8	15.51			Error	0.046		
<i>Within-subject</i>								
Time (T)	3	7462.03	177.59	0.001	Time (T)	0.115	20.601	0.001
P x T	9	1210.45	28.80	0.001	P x T	0.114	25.811	0.001
Error	24	42.02			Error	0.006		
<i>Significant Tukey HSD Tests</i>					<i>Significant Tukey HSD Tests</i>			
Treatment: All pairwise comparison significant					C x I10			
					C x I20			
					C x I30			

Observation of THC showed significant differences between the inversion treatments and the control treatment (Fig. 3.4.; Table 3.5.). The THC measurements across treatments ranged from 1.96×10^6 to 2.61×10^6 cells.mL⁻¹, compared to controls that ranged from 3×10^5 to 2.76×10^6 cells.mL⁻¹ throughout the experiment.

3.4. Discussion

Currently, there is limited information on the factors that affect the *in vitro* conditions of abalone haemocytes. Upon withdrawal, haemocyte cells are faced with radical changes, in addition to their interactive relation to one another, which clearly affects the cells (Rubin, 1997). Previous studies have shown that calcium ions trigger cell adhesion and aggregation in haemolymph of many molluscs by activating recognition molecules on the haemocyte surface (Renwrantz and Stahmer, 1983; Auffret and Oubella, 1997). Findings in previous studies (Travers *et al.*, 2008a; Grandiosa *et al.*, 2016; Hinzmann *et al.*, 2013; Torreilles *et al.*, 1999; Allam and Ford, 2006; Carballal *et al.*, 1997; Kanungo, 1982) have led to the routine use of Alsever's Solution or Modified Alsever's Solution as a haemocyte antiaggregant for marine invertebrate immunological studies. However, the effectiveness of these antiaggregants still requires evaluation, especially with regard to their abilities to maintain cell morphology and viability until physiological and immunological studies can be undertaken. In the present study, an effective antiaggregant and preparation protocol was successfully developed for abalone (*H. iris*) haemocyte cell count and viability analyses. The use of a portable flow cytometer as a method to provide rapid results of THC and viability provided effective preparation and simple application. Previously, we demonstrated that results of THC and viability are comparable to those obtained from Trypan blue exclusion tests and cell counts through a haemocytometer (Grandiosa *et al.*, 2016). The MUSE® flow cytometer platform has great potential to be used as a quantitative method for a wide range of cell features, such as apoptosis detection, changes in mitochondrial potential, oxidative stress, and cell cycle distribution that are routinely needed for cell analysis.

This study confirms that the K₂EDTA microtainers (BD) and Alsever's Solution (Sigma-Aldrich) were suitable antiaggregant media and have the ability to maintain cell integrity without significant loss to cell viability when used at room temperature (21°C).

The K₂EDTA microtainer and AS contain EDTA and citric acid respectively, which are chelating agents that sequester Ca²⁺ and Mg²⁺ ions from the haemolymph of molluscs (Chen and Bayne, 1995; Macey *et al.*, 2003). The effect of chelators may also possibly influence the membrane permeability to divalent cations (Kanungo, 1982).

Ca²⁺ and Mg²⁺ in haemocyte are important for normal behaviour of cells (Chen and Bayne, 1995; Macey *et al.*, 2003) and in supporting ligand-receptor binding mechanisms (Macey *et al.*, 2003). Our study revealed that weak aggregation consisting of 3–5 cell clusters still occurred during the first 30 minutes even in the AS treatments. It is possible that the aggregation in the early phase does not depend upon divalent cations since these clumps are still found. Previous studies have found that there are two-stage reactions related to aggregation which are Ca⁺⁺/Mg⁺⁺- independent and dependent respectively (Kanungo, 1982; Davies and Partridge, 1972).

Among the potential antiaggregants tested in this study, heparin was not an effective solution. Heparin-based antiaggregants are commonly used in human blood immunoassays and act primarily through an anti-thrombin mechanism by inhibiting the production of fibrin thus thinning the blood concentration (Yip *et al.*, 2006). In human blood, fibrin molecules combine to form long fibrin that entangles blood platelet (Yip *et al.*, 2006).

Our study found that in order to prevent coagulation and maintain a high viability of haemocytes, haemolymph samples contained in the K₂EDTA microtainers require proper mixing. Our study also showed that the technique of mixing is critically important to maintain high cell viability and cell count of *H. iris* haemocyte. The decreasing viability of haemocytes from the unmixed haemolymph in K₂EDTA microtainer may have been due to the reduced contact of haemocytes with K₂EDTA. Mixing haemolymph in K₂EDTA microtainer tubes successfully maintained cell viability for up to 6 hrs at room temperature (21°C), while haemolymph in K₂EDTA without mixing resulted in low

viability. Results also showed that the mixing method influenced the cell viability and THC. Vortex mixing resulted in a slightly higher cell viability compared to the other two methods. The optimum duration of vortex mixing was 10 sec, since additional exposure to vortex (15 and 20 sec) caused a slightly lower cell viability, which indicates a negative effect on mechanical stability. Similar findings were also found for human blood samples, where inverted K₂EDTA tubes resulted in higher red blood cell counts, haemoglobin concentration, and haematocrit and platelet counts compared to unmixed samples that tended to clot (Lippi *et al.*, 2007). In the present study, the tube inversion technique was also an effective haemolymph mixing method when K₂EDTA microtainer was inverted 20–30 times, resulting in relatively high cell viability. Conversely, pipette mixing resulted in lower cell viability compared to vortexing and tube inversion. There is a possibility that factors associated with pipetting might have affected cell viability, such as the bore of the pipette tip, speed at which the haemolymph suspension passes through the opening, size and rigidity of the cell (or cell clumps) and concentration of cells. Micropipette suction apparatuses may provide a versatile method for live cell sampling (Hochmuth, 2000). However, the use of micropipette for mixing might not be effective to ensure homogenous cell condition of haemocyte cells in K₂EDTA microtainers.

In this study, flow cytometry was used to demonstrate the effect of temperature and storage time on the haemocyte parameters of *H. iris*. We observed that 24 hrs storage of haemocytes in K₂EDTA and AS at room temperature (21°C) was able to preserve viability, while low temperature (4°C) storage reduced the viability of *H. iris* haemocytes. Similar conditions were found where low temperatures (e.g., 10 °C) present a stressful condition for *Perna viridis* haemocytes (Donaghy and Volety, 2011). However, in other species such as *C. gigas*, haemocytes maintained high viability when stored at low temperature in sterile sea water media (Gagnaire *et al.*, 2006). Meanwhile, temporary storage of blue mussel (*Mytilus edulis*) haemocytes in Hanks Balanced Salt Solution

(HBSS) and Leibovitz Media (L-15) at 4°C did not affect the viability (Hartl *et al.*, 2010). In addition, low temperatures (e.g., 10 °C) present a stressful condition for *Perna viridis* haemocytes (Donaghy and Volety, 2011). These contrasting findings may be somewhat limited by the fact that haemocytes are stored in different media. However, it is possible that the functional activities and metabolism of haemocytes in *in vitro* conditions are highly influenced by temperature (Donaghy and Volety, 2011), with temperatures above or below a certain threshold may result in stressful conditions for haemocytes of certain molluscan species (Monari *et al.*, 2007).

Previous studies raised the concern about using EDTA-based antiaggregants since it is thought to interfere with the immune capacity of invertebrate haemocytes by inhibiting release of oxygen radicals (Xu *et al.*, 2010). Although MAS and K₂EDTA are useful for haemocyte handling, these antiaggregants should be used carefully for quantitative studies related to calcium-dependent mechanisms of the cells.

3.5. Conclusions

This paper presents results on alternative antiaggregant applications for abalone haemocyte studies by cell viability, total haemocyte counts and stability analysis using the rapid MUSE Cell Analyser. Storage of haemocytes in low temperature using antiaggregant media is not recommended. The results presented in this paper showed that K₂EDTA and Alsever's solution are technically feasible and cost-competitive. The K₂EDTA tubes is preferable since it is easier to use and performed better. The findings of this study highlight the need to optimize and standardize approaches and techniques for observation of haemocyte.

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CHAPTER 4

Multi-strain probiotics enhances immune capacity and alters metabolic profiles in the New Zealand Black footed abalone (*Haliotis iris*)

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Abstract

We assess whether dietary administration of a multi-strain probiotic (*Exiguobacterium* JHEb1, *Vibrio* JH1 and *Enterococcus* JHLDc) led to enhanced immune responsiveness in juvenile New Zealand black-footed abalone (*H. iris*). Two groups of abalone were fed (1% body weight per day) over a four-month period with different diets. The control diet consisted of a standard commercial pellet feed (AbMax 16), whereas the treatment diet was additionally enriched with the probiotic mix.

At the end of the experiment, probiotic-fed animals showed improved growth compared with control-fed abalone in (length (32.3% vs 22.3%), width (31.9% vs 20.7%) and wet weight (109.6% vs 72.8%), respectively. Haemolymph sampling was conducted at the beginning of the experiment and after 2 and 4 months. Haemolymph samples were analysed for total haemocyte count (THC) and viability, presence of apoptotic cells and production of Reactive Oxygen Species (ROS). Compared with control abalone, probiotic-fed abalone had significantly higher THC (1.9×10^6 vs 5.6×10^5 cells), higher viability (90.8% vs 75.6%), higher percentage of ROS-positive cells (19.4% vs 0.5%) and higher numbers of non-apoptotic cells (88.0% vs 78.0%), respectively. These results indicate that the probiotic-enriched diet enhanced the immunostimulatory mechanisms, with a simultaneous low-level up-regulation of ROS production as a priming mechanism of the antibacterial defence system. Metabolomics-based profiling of foot muscle tissue additionally revealed that probiotic-fed abalone differentially expressed 16 unique metabolites, including amino acids, fatty acids and tricarboxylic acid related compounds. These data suggest that the probiotic-supplemented diet can also alter central carbon metabolic processes, which may improve the survival, as well as the growth of abalone.

Keywords: Probiotics; Abalone; Apoptosis; *Haliotis iris*; Oxidative stress, Metabolomics; Immunology; Haemolymph

4.1. Introduction

A key focus to develop the New Zealand black-footed abalone (*H. iris*) aquaculture sector is to improve health and enhance growth. New Zealand is well known for its eco-friendly production and strict quality assurance of shellfish aquaculture suitable for the development of abalone aquaculture (Alfaro *et al.*, 2014). Previous studies have observed the optimal nutrition and environmental parameters for the growth and health of cultured *H. iris* juvenile (Tung and Alfaro, 2011 a; 2011 b; 2011 c; Stuart and Brown, 1994; Allen *et al.*, 2006; Searle *et al.*, 2006; Preece and Mladenov, 1999; Bewick *et al.*, 1997). However, risks of disease outbreaks caused by pathogenic bacteria and viruses continuously pose a potential threat to this growing industry (Webb, 2013). Indeed, huge economic losses have been experienced around the globe due to frequent occurrences of disease outbreaks in farmed shellfish, such as abalone (Wang *et al.*, 2004; Hooper *et al.*, 2007; Sawabe *et al.*, 2007). Antibiotics can be used as a valuable short-term treatment to control bacterial infections. However, antibiotics come with food safety concerns and negative health and environmental impacts, such as the emergence of antibiotic resistant bacteria (Handler *et al.*, 2005). A scientifically demonstrable and ‘environmentally friendly’ solution to overcome pathogenic infections lies in the application of probiotics (Kesarcodei-Watson *et al.*, 2008; Newaj-Fyzul *et al.*, 2014; Verscuere *et al.*, 2004).

Probiotics are live microbial species, which can be incorporated as feed supplements and function to enhance intestinal balance and enhance food digestibility thus indirectly benefiting the growth and the health of the host (Fuller *et al.*, 1989; Irianto & Austin, 2002). Probiotic bacteria may be isolated from the aquatic environment (water column or substrate) or from the aquatic animal itself by scraping the skin mucus or isolating the probiotic candidate from the digestive tract of a healthy animal (Hadi *et al.*, 2014). Bacterial species used as probiotics in aquaculture comprise of a wide range of Gram-positive and Gram-negative species (Cross *et al.*, 2004) that have been suggested to

possess some kind of host specificity (Irianto & Austin, 2002). In most cases, the potential microbial organisms are isolated from the host of the intended aquaculture species. Putative probiotic species used in aquaculture are generally selected based on their abilities to colonize the intestine, assist in catabolism and uptake of nutrients (i.e. proteins, starch and alginate), produce acid, resist bile salts, and/or improve survival through presumed immunostimulant activity (Irianto and Austin, 2002; Hadi *et al.*, 2014).

Although molluscs rely primarily on non-specific immunity, there is increasing evidence to suggest that abalone immune systems can be modulated by probiotic bacteria via an immuno-prophylactic control mechanism against pathogenic bacterial strains (Cross *et al.*, 2004; Jiang *et al.*, 2013). Thus, the application of probiotics in abalone aquaculture has been successful for various species with significant benefits in improving health and disease resistance (Jiang *et al.*, 2013; Macey and Coyne *et al.*, 2005; Iehata *et al.*, 2010; Silva-Aciares *et al.*, 2013). For example, in *H. discus*, specific immune enhancements in probiotic fed abalone include an increase in haemocyte numbers, elevated haemocyte respiratory burst activity, enhanced serum lysozyme activity, and higher levels of total protein concentration in centrifuged haemolymph (Jiang *et al.*, 2013). In *H. rufescens*, a probiotic-supplemented diet resulted in a reduction of haemocyte apoptotic processes (Silva-Aciares *et al.*, 2013). In addition, a transcriptomic study in *H. rufescens* (Silva-Aciares *et al.*, 2013) was conducted to observe caspase-8, which is known to be an initiator of the caspase cascade involved in apoptosis as a process for maintaining cellular and tissue homeostasis (Hildeman *et al.*, 2007). Observations through proteomics analysis of haemocytes also showed that probiotic-fed *H. midae* had stimulated haemocytes based on the higher expression of Ras-related proteins and V⁺ ATPase proteins (Dias, 2016). These components are involved in the haemocyte maturation pathway (Dias, 2016).

Previous research by our group resulted in the development of multi-strain bacterial supplemented feeds, which display probiotic activity in the New Zealand black-footed abalone (Hadi *et al.*, 2014). Three probiotics (*Exiguobacterium* JHEb, *Vibrio* JH1, and *Enterococcus* JHLDc) isolated from the digestive organs of adult *H. iris* increased growth and survival of juvenile animals, and enhance digestibility of proteinaceous components. The observed improvement in health and reduced mortality suggested that the beneficial probiotic activity might be related, in part, to an immunostimulatory mechanism (Hadi *et al.*, 2014).

In the abalone, the haemocyte is the main cell that is involved in immune response such as the production of mediators of stress (Hooper *et al.*, 2007) and phagocytosis activity (Grandiosa *et al.*, 2016). According to their morphology and functions, *H. iris* haemocytes are classified into lymphocyte-like and monocyte-like haemocytes (Grandiosa *et al.*, 2016). The development of an innovative approach to characterize abalone haemocytes using a miniaturized fluorescent detection and micro capillary technology flow cytometer platform (Muse® Cell Analyser [Merck Millipore]) (Grandiosa *et al.*, 2016), made an important contribution to the study, since we could measure non-specific immune parameters of haemocytes in an efficient and quantitative manner.

The methodological approach taken in this study to investigate the effect of probiotic supplemented diets on the growth and health of abalone is a mixed methodology based on measurements of a variety of non-specific cellular immune parameters of haemocytes, including total haemocyte cell counts, haemocyte viability, generation of reactive oxygen species (ROS), and different stages of apoptosis. We also applied untargeted metabolite profiling, or metabolomics-based analysis (Young and Alfaro, 2016; Alfaro and Young, 2015; Alfaro and Young, 2016) of foot muscle tissues in an attempt to gain additional insights into the health status and nutritional condition of the animals. This study aims to

advance the understanding of the role of probiotics to support the development of *H. iris* aquaculture in New Zealand.

4.2. Methods

4.2.1. Experimental animals

About a total of 160 juvenile abalone (*H. iris*) (shell length = 27.5 ± 5 mm; weight = 2.14 ± 1.19 g) were obtained from a commercial hatchery (Moana Ltd: Ruakaka, Northern New Zealand). Animals were acclimatized at the Auckland University of Technology aquaculture facility for one week prior to commencement of the feeding trial. Animals were individually labelled with waterproof tags and the population were divided into four 40-L tanks (40 animals per tank) on a recirculating seawater system (5 μ m filtered and UV-treated seawater [35 ppt]), maintained in dark conditions at 16°C, and fed daily to satiation thrice per day with feeding at 1% of body weight.

4.2.2. Probiotic bacteria preparation

The probionts used in this study were previously isolated from the gastrointestinal tract of healthy abalone (selected for their proteolytic and amylolytic capabilities, and ability to alter the acid environment (Hadi *et al.*, 2014)) and comprised the strains *Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc. Bacteria isolates were previously stored at – 80°C in sterile marine broth (Difco) containing 20% [v/v] glycerol (– 80°C). The isolates were re-cultured in suitable enriched media for 48 hr at 22°C. *Exiguobacterium* JHEb1 was propagated in 200 mL marine broth (Difco) supplemented with 1% yeast extract; *Vibrio* JH1 was grown in 200 mL marine broth (Difco) supplemented with 0.5% glucose; *Enterococcus* JHLDc was prepared anaerobically in 100 mL MRS broth (Difco) containing 2% NaCl. The three strains of bacteria were prepared and cultured weekly.

4.2.3. Experimental Diets

The control diet consisted of a commercial pelletized abalone feed (AbMax 16; E.N. Hutchinson Ltd, Auckland, New Zealand). The feed was shown to be free of culturable bacteria via microbiological evaluation every two weeks using general nutrient agar (Difco).

The probiotic-enhanced diet was prepared weekly by supplementation of commercial feed (AbMax 16) with the probiotic mix. The bacterial strains cultured in broth media were harvested via centrifugation (Sorvall RC- 5C; Sorvall Instruments) at 5000 rpm at 10°C for 10 minutes. Bacterial pellets (probiotic biomass) were collected, transferred to a single bottle containing 25 mL of sterile nutrient broth (Difco), and then vortexed to get a homogeneous suspension. The probiotic mixture was inoculated on 20 g of commercial feed by spraying the feed pellets spread on plastic trays. The pellets were air-dried for 5 hours in a sterile microbiological cabinet at room temperature (19°C), then placed in sterile Petri dishes and stored at 10°C. Using the marine agar plating method (Hooper *et al.*, 2007), the average total viable bacterial count in the pellets was found to be around 2×10^9 CFU.g⁻¹. New batches of feed were prepared weekly and stored at 10°C until use. The stability of the probiotic-supplemented diet during semi-long term storage was tested by assessing bacterial viability after three months of storage at 10°C to assure that the probiotics were still active in the feed.

4.2.4. Experimental Design

After acclimation, shell length, shell width and total body wet weight of all animals were measured. Allometric data were then collected monthly for the four-month feeding trial, and cumulative daily mortalities recorded. Six static 20 L tanks (5 µm filtered seawater; salinity 35 ppt; temperature 16°C) were allocated, with triplicate tank systems for each of the two dietary treatments. Twenty healthy abalone were randomly distributed to each tank. Remaining animals were divided into two additional tanks and maintained

under similar experimental conditions to be used as replacements for dead individuals in the feeding trial to maintain the same densities, but these animals were not used in the analyses.

Water exchanges (50% by volume) were performed daily, while 100% water exchanges and thorough tank cleaning were performed every three days. Uneaten food was siphoned prior to each feeding event. Each tank contained a black coloured plastic tray that provide the abalone with shelter for favourable living conditions.

4.2.5. Microbial Analysis

Microbial quantification of probiotic strains within the digestive tracts were determined after one and three months by sacrificing three animals per treatment (one animal per tank) for each microbial sampling. Digestive tracts were aseptically excised with sterile forceps and blades, homogenized and 10-fold serially diluted with peptone water. From each dilution, 100 μ L aliquots were spread plated on selective agar. ChromAgarTM *Vibrio* (CHROMAgar, France), OxoidTM Kanamycin Aesculin Azide Agar Base (Thermo ScientificTM) and Marine Agar pH 8 (Fort Richard Laboratories Ltd) were used for the selective cultivation and enumeration of *Vibrio* sp., *Enterococcus* sp., and *Exiguobacterium* sp., respectively. Difco Marine Agar (Thermo ScientificTM) was used for the enumeration of the mixed probiotic species.

4.2.6. Haemocyte Analysis

Haemocyte parameters, including counts and viability, apoptosis and percentage of cells exhibiting ROS were assessed in haemolymph samples with the Muse® Cell Analyzer at the beginning of the trial and after two and four months. At each sampling event, three abalone per tank (nine abalone per treatment) were used to obtain haemolymph. Haemolymph was collected from the anterior arterial sinus of the abalone

for each sampling event using a 1 mL sterile syringe (25 G x 5/8 in), and immediately mixed with Alsever's solution in a 1:1 ratio.

4.2.6.1. *Haemocyte Cell Count and Viability*

Haemocyte concentration and viability were measured using the Muse® Cell Cycle Assay Kit (Merck Millipore) according to the manufacturer's instructions. Twenty microliters of diluted sample in Alsever's solution (SIGMA) were mixed with 380 µL assay reagent in 1.5 mL micro-centrifuge tubes, incubated for 5 min in the dark at room temperature, briefly vortexed and analysed.

4.2.6.2. *Haemocyte Oxidative Stress*

Production of Reactive Oxygen Species (ROS) in haemocytes were evaluated using the MUSE® Oxidative Stress Kit (Merck Millipore), with minor modifications to the manufacturer's instructions. Briefly, diluted haemocyte samples were prepared by mixing 100 µL solution containing 1:1 of haemolymph to Alsever's solution with 100 µL 1X Assay Buffer (Merck Millipore). The Muse® Oxidative Stress working solution was first diluted 100X with the Assay Buffer to make an intermediate solution. This intermediate solution was further diluted 80X with Assay Buffer to make Muse® Oxidative Stress working solution. The final sample solution to be processed via flow cytometry was prepared by mixing 190 µL of Muse® Oxidative Stress working solution with 10 µL of the diluted haemocyte sample in 1.5 ml micro-centrifuge tubes, followed by incubation at 37°C for 30 minutes.

4.2.6.3. *Haemocyte Apoptosis*

Haemocyte apoptosis profiling was performed using the Muse® Annexin V and Dead Cell Assay Kit (Merck Millipore), with minor modifications to the manufacturer's instructions. A total of 100 µL of diluted sample in Alsever's solution was mixed with

100 µL of assay reagent in 1.5 mL micro-centrifuge tubes, incubated for 20 min in the dark at room temperature, briefly vortexed and analysed.

4.2.7. Metabolomic Analysis

Nine randomly selected animals from each dietary treatment group (probiotics and control) were sacrificed at the end of the feeding experiments to obtain tissue samples for metabolomics analysis. Approximately 150 mg wet weight of foot muscle were dissected from each individual, placed in 2 mL cryogenic vials, snap frozen in liquid nitrogen, and stored at -80°C until metabolite extraction. Tissue samples were lyophilised overnight then ground in liquid nitrogen using a mortar and pestle. Approximately 10 mg of powdered tissue were placed in 1.5 mL Eppendorf tubes and co-extracted with an internal standard (L-Alanine-2,3,3,3-d₄) using a cold methanol-water method according to Villas-Bôas *et al.* (2011), with modifications. L-Alanine-2,3,3,3-d₄, sodium hydroxide, pyridine and methyl chloroformate (MCF) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol and chloroform were purchased from Merck (Darmstadt, Germany). Sodium bicarbonate and anhydrous sodium sulphate were purchased from Ajax Finechem (NSW, Australia). One control sample was accidentally contaminated during the extraction process, and thus excluded from further processing. Polar extracts were lyophilised, re-suspended in NaOH followed by derivatization via MCF alkylation (Supplementary Methods). A solvent blank was similarly processed. Derivatised extracts were analysed using a gas chromatograph GC7890 (Agilent Technologies, USA) coupled to a quadrupole mass spectrometer MSD 5975 (Agilent Technologies, USA) according to the protocol described by Smart *et al.* (2010). Deconvolution of chromatographic data was performed using the Automated Mass Spectral Deconvolution and Identification System. (AMDIS v2.66) software. According to the electron impact fragmentation mass spectrum and retention time, metabolites were identified using Chemstation software (Agilent Technologies) and customised R xcms-based scripts (Aggio *et al.*, 2011) to interrogate

an in-house library of 227 MCF derivatised compounds. Data were manually checked for the presence of contaminants (i.e., derivatization artefacts and non-biologically derived compounds) with and aberrant records being removed, and remaining data were blank corrected to control for background contamination (Smart *et al.*, 2010). The resulting QC-filtered peak intensity values were normalised by the internal standard to compensate for potential technical variations (e.g. variable metabolite recoveries), and then by sample-specific biomass prior to statistical analyses.

4.2.8. Statistics

Differences between bacterial cell counts, treatments of total haemocyte cell counts, cell viability, oxidative stress and apoptosis were identified with the Student's *t*-test ($p < 0.05$) and ANOVA with Duncan post hoc analysis using the SPSS Statistics for Windows (Version 19.0. Armonk, NY: IBM Corp.). Bacterial cell count data were log transformed prior to analysis to meet parametric assumptions.

The normalised data matrix obtain from the GC – MS analysis was auto scaled and analysed using Metaboanalyst 3.0 (Xia *et al.*, 2013). A Student's *t*-test with an alpha level of 0.05 was performed to identify differences in foot muscle metabolite profiles between abalone fed with the two experimental diets. Combined heat map and hierarchical cluster analysis (Euclidian distance; Wards criterion) of the top 20 metabolites ranked by their *t*-test statistics was performed to assist visualisation of major group differences and between-sample variability.

4.3. Results

4.3.1. Microbial analysis of probiotic bacteria in the feed post storage

The viability of putative probiotic of three selected bacterial species was observed by plate culture and remained viable at a concentration optimal for probiotic function after three months of storage at 10°C (Fig. 4.1.). *Enterobacterium* spp., *Vibrio* spp. and

Exiguobacterium spp. were detected at an average bacterial count of 4.15×10^8 CFU g⁻¹, 7×10^7 CFU g⁻¹ and 2.25×10^7 CFU g⁻¹, respectively, while the count for mixed species in marine agar was 1.3×10^9 CFU g⁻¹. Observation of non-probiotic supplemented control feed showed no presence of microbial contamination.

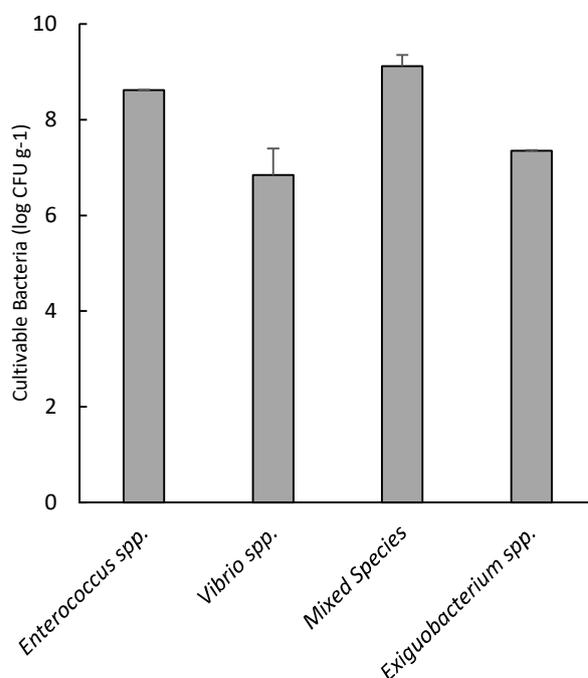


Figure 4.1. Viability of probiotic bacteria in the probiotic supplemented feed after three months storage at 10°C.

4.3.2. Microbial analysis of digestive tracts

After one month and three months of feeding, probiotic bacteria had already been established within the digestive tracts of abalone fed with probiotic-supplemented feed in substantially higher concentrations than those found in abalone fed the control diet (except for *Vibrio* spp. after one month) (Fig. 4.2.). *Enterococcus* spp. nor *Exiguobacterium* spp. were detected in the non-probiotic fed control animals at either of the time points assessed. Since our culture-based techniques could not distinguish the specific-species of *Vibrio*, it is unknown if control animals had our probiotic *Vibrio* JH1 or another commonly found *Vibrio* species in the digestive tracts of the animals.

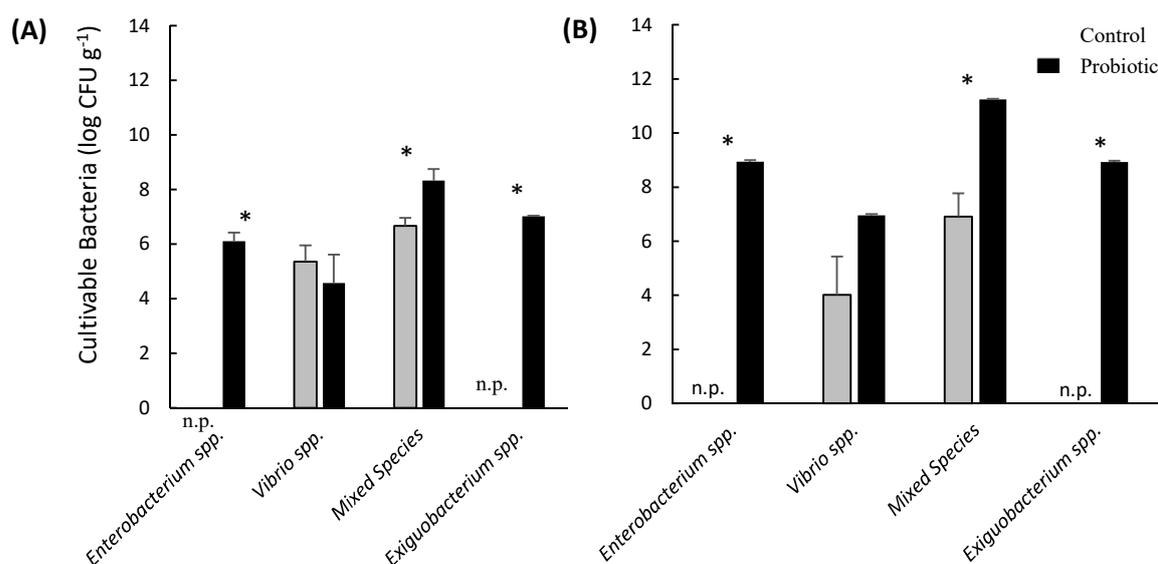


Figure 4.2. Quantity of probiotic bacteria present in the digestive tracts of *H. iris* after 1 month of feeding (A), and 3 months of feeding (B). Asterisks indicate statistical significance ($p < 0.05$) between the different dietary groups (n.p. = bacteria not present).

4.3.3. Effect of probiotics on mortality

Survival of abalone was monitored throughout the experiment. All animals fed with the probiotic-supplemented diet survived after four months of feeding, whereas five animals from the control treatment died after the first three months (ca. 10% mortality). All of the abalone that died showed signs of poor growth, appearance of pigment loss in the foot tissue and epithelial erosion of the foot organ.

4.3.4. Effect of probiotic feeding on growth

A general increase in length, width and weight of the probiotic-fed *H. iris* was observed throughout the four-month feeding period (Fig. 4.3.). In general, the probiotic-fed had higher values of length, width and weight compared to the control animals. However, the only statistically significant growth difference between the probiotic-fed and the control animals was recorded in length and width after 3 months of feeding. At

the end of the four-months feeding experiment, the mean growth increase length, width and wet weight percentage (\pm SD) of the probiotics fed animals were 32.28 ± 1.11 %, 31.88 ± 0.25 %, 109.58 ± 6.54 %, respectively, while the values for the control fed abalone were 22.31 ± 9.64 %, 20.65 ± 11.48 % and 72.77 ± 41.31 % respectively.

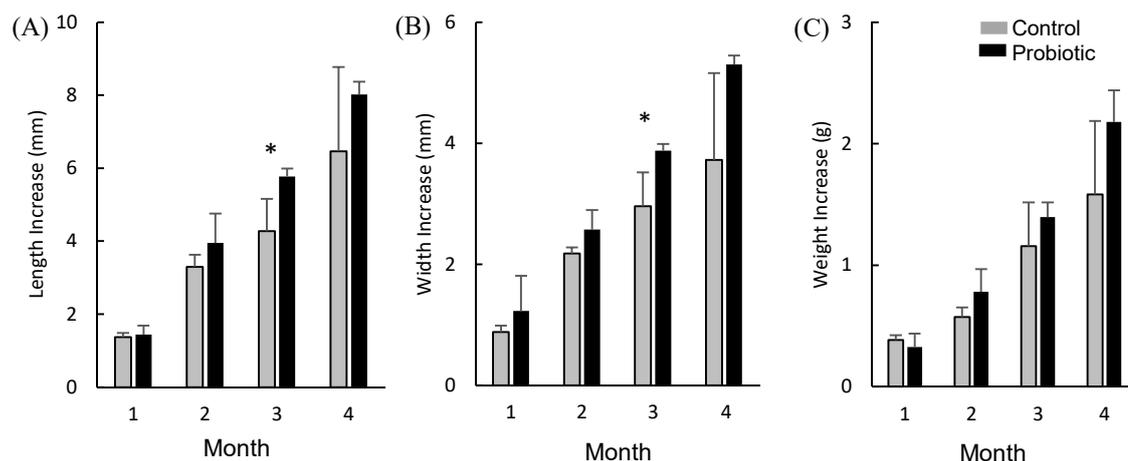


Figure 4.3. Abalone growth in shell length (A), shell width (B) and wet weight (C) for control (grey bars) and probiotics (black bars) fed animals over four months. Data represents the mean \pm SD ($n=60$ per treatment) from nine abalone per treatment. Asterisks indicate statistical significance ($p < 0.05$) between the different dietary treatment of control and probiotic.

4.3.5. Immune responses after probiotics feeding

Total haemocyte counts ($\times 10^6$ cell ml^{-1}) and cell viability (%) showed no statistical differences ($p > 0.05$) between control fed and probiotic fed animals at week 1 and week 8 (Figure 4.4.). However, after 16 weeks (fourth months), total haemocyte counts and cell viability were significantly higher ($p < 0.05$) than those in the control group, with relative mean fold changes of 3.4 and 1.2, respectively.

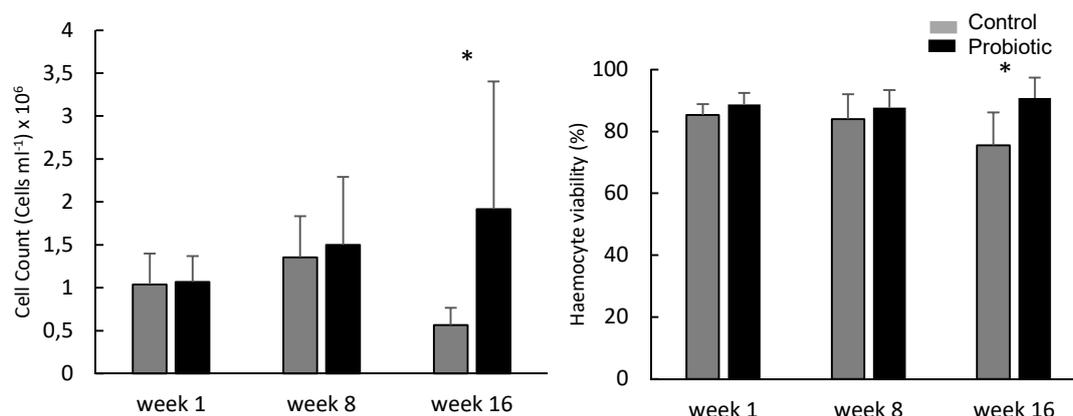


Figure 4.4. Immune responses of abalone (*H. iris*) fed with probiotic supplemented and non-supplemented control diets, including A) total cell counts and B) haemocyte viability. Data represents the mean \pm SD from nine abalone per treatment. Asterisks indicate statistical significance ($p < 0.05$) between the different dietary groups of control and probiotic within the same sampling week.

Direct quantitative measurements of the percentage of haemocyte cells exhibiting ROS (namely superoxide radicals) showed a low percentage (ca. 1%) of ROS positive cells at the beginning of the trial (Fig. 4.5.). After 4 months of feeding, the probiotics fed abalone exhibited a significantly higher percentage (19.41 ± 23.31 %) of ROS positive cells compared to those of the control group (0.5 ± 0.7 %) (Student's t-test, $p < 0.05$).

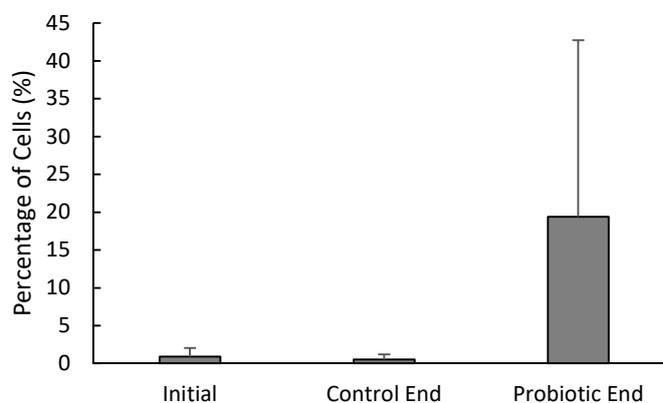


Figure 4.5. Percentage of abalone haemocytes exhibiting Reactive Oxygen Species (ROS) before and after feeding with probiotic supplemented and non-supplemented control diets. Data represents the mean \pm S.D from nine abalone per treatment.

Haemocytes at different stages of apoptosis were measured for each treatment. An example of an apoptosis profile (Fig. 4.6.), presents the population cells based on their apoptotic status; non-apoptotic live cells (lower left quadrant: Annexin V⁻/7-AAD⁻), cells exhibiting early stages of apoptosis (lower right quadrant: Annexin V⁺/7-AAD⁻), cells during late stages of apoptosis (upper right quadrant: Annexin V⁺/7-AAD⁺), and dead cells (upper left quadrant: V⁻/7-AAD⁺). Apoptosis were quantitatively analysed and the results are presented in a bar graph format (Fig. 4.7.).

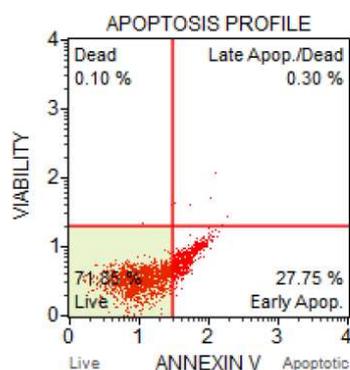


Figure 4.6. Apoptotic analysis of abalone (*H. iris*) haemocyte using the MUSE[®] Cell Analyzer produced cellular profiles, which displayed various stages of apoptosis. Red lined quadrants indicate boundaries of the four cell populations (live, early apoptotic, late apoptotic, and dead cells).

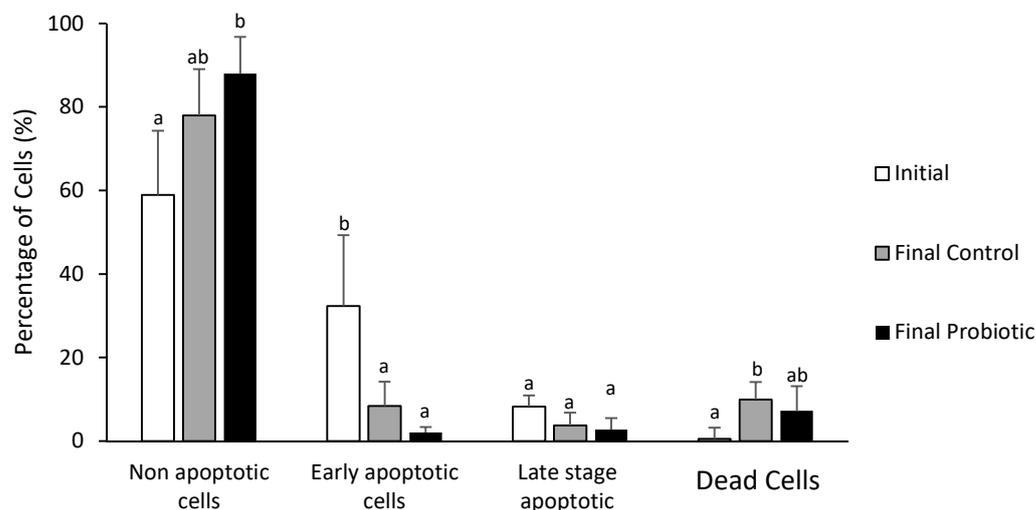


Figure 4.7. Percentage of non-apoptotic, early apoptotic, late apoptosis and dead cells (mean \pm S.D from 9 abalone). Difference in notation above bar graphs indicate statistical significance ($p < 0.05$).

Apoptosis analyses was conducted at the beginning of the experiment and at the end of the experiment. Early apoptotic haemocyte percentage were present at a significantly higher value (analysis of variance, $p < 0.05$) during the initial stage of the experiment (32.4%). The early apoptotic cell values obtained after four month feeding from both probiotic supplemented and non-supplemented control diets were 8.4% and 2.0% respectively. The percentage of non-apoptotic haemocyte cells in control fed animals (78.0%) and in probiotic fed animals (88.0%) did not differ significantly upon each other. However, it was observed that the percentage of non-apoptotic haemocyte cells in the initial stage of experiment was significantly lower (58.9%).

4.3.6. *Metabolomics*

GC-MS analysis of foot tissue polar extracts detected 102 unique metabolites after QC filtering of the data. Of these, 82 were attributed specific chemical identities by matching chromatographic and mass-spectral information against our in-house metabolite library. The remaining 20 features are currently listed as 'unknowns' since no matches were found. Univariate statistical analysis showed a number of differences in the metabolite profiles between animals receiving the two experimental diets (Fig. 4.8. and Table 4.1.). Five amino acids (serine, glutamine, asparagine, lysine, proline), two fatty acids (palmitoleic acid, adrenic acid), four organic acid derivatives (alpha-ketoglutarate, succinic acid, lactic acid, oxalic acid), and six unknowns, but unique, metabolites were over-expressed (t-test; $p < 0.05$) in the probiotic-fed abalone, compared to those which received the non-probiotic supplemented control feed.

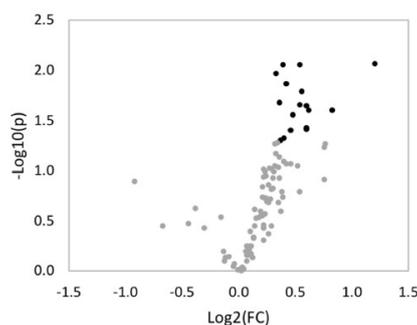


Figure 4.8. Volcano plot of metabolites comparing probiotic supplemented diet vs control diet. Each point represents a metabolite. Points in black are significantly different between treatments (t-test; $p < 0.05$).

Table 4.1. List of significantly (t-test) altered metabolites (Fold Change = Probiotic/Control).

Metabolite	^a Foldchange	p-value
Lysine	1.45	0.009
Proline	1.47	0.016
Asparagine	1.51	0.023
Glutamine	1.52	0.038
Serine	1.31	0.046
Adrenic acid	1.45	0.022
Palmitoleic acid	1.29	0.049
Lactic acid	1.78	0.025
Succinic acid	1.52	0.038
Oxalic acid	1.31	0.009
α - ketoglutaramate	1.33	0.013
Unknown 1	2.30	0.009
Unknown 2	1.26	0.011
Unknown 3	1.28	0.021
Unknown 4	1.53	0.025
Unknown 5	1.39	0.027
Unknown 6	1.37	0.039

A heatmap with combined hierarchical cluster analysis of the top 20 metabolite ranked by their t -test statistics was used to assist visualisation of the major group differences (Figure 4.9.). Although variations in levels of some metabolites were

observed among individuals, a general pattern of enhanced metabolite expressions in probiotic fed abalone can easily be identified.

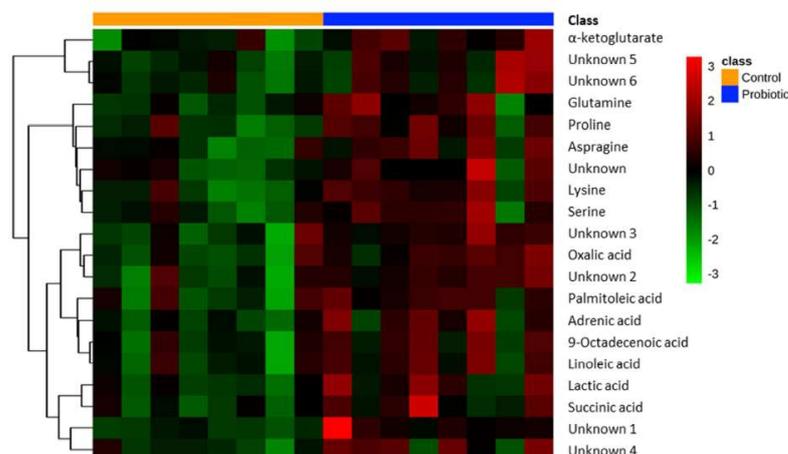


Figure 4.9. Heatmap with combined hierarchical cluster analysis (Euclidian distance; Ward's criterion) of metabolites. *Columns* represent samples (*orange* = Control diet, *blue* = Probiotic diet) and rows represent metabolites. The *green/black/red* colour scale represents standardised (autoscaled) abundance data, where *red* = higher values, and *green* = lower values.

4.4. Discussion

Our study is the first to report the effect of probiotic in modulation of various properties of the immune system of *H. iris* abalone fed with probiotics compared to control abalone. Following the dietary inclusion of multi-strain probiotics (*Exiguobacterium* JHEb, *Vibrio* JH1, and *Enterococcus* JHLDe) in the feed, there was evidence of the enhanced innate-immunity in the probiotic-fed animals observed from the total haemocyte count, cell viability, apoptosis and production of reactive oxygen species (ROS) parameters. In addition, metabolic profiles from foot tissues of abalone showed differences between treatments. After 3 months of feeding, growth and survival rates of probiotic-fed abalone were also found to be higher than that in control-fed animals.

The high numbers of *Exiguobacterium* JHEb, *Vibrio* JH1 and *Enterococcus* JHLDc found in the gastrointestinal tract (GIT) of *H. iris* was suggested to contribute upon the growth and health of juvenile abalone by the bacterial-mediated improvement on feed digestibility and feed utilization (Hadi *et al.*, 2014). The active role of the multi-strain probiotic may have reduced their energy consumption to digest feed, hence providing improved growth and immunologic condition. A previous study in *H. rufescens* (Silva-Aciaras *et al.*, 2013) showed that probiotic-fed abalone was associated with a decrease in expression of genes related to energy metabolism. Probiotic may provide additional digestive enzymes, which may increase the growth and immune state by reducing the energy needed for digestion.

Haemocytes are the main defence cell of molluscs and are capable of antigen recognition, attachment followed by agglutination, phagocytosis, and elimination of antigens by respiratory burst or exocytosis of antimicrobial factors (Adema *et al.*, 1991). Our study have found an increase of haemocyte number circulating inside the body possibly due to the effect of probiotic enriched in the abalone feed. Previous studies confirms our finding where probiotic feeding also increased the total haemocyte count (THC) in *H. discus hannai* (Jiang *et al.*, 2013), *H. midae* (Macey & Coyne, 2005) and *H. rufescens* (Silva-Aciaras *et al.*, 2013). These increases also occurred in other invertebrate phyla such as *Litopenaeus stylirostris* where animals were fed probiotic daily (Castex *et al.*, 2010). In some cases, probiotic feeding did not necessarily increase the THC in abalone (*H. midae*), but provided an enhanced ability for haemocyte proliferation to occur post-challenge to pathogenic *Vibrio anguillarum* (Macey and Coyne, 2005). Increased THC is associated with haematopoiesis processes, that involves components such as the haemocyte precursor and the haematopoietic tissue. However, the haematopoiesis in the abalone is still unclear. Previous studies in molluscs have indicated that the haematopoiesis process involves a complicated mechanism of signalling pathways,

receptors, cytokines, growth factors, and transcription factors (Pila *et al.*, 2016). Given that the haemocyte proliferation remain high after probiotic feeding and considering haemocytes still remains in the circulation for a certain period of shelf life, this may indicate a process of immunological priming to protect the animal against future pathogenic stress (Little & Kraaijeveld, 2004). However, the mechanisms and consequences of immune priming in invertebrates still need to be properly demonstrated as to whether these immune responses are stable following immune system stimulation.

The lower cell viability observed in the control-fed animals in the end of the experiment may reflect stress status, and suggests that the dietary probiotics provide a protective effect. Haemocyte viability in invertebrates can be influenced by environmental factors, such as sudden temperature fluctuation (Hégaret *et al.*, 2003), pH stress (Wang *et al.*, 2009), handling (Cardinaud *et al.*, 2014), air exposure (Cardinaud *et al.*, 2014) and presence of heavy metals (Mottin *et al.*, 2010). However, it is thought that the low viability observed in the control animals was caused by stress from an opportunistic pathogen, since animals that died in the control treatment showed symptoms of a disease caused by a bacterial pathogen. The effect of antigens on invertebrate haemocyte viability have been studied *in vitro* where bacterial pathogens, bacterial extracellular product and LPS has reduced the cell viability (Mottin *et al.*, 2010; Prado-Alvarez *et al.*, 2012). Furthermore, *in vivo* pathogenic bacterial challenge can also reduce the haemocyte viability (Allam *et al.*, 2006). The reduction of invertebrate haemocyte viability might be induced by oxidative damage or by other pathways leading to apoptosis (Franco *et al.*, 2009). It is possible that the multi-strain probiotic actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and/or space, alteration of microbial metabolism (Irianto and Austin, 2002).

Most of the previously mentioned studies regarding haemocyte cell viability above did not report the specific necrotic status, since cell death could manifest non-apoptotic

features (Sokolova, 2009). Although a difference of apoptotic status of haemocyte cells was not found between control fed and probiotic fed animals, we detected apoptotic symptoms from animals during the initial sampling. Activation of apoptosis in mollusc haemocytes can be stimulated by various inducing factors, for different purposes. For example, apoptosis provides a protective host defence mechanism during viral infections by reducing cell longevity to stem viral proliferation, it can be induced by phagocytised bacterial pathogens to impair the host defence system, and it can be activated as a cell death mechanism during exposure to environmental stressors to reduce pro-inflammatory necrosis and maintain good health (Kiss, 2010; Terahara and Takahashi, 2008). A higher percentage of early apoptotic haemocyte cells at the start of the experiment may indicate that the abalone were mildly stressed.

Probiotics-fed abalone showed higher production of ROS compared with control-fed animals. The presence of ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) may indicate an immune response mechanism specifically related to the phagocytosis activity of the haemocyte (Nappi and Ottaviani, 2000). The specific signal transduction pathways that mediates this production are not been thoroughly understood. However, a recent proteomic study in *H. midae* observed that the haemocyte in probiotic-fed abalone showed phagosomal maturation and also exhibiting significant expression of fundamental proteins such as V-type proton ATPase and Ras-related protein Rab in haemocytes (Dias, 2016). Although production of cytotoxic ROS molecules is employed by the abalone, it does not necessarily indicate oxidative stress and may only be a sign that a stimuli factor is threatening the homeostasis status of the abalone (Nappi and Ottaviani, 2000). Environmental stressors may also have a role in the production of these molecules (Martello *et al.*, 2000). However, it is unlikely to be the cause of ROS production in haemocyte of probiotic-fed animals since environmental parameters were similar in both control- and probiotic-fed abalone tanks. The multi-probiotic

supplemented diet may enhance the immune capacity potentially by influencing or priming the ROS regulatory system. However, further work is needed to test this hypothesis and should include an analysis of ROS-regulatory enzyme expression and/or activities.

Metabolite profiling of foot tissue revealed variations in the relative abundance of 17 unique metabolites between control-fed and probiotics-fed abalone. Enriched levels of FFA's (e.g., lysine, proline, asparagine, glutamine and serine) in probiotics-fed abalone reflect differential capacities for protein turnover. These observations could be a result of enhanced digestibility of dietary protein attributable to the high proteolytic abilities of the selected probiotics, as reported by Hadi *et al.* (2015). Irrespective of their origin, higher levels of FAA's in abalone foot muscle tissue reflect good animal health status (Viant *et al.*, 2003; Rosenblum *et al.*, 2005) and some specific amino acids are associated with non-specific immunity. For example, proline appears to play a role in modulating immune responses of fish towards bacterial infections and can be used as a therapeutic agent to improve survivability (Zhao *et al.*, 2015). Free proline also serves an important function in protecting organisms through its ability to act as a free radical scavenger (Paniello *et al.*, 1988), and by stabilizing proteins from *in vivo* aggregation (Fisher, 2006). Up-regulation of glutamine and asparagine in probiotics-fed abalone may stimulate growth and improve immunocapacity (Hochachka, 2014). Glutamine is a key nutrient for dividing cells since it functions as non toxic nitrogen vehicle and respiratory fuel (Aledo, 2004; Carrascosa *et al.*, 1984; Moeradith and Lehninger, 1984) and serves as a precursor for purine and pyrimidine synthesis, which is essential for immune cells to replicate (Walsh *et al.*, 1988). Glutamine synthase in abalone muscle was down-regulated following environmental stress (Cardinaud *et al.*, 2014) and bacterial infections (Travers *et al.*, 2010). Thus, glutamine content in foot muscle may indicate the health status of abalone. Elevated levels of free asparagine also appear to be beneficial for mounting a

successful immune response in higher taxa (Li *et al.*, 2007). However, the mechanisms involved are poorly understood, and a functional role in molluscan immunity is yet to be investigated.

Alterations in metabolites involved in glycolysis and the tricarboxylic acid (TCA) cycle likely reflect differential energetic statuses. Alpha-ketoglutarate is a rate-determining intermediate in the TCA cycle and has a crucial role in cellular energy metabolism. Succinate is also a crucial intermediate and is positioned at the crossroads of several metabolic pathways (Tannahill *et al.*, 2013), including the formation and elimination of ROS (Tretter *et al.*, 2016). Although higher levels of lactic acid in tissues of probiotics-fed abalone could signal differences in glycolytic metabolism, the relative increase in this metabolite may reflect input from the selected lactic acid producing probiont (*Enterococcus* JHLDc) which was incorporated into the treatment diet. Other differential metabolite signatures in probiotics-fed abalone tissue included increased levels of the FFAs palmitoleic acid and adrenic acid, and higher levels of oxalic acid. Palmitoleic acid is a common mono-unsaturated fatty acid produced from desaturation of palmitic acid, whereas adrenic acid is an eicosanoid derived from elongation of arachidonic acid and is involved in prostaglandin synthesis (Campbell *et al.*, 1985). Although defined functional roles for oxalic acid in animals are not yet firmly established, this metabolite has been marked as a key regulator or precursor of various branches of amino acid synthesis, an antioxidant, and a mediator of apoptotic processes (Kayashima & Katayama, 2002; Gregorc *et al.*, 2004; Lehner *et al.*, 2008).

4.5. Conclusions

The present study was designed to determine the effect the enrichment of feed with multi-strain probiotic upon the survival, growth, selected non-specific immune parameters and metabolite profile of the New Zealand black-footed abalone. The results

of this investigation shows that probiotic feeding has increased the survival rate and growth and stimulate haemocyte immune parameters. In addition, significant metabolites in the probiotic-fed abalone were assumed to be associated with enhancement of the non-specific immune response. These findings have significant implications for the understanding of how probiotic enrichment affects the abalone. Therefore, the findings of this study strengthen future development and application of probiotics in aquaculture of New Zealand black-footed abalone (*H. iris*).

Acknowledgement

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CHAPTER 5

Immune response in probiotic-fed New Zealand black-footed abalone (*Haliotis iris*) under *Vibrio splendidus* challenge

Note: This chapter will be submitted:

Abstract

New Zealand black-footed abalone (*H. iris*) were fed a commercial diet enhanced with multi-strain probiotics (*Exiguobacterium* JHEb1, *Vibrio* JH1 and *Enterococcus* JHLDe) for four months, then challenged with an injection of pathogenic *Vibrio splendidus*. Host immune responses in haemocytes were characterized using flow cytometry by measuring total haemocyte count (THC) and viability, degree of apoptosis, and production of reactive oxygen species (ROS) 48 h post-challenge. Probiotic-fed abalone had significantly higher survival rates compared to control animals after the bacterial challenge. Infected probiotic-fed abalone also had significantly higher THCs, slightly lower proportions of haemocytes undergoing early apoptosis, and lower percentages of ROS-producing haemocytes compared to infected control-fed abalone. In addition, metabolite profiles of muscle tissues generated via GC-MS provided suggestions of a perturbed ROS-regulatory system in infected abalone through changes in key metabolites associated with glutathione biosynthesis. The results of this study provide valuable information to assist in farm management practices, leading to enhanced production and sustainability of the growing New Zealand abalone industry.

Keywords: *Haliotis iris*: *Vibrio splendidus*: probiotics: haemocytes: metabolomics: immunostimulation

5.1. Introduction

Bacterial pathogens are ubiquitous in marine systems, and continuously threaten the health of key aquaculture species. In particular, Vibriosis disease is a major obstacle for molluscan culture, with several *Vibrio* spp. reportedly being causal agents for mass mortality outbreaks in abalone worldwide. For example, *V. parahaemolyticus* was responsible for substantial stock losses of *H. diversicolor supertexta* in Taiwan (Liu *et al.*, 2000) and China (Cai *et al.*, 2007). Infections of *V. carchariae/harveyi* have been observed in *H. tuberculata* along the French coast (Nicolas *et al.*, 2002) and in *H. discus hannai* in Japan (Sawabe *et al.*, 2007), whereas *V. splendidus* was isolated from moribund *H. rubra* and *H. laevigata* during disease outbreaks in Australia (Handlering *et al.* 2005). In New Zealand, abalone aquaculture with poor health are often affected by bacterial infections such as *Vibrio splendidus* (Diggles *et al.*, 2002; Diggles & Oliver 2005). Stress factors (e.g., high temperatures, grading trauma, anaesthetics, and altered salinity) typically tend to precipitate such diseases. One potential method which can increase the health of abalone and overcome susceptibility to bacterial pathogens is the administration of dietary probiotics (Grandiosa *et al.* 2018).

The application of probiotics as feed supplements can enhance intestinal balance and food digestibility, which ultimately result in health and growth benefits for the host (Fuller, 1989; Irianto and Austin, 2002). Maintenance of good health status ideally ensures that host mechanisms of immunity are sufficiently primed to mount a defence against foreign antigens, safeguarding against pathogen infections. Immunological benefits of dietary probiotics against bacterial infections in abalone have specifically been demonstrated. For example, Jiang *et al.* (2013) and Park *et al.* (2016) showed that *Vibrio*-infected *H. discus hannai* Ino fed with commercial diets containing probiotics had significantly improved survival rates compared to non-probiotic fed abalone. Similarly, Macey and Coyne (2005) showed that survival and immunocapacity of haemocytes can

be enhanced in *Vibrio*-infected *H. midae* after being fed a multi-strain probiotic-enriched diet. Incorporating evidence at a genetic level, Silva-Aciares *et al.* (2013) further demonstrated that probiotic-enriched diets promoted greater survival and differential expressions of immune-related genes in *Vibrio*-infected *H. rufescens* compared to their non-probiotic fed counterparts.

In New Zealand, application of dietary probiotics has provided promising results to improve commercial production of the endemic black-footed abalone, *H. iris* (Hadi *et al.*, 2014; Grandiosa *et al.* 2018). A selected three-strain probiotic mix (*Exiguobacterium JHEb1*, *Vibrio JH1* and *Enterococcus JHLDc*) was initially isolated from gastrointestinal tracts of healthy *H. iris* adults and screened as potential probionts. After a two-month feeding experiment, enhanced growth and higher survival in probiotic-fed juvenile abalone were observed compared to controls (Hadi *et al.*, 2014). In a follow-up trial, Grandiosa *et al.* (2018) found that the same multi-strain probiotics also altered innate immunity parameters of *H. iris* haemocytes and imparted metabolic alterations in muscle tissue, thus improving their health status. However, whether such apparent immune-enhancement infers greater resistance towards pathogen attack is unknown. In the present study, we investigated the immune responses of *Vibrio*-infected (*V. splendidus*) *H. iris* after being fed for four months with a multi-strain probiotics diet through an *in vivo* pathogen challenge experiment. We quantified non-specific immune responses in haemocytes, including total cell count (THC) and viability, degree of apoptosis, and production of reactive oxygen species (ROS). Metabolite profiles of foot muscle tissues were simultaneously assessed using a GC-MS-based metabolomics approach to investigate whether complimentary information could be obtained at the metabolic level.

5.2. Material and Methods

5.2.1. Abalone and Feeding Experiment

Healthy juvenile abalone (*H. iris*) with the average size of 27mm were obtained from a commercial hatchery (Moana Ltd., New Zealand), transported to the AUT aquaculture facility (Auckland University of Technology, New Zealand), and acclimated for one week as described by Grandiosa *et al.* (2018). A feeding experiment was then conducted for four months by feeding abalone with an experimental diet containing a multi-strain probiotic mix incorporated into a commercial abalone feed (Abmax 16; E.N. Hutchinson Ltd. Auckland), and a control diet (Abmax 16) without probiotics. Animals were provided with a 1% body weight feed ration per day. Dietary treatments were administered in triplicate 20 L tanks, with 20 abalone per tank, and faeces/leftover food were siphoned daily. Water exchanges (50% by volume) with 5 µm filtered and UV-treated seawater were performed daily, while 100% water exchanges and thorough tank cleaning were performed every three days. The culture water was maintained at $\pm 16^{\circ}\text{C}$, 33-35 ppt salinity, pH 8.0, and a dissolved oxygen content above 7.0 mg L⁻¹.

5.2.2. Probiotic Strains

6. The multi-strain probiotics comprised of *Exiguobacterium* JHEb1, *Vibrio* JH1 and *Enterococcus* JHLDe, which were part of the autochthonous microbiota of abalone screened and selected by Hadi *et al.* (2014) based on their abilities to hydrolyse protein, starch and alginate, and for their acid tolerances. The strains were cultured in marine agar supplemented with 20 g L⁻¹ NaCl and incubated at 26°C for 24 h. Stock cultures were stored in sterile (121°C for 15 min) marine broth (Difco, Becton Dickinson, USA) containing 20% [v/v] glycerol at -80°C.

5.2.3. Experimental Diets

The probiotic-enriched diet was prepared weekly by culturing bacterial strains in each respective enriched media, following the preparation protocols of Hadi *et al.* (2014). *Exiguobacterium* JHEb1 was propagated in 200 mL marine broth supplemented with 1% yeast extract for 48 h at 22°C, *Vibrio* JH1 was grown in 200 mL marine broth supplemented with 0.5% glucose for 48 h at 22°C, while *Enterococcus* JHLDC was prepared anaerobically in 100 mL MRS broth (Difco, Becton Dickinson, USA) containing 2% NaCl for 48 h at 22°C. Cultures were harvested by centrifugation at 5000 rpm, then mixed together in 100 mL marine broth as described by Hadi *et al.* (2014). Inoculation of Abmax 16 was conducted by spraying the mixture on feed pellets and air drying at 24°C under sterile conditions. Feed containing the multi-strain probiotics was stored at 10°C until needed. The viability of bacteria in the probiotics feed were evaluated using the spread plate count method. Bacterial counts from the inoculated probiotics feed showed a constant population of 3×10^9 CFU g⁻¹ after 1-week storage.

5.2.4. Bacterial Challenge Experiment Design

Four treatments were set up in the challenge experiment: probiotic-fed abalone injected with *Vibrio splendidus* (P.I.), probiotic-fed abalone injected with PBS (P.C.), non-probiotic-fed (control) abalone injected with *V. splendidus* (C.I.), and non-probiotic-fed (control) injected with PBS (C.C.). Three tank replicates were used for every treatment, and each tank was stocked with 20 abalone. A frozen culture of *V. splendidus* (obtained from Cawthron Institute, Nelson, New Zealand) was revived from -70°C stores according to Kesarcodi-Watson *et al.* (2009), and re-cultured in marine broth at 22°C for 24h. Infection with *Vibrio splendidus* was achieved by injecting 50 µL of bacterial suspension (containing 5×10^7 cfu in PBS) into the pedal sinus using a 1 mL syringe with 25G x 5/8" needle. Abalone not receiving the bacterial challenge were injected with 50 µL PBS solution. After injections were administered, animal health condition was

observed based on shell turnover rate and subsequent feeding activity (adapted from Takami *et al.*, 1995). Signs of bacterial disease (Diggles *et al.*, 2005) and survival were assessed every 6h for 48h after injections were administered, with dead animals immediately being removed from tanks.

5.2.5. Analysis and Measurements

Measurements of non-specific immune parameters in haemocytes were conducted 48 h post- challenge on three randomly selected abalone from each of the treatment. Haemolymph samples (100 μ L) were collected from the pedal sinus (1 mL syringe; 25G x 5/8" needle) and immediately mixed with 100 μ L Alsever's solution to prevent aggregation, as recommended by Grandiosa *et al* (2016). Total haemocyte counts (THC), viability, stage of apoptosis, and proportion of cells producing ROS (namely superoxide anion) were recorded using a flow cytometry platform (Muse® Cell Analyser; Merck KGaA; Darmstadt, Germany) and commercial assay kits (Merck KGaA), following the manufacturer's specifications.

5.2.5.1. Total haemocyte count and viability

20 μ L of haemolymph mixed with Alsever's solution were added to 380 μ L of reagent assay from the Muse® Cell Count and Viability Assay Kit (MCH100102; Merck KGaA) in 1.5 mL micro-centrifuge tubes. Solutions were incubated for 5 min in the dark at room temperature and briefly vortexed.

5.2.5.2. Oxidative stress

Haemocytes producing ROS (i.e., superoxide) were evaluated using the Muse® Oxidative Stress Assay Kit (MCH100111; Merck KGaA). Briefly, 100 μ L of haemolymph mixed with Alsever's Solution was added to 100 μ L of 1X Assay Buffer. An intermediate assay reagent solution was prepared 1:100 with 1X Assay Buffer. This was further diluted 1:80 with 1X Assay Buffer to make a working solution (WS). 190 μ L of WS was mixed

with 10 μL of haemocyte samples (previously mixed with the Assay Buffer) in 1.5 mL micro-centrifuge tubes. These solutions were then incubated at 37°C for 30 minutes before the ROS fluorescence probe intensity was quantified via flow cytometry.

5.2.5.3. Cell Apoptosis

The degree of apoptosis in the haemocyte population reflects the importance of cell health and is characterized by distinct morphological changes. Cells were treated using the Muse® Annexin V & Dead Cell Assay Kit (MCH100105; Merk KGaA) and were categorized into live cells, cell undergoing early apoptosis, late apoptosis and dead cells. Percentages of haemocytes undergoing different stages of apoptosis were assessed using the Muse® Cell Analyser. As much as 100 μL of haemolymph mixed with Alsever's Solution were added to 100 μL of assay reagent in 1.5 mL micro-centrifuge tubes, incubated for 20 min in the dark at room temperature, briefly vortexed, then processed via flow cytometry.

5.2.6. Metabolomics

Foot muscle (~150 mg wet weight) was dissected from abalones surviving the bacterial challenge treatment (10 abalone per treatment), placed in 2 mL cryogenic vials, snap frozen in liquid nitrogen, and stored at -80°C until metabolite extraction. Tissue samples were lyophilised then ground using a mortar and pestle. Approximately 10 mg of powdered tissue were placed in 1.5 mL Eppendorf tubes and co-extracted with an internal standard (L-Alanine-2,3,3,3-d4) using a cold methanol-water method according to Villas-Bôas *et al.* (2010). Polar extracts were lyophilised in a SpeedVac Concentrator with a Refrigerated Vapor trap (Savant™ SC250EXP, Thermo Scientific) for 4 h (0°C; vacuum level 3), re-suspended in NaOH, and derivatized via methylchloroformate (MCF) alkylation (Smart *et al.*, 2010). Derivatized metabolites were analysed according to the protocol and instrument settings described by Smart *et al.* (2010), using a gas chromatograph GC7890 (Agilent Technologies, USA) coupled to a quadrupole mass

spectrometer MSD 5975 (Agilent Technologies, USA). Metabolites were first identified using an in-house library of MCF derivatives according to their electron impact fragmentation mass spectrum and retention time. All metabolite annotations are based on four proposed levels of reporting standards for chemical analysis which were identified compounds, putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries), putatively characterized compound classes (e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class) and unknown compounds which could be differentiated and quantified based upon spectral data (Sumner *et al.*, 2007). The data in this study were designated as ‘Level 2’ according to these standards. Data were manually checked for the presence of contaminants and aberrant records were removed. The resulting QC-filtered peak intensity values were normalised by the internal standard to compensate for potential technical variations (e.g., variable metabolite recoveries) normalised by sample-specific biomass, then glog-transformed and mean-centered.

Pathway mapping of metabolites able to be matched to entries within the KEGG database (gastropod mollusc reference organism: *Lottia gigantea*) (Aoki & Kanehisa, 2005) was performed using Interactive Pathway Explorer v3 (iPATH3) (Darzi *et al.* 2018). Metabolite data analysis pipeline was conducted using the web-based Metaboanalyst 4.0 open source program (Chong *et al.* 2018). ANOVA-Simultaneous Component Analysis (ASCA) was applied to determine major metabolic patterns in foot muscle tissue according to dietary treatment and infection status. Main effects of diet and infection status were modelled using the first component, whereas their interaction was modelled using the first two components (leverage threshold 0.9; alpha threshold 0.05). These models were validated through permutation tests ($n=1000$) (Vis *et al.* 2007). Based

on the outcome of ASCA, samples were combined by their main effects group labels and further investigated via combined Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA) to determine differential regulation of pre-defined metabolic pathways within the KEGG database (QEA = Global Test; NTA = Relative Betweenness Centrality). An integrated summary of immune-related pathways identified as being impacted ($p < 0.05$) by infection status was manually constructed with metabolites colour-coded by their Log₂ foldchange values (case/control).

5.2.7. Data analysis and visualisation

Differences among THC, cell viability, ROS production, and apoptosis profiles were analysed using 2-Way ANOVA with Duncan post hoc tests (SPSS Statistics for Windows v 19.0; IBM Corp., Armonk, NY).

5.3. Results

5.3.3. Challenge Experiment

All non-infected abalone (receiving PBS injections only) survived the 48h trial and did not display any visual signs of disease. However, those challenged with *V. splendidus* injections resulted in 33.3% and 11.1% mortality in non-probiotic-fed and probiotic-fed animals, respectively (Fig. 5.1.). Furthermore, infected abalone typically presented with discoloration of mantle, and swelling of the foot muscle.

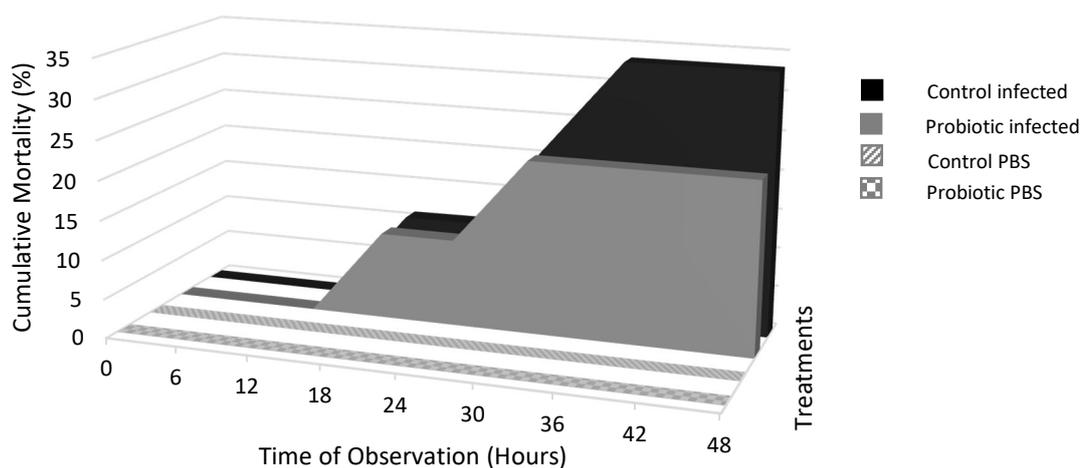


Figure 5.1. Cumulative mortality (%) from the treatments of probiotic-fed abalone injected with *Vibrio splendidus* (P.I.), probiotic-fed abalone injected with PBS (P.C.), non-probiotic-fed (control) abalone injected with *Vibrio splendidus* (C.I.), and non-probiotic-fed (control) injected with PBS (C.C.); n=9.

5.3.4. Cellular Immune Response

Measurements of total haemocyte count (THC) did not show any significant difference ($p > 0.05$) among treatments. However, THCs in control-fed infected abalone (C.I.) were lower than those in all other groups (Fig. 5.2. A). Haemocyte cell viabilities in the P.C. group (59.93 ± 7.47 %) were significantly higher than those in all other treatments. Haemocyte viability from the P.I. (36.6 ± 6.16 %), C.C. (37.75 ± 12.00 %) did not differ among one another (Fig. 5.2. B), while the value obtained from C.I. treatment (10.6 ± 2.96 %) was significantly lower compared to P.C., C.C. and P.I.

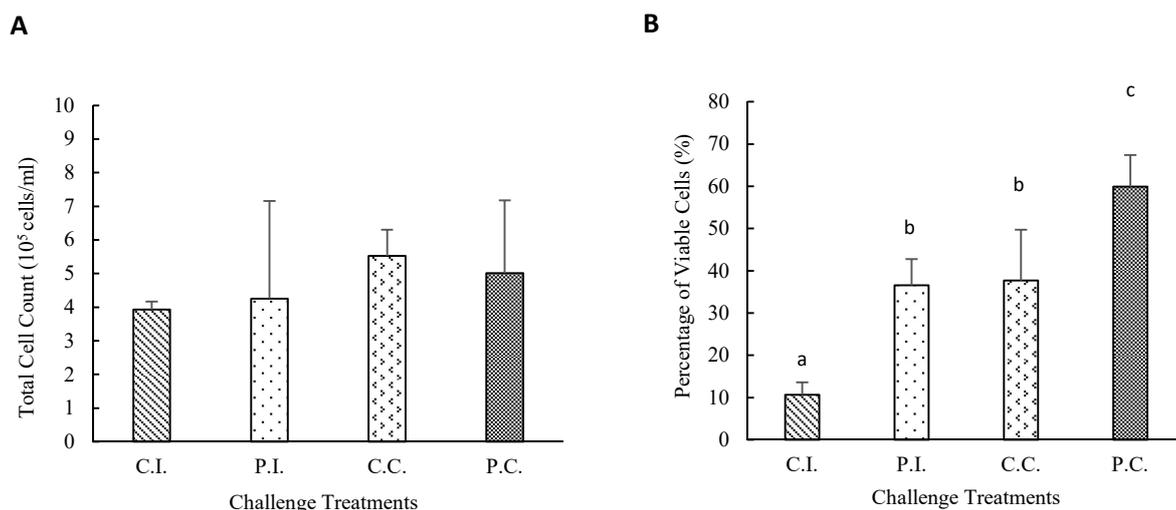


Figure 5.2. The A) total haemocyte cell count and (B) haemocyte viability of abalone (*H. iris*) from the treatments of probiotic-fed abalone injected with *Vibrio splendidus* (P.I.), probiotic-fed abalone injected with PBS (P.C.), non-probiotic-fed (control) abalone injected with *Vibrio splendidus* (C.I.), and non-probiotic-fed (control) injected with PBS (C.C.). (Data represent the mean S.D from 3 abalone per treatment (ANOVA with post-hoc Tukey HSD; $p < 0.05$).

Measurements of ROS-producing cells indicated that abalone from the C.I. treatment had a significantly higher portion of ROS positive cells ($14,81 \pm 3$ %) compared to the other treatments (Fig. 5.3.).

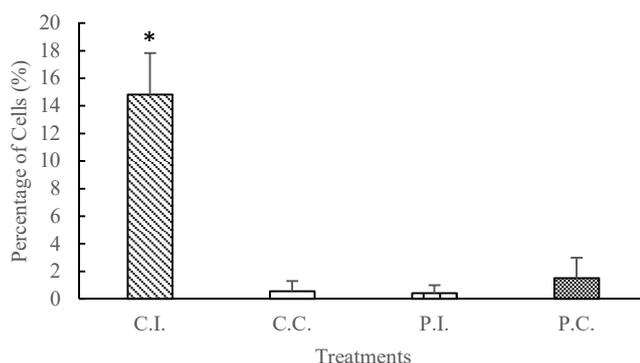


Figure 5.3. Percentage of abalone haemocytes exhibiting Reactive Oxygen Species (ROS) from the treatments of probiotic-fed abalone injected with *Vibrio splendidus* (P.I.), probiotic-fed abalone injected with PBS (P.C.), non-probiotic-fed (control) abalone injected with *Vibrio splendidus* (C.I.), and non-probiotic-fed (control) injected with PBS (C.C.). (Data represent the mean S.D from 3 abalone per treatment (ANOVA with post-hoc Tukey HSD; $p < 0.05$).

The average values of the category of apoptotic, non-apoptotic, early apoptotic and late stage apoptotic cells were measured from three abalone per treatment and were presented in Fig. 5.4. Abalone from the probiotic-fed and non-infected treatment (P.C.) had a significantly higher number of non-apoptotic cells (75.6%) compared to the C.I. and the P.I. treatments. However, the percentage of non-apoptotic cells of the C.C. treatment did not differ significantly from the P.C. treatment. Also, the C.I. treatment had a significantly higher percentage of early apoptotic cells (11.45%) compared to the other treatments. The percent of late stage apoptotic and dead cells did not differ significantly across all treatments.

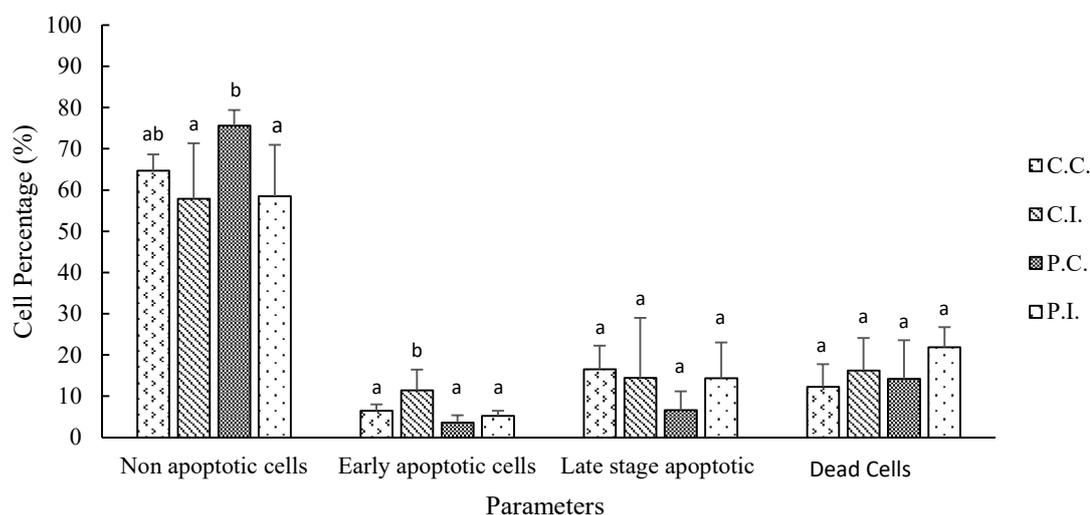


Fig. 5. 4. Quantitative results of Muse™ Annexin V and Dead Cell Assay as an immune response of abalone (*H. iris*) from the treatments of probiotic-fed abalone injected with *Vibrio splendidus* (P.I.), probiotic-fed abalone injected with PBS (P.C.), non-probiotic-fed (control) abalone injected with *Vibrio splendidus* (C.I.), and non-probiotic-fed (control) injected with PBS (C.C.). This figure shows the percentage of non-apoptotic, early and late apoptosis and dead cells. Mean \pm S.D. from 3 abalone per treatment. Difference in notation above bar graphs indicate statistical significance ($p < 0.05$).

5.3.5. Metabolite Profiles

A total of 99 metabolites were detected in abalone foot muscle by GC-MS. Sixty seven of these were reliably annotated, broadly covering a number of modules (collection of manually defined functional units) in the KEGG global metabolic network. ASCA was

used to identify metabolites with significantly different ($p < 0.05$) abundances among groups of abalone which received different diets (commercial feed pellets with or without a probiotic cocktail) for four months before undergoing an infection challenge (injected with *V. splendidus* or with PBS carrier only). ASCA applies univariate statistics to experimental factors (diet and infection status), whilst also accounting for multivariate interactions by applying a Principal Components Analysis to the estimated parameters in each source of variation of an ANOVA model. Validated by permutation tests, main effects were distinguished with metabolite profiles differing significantly according to diet type ($p=0.046$) and infection status ($p=0.037$), but with no evidence of an interaction between factors ($p=0.756$) (Table 5.1.).

Table 5.1. List of perturbed metabolic pathways in *H. iris* foot muscle tissues caused by *V. splendidus* infection.

Pathway Name	Metabolite Matches	<i>p</i> -value	FDR	Impact
Glutathione metabolism	Glutathione; Glycine; Cysteine; Glutamate; Pyroglutamate; Ornithine	<0.001	0.009	0.54
Cysteine & methionine metabolism	Cystathionine; Cysteine; Serine; Methionine	0.001	0.011	0.50
Taurine & hypotaurine metabolism	Cysteine	<0.001	0.009	0.00
Thiamine metabolism	Cysteine	<0.001	0.009	0.00
Glycine, Serine & threonine metabolism	Glycine, serine, threonine, glyoxylate,	0.007	0.045	0.60

The main effect of the *V. splendidus* injections were further evaluated through pathway analyses (QEA and NTA) of the normalised metabolite abundances to distinguish altered metabolic pathways in foot muscle tissues during the infection. Five pathways were identified as being most differentially regulated, comprising glutathione metabolism, cysteine and methionine metabolism (transulphuration pathway), taurine–hypotaurine metabolism, thiamine metabolism, and glycine, serine & threonine metabolism (Table 5.1). The immune-relevant modules glutathione metabolism and the transulphuration pathway had impact factors (≥ 0.5) which may suggest key metabolites

in these pathways were present in the analysis. The pathways identified were indicative that infected abalone may use these metabolites differently relative to each other. Taurine–hypotaurine metabolism and thiamine metabolism were also significantly enriched, but had zero impacts, indicating that the relative between-group abundances of matched metabolites occurred in marginal or relatively isolated positions in these pathways. The summary of pathways affected by *V. splendidus* infection (identified via QEA and NTA [$p < 0.05$]) are illustrated in Figure 5.5.

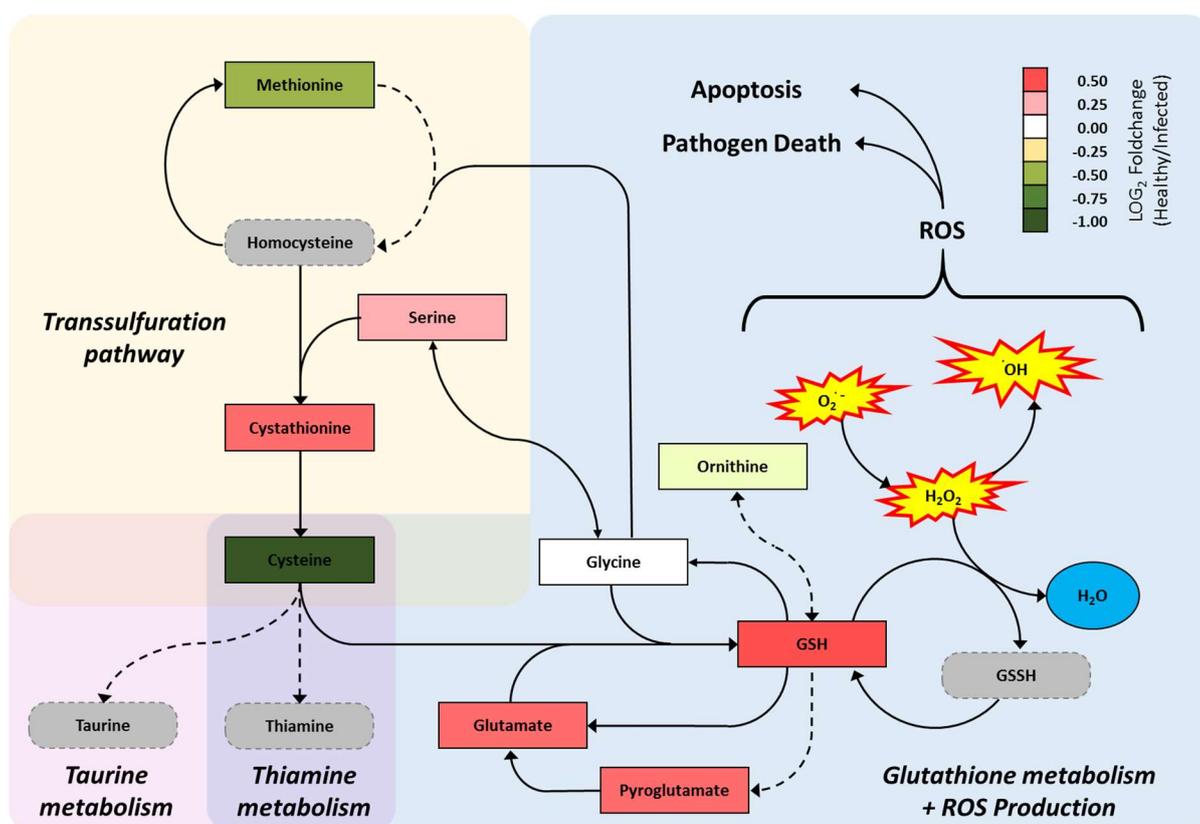


Figure 5.5. Summary of immune-related pathways affected by *V. splendidus* infection (identified via Quantitative Enrichment Analysis and Network Topography Analyses [$p < 0.05$]). Rectangles represent detected metabolites, and their colour indicate relative fold changes (see scale on image). Rounded rectangles represent important metabolites within networks that were not detected in our study. Solid and dashed arrows represent single and multi-step reactions, respectively.

5.4. Discussion

The current study presents the first report on the effect of a bacterial pathogen upon the immunological parameters of the New Zealand black-footed abalone which was fed an enrichment of multi-strain probiotics. The study revealed enhanced non-specific immune responses on probiotic-fed abalone following a bacterial infection experiment on control-fed and probiotic-fed abalone. This study also revealed metabolic responses on the foot tissue as a result of probiotic activity and the introduction of bacterial pathogen, however metabolomics data analysis indicated no interaction between probiotic feeding and the pathogenic challenge was shown.

5.4.3. Mortality

The probiotic-fed *Vibrio* challenged animals had significantly lower mortality compared to the control fed *Vibrio* challenged animals after the bacterial challenge. The protection effect of the dietary enhancement by probiotic bacteria was also shown by non-specific immune stimulation. It is possible that the lower mortality may be a result of an enhanced resistance from simple defence mechanisms sustaining their activity during the period of probiotic feeding. Abalone lack a specific immune response to pathogens, however an enhanced resistance referred to as the immunological loitering effect explained by McTaggart *et al.* (2012) may have occurred by persistent production of immune related molecules in the haemolymph throughout the period of the probiotic feeding activity (Little and Kraaijeveld, 2004). The constant exposure to probiotics prior to infection was thought to activate different pattern recognition molecules (Nappi and Ottaviani, 2000).

The outcome of this study was similar to previous studies in other abalone species in terms of survival and disease resistance in probiotic-fed abalone challenged with bacterial pathogen (Macey and Coyne, 2005; Silva-Aciaras *et al.*, 2013; Jiang *et al.*, 2013; Park *et al.*, 2016). It has been known that the effect of probiotic feeding on invertebrate organisms

may highly depend upon various factors, including the type and species of probiotic bacteria, the dose and duration of exposure of probiotics (Silva-Aciares *et al.*, 2013; Jiang *et al.*, 2013; Park *et al.*, 2016; Dias, 2016; Tseng *et al.*, 2009; Yan *et al.*, 2014; Anguiano-Beltrán, 2012).

5.4.4. Total Haemocyte Counts

The observation of THC from the post-injected abalone in this study showed that the average THC values 48 hours from the treatments did not differ significantly and were found at an average of 10^5 cells/ml. Abalone haemocyte cells have been directly related to immune competency and are consistently affected by stress (Hooper *et al.* 2007). The variation of haemocyte counts in this study could not be attributed to the presence of *Vibrio splendidus* since the injection of PBS also reduced the number of THC. A similar situation was found in other invertebrates (*P. elegans*) where antigens (LPS) and saline injection both caused a decrease in the number of circulating haemocytes. This event may be described as a generalized response in the presence of foreign substances (Lorenzon *et al.*, 1999). In our study, we did not observe the recovery time of the abalone towards the normal range of THC. However, it is known that the haemocytes may be replenished through the haematopoiesis process although currently the specific mechanism is not well understood (Pila *et al.*, 2016).

5.4.3. Haemocyte Viability

The significant reduction of the haemocyte viability in the infected control-fed and probiotic-fed abalone compared to the non-infected probiotic-fed abalone clearly indicate the effect of a sudden increase of pathogenic bacteria on the host's immune system. The pathogenic *V. splendidus* were known to be present in the abalone haemolymph and the peri-gut haemocyte region (Handlering *et al.*, 2005). Although the virulence and mode of action of *V. splendidus* strain used in this study was not known, it was suggested that the bacterial pathogen may have infected the intracellular part of the haemocyte similar to

the virulence characteristic of the *V. splendidus* LGP32 (Duperthuy *et al.*, 2011). This study showed that the percentage of viable haemocytes from the probiotic-fed infected abalone treatment was higher than the control-fed infected abalone haemocytes possibly caused by an increase of immunological loitering effect. The haemocytes of probiotic-fed abalone may have had different characteristics compared to the control-fed abalone since a study on *H. midae* showed phagosomal maturation and also exhibited a significant expression of fundamental proteins such as V-type proton ATPase and Ras-related protein Rab in haemocytes on probiotic-fed *H. midae* (Dias, 2016).

During this study, the PBS injection lowered the cell viability in the non-probiotic-fed animals. However, there were no signs of stress (normal behaviour), infection or mortality in these animals. It is likely that the reduction in haemocyte viability may have resulted from the physical injection stress since it may correlate with the reduction of THC explained previously (Lorenzon *et al.*, 1999).

5.4.4. Reactive Oxygen Species

Post-bacterial challenge measurements showed that non-probiotic-fed infected abalone had significantly higher numbers of ROS-producing cells than those of probiotic-fed infected abalone. Cells exhibiting ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) were identified as one of the immune response mechanisms specifically related to the phagocytosis activity of haemocytes (Nappi and Ottaviani, 2000). Before the pathogenic challenge, it was suggested that the balance exists between the ROS production during normal cellular metabolism and the level of endogenous antioxidants, whose function is to protect tissues from oxidative damage (Labreuche *et al.*, 2006). Bacterial pathogens through their virulent factors (Cross, 2008), may have triggered the innate immune system of the abalone, which is composed mainly of humoral and cellular defence mechanisms (Hooper *et al.*, 2011; Zong *et al.*, 2008). The probiotic feeding activity in abalone may alter the metabolite production and enhance metabolites that

could possibly assist in cell functions (Grandiosa et al., 2018). Haemocytes in probiotic-fed abalone are known to have higher levels of phagosomal maturation and fundamental proteins (Dias, 2016) which may assist during crucial host defence responses (Zong *et al.*, 2008); thus reducing the negative impact of the pathogenic challenge and potential production of ROS.

5.4.5. Apoptosis

The apoptosis results in this study were consistent with those of the other immune parameters measured. The non-probiotic-fed and infected animals (C.I.) had a significantly higher number of early apoptotic cells, although late stage apoptosis did not differ between treatments. The presence of early apoptosis cells due to bacterial infection has been reported in other molluscan haemocytes (Terahara and Takahashi, 2008). Apoptosis is an orchestrated process where cells are disposed to die without inducing inflammation and observed to have characteristics, such as chromatin condensation, membrane protrusion and cell shrinkage (Kerr *et al.*, 1972). The apoptotic process caused by bacterial pathogens was beneficial to protect the host and eliminate the infection (Böttger *et al.*, 2008) and may have been induced by oxidative damage to the haemocytes during the bacterial killing (Sokolova, 2009).

5.4.6. Metabolite Profiles

Metabolite profiles from abalone foot tissues indicate that major differences between infected and non-infected abalone involved differential regulation of glutathione metabolism and the transulphuration pathway. Both glutathione and cysteine are important metabolites involved in regulating ROS (Mailloux *et al.*, 2013; Manduzio *et al.*, 2005) and glutathione production has been reported to keep oxidative damage within tolerable limits (Mailloux *et al.*, 2013). Previously it was known that ROS in a healthy organism were produced as a natural by-product of the normal metabolism of oxygen and

have important roles in cell signalling and homeostasis (Manduzio *et al.*, 2005; Sundaresan *et al.*, 1995). Pathogen response mechanisms in infected abalone may have resulted in a change in cellular glutathione metabolism and possibly leads to increase of oxidative stress in other tissues.

The effect of a probiotic diet was previously shown to influence the foot muscle metabolome (Grandiosa *et al.* 2018) while the results of this current study may confirm that the effect of the probiotic diet had further influence on the metabolism of the bacterial challenged probiotic-fed animals. These bacterial strains may participate in the digestion processes of molluscs by producing extracellular enzymes, such as proteases, lipases and necessary growth factors (Hadi *et al.*, 2014; Prieur *et al.*, 1990). In addition, probiotic enhanced diets have also been reported to supply additional fatty acids and vitamins to hosts, which may alter the immune system (Sakata, 1990). Although we did not identify an interaction between diet and infection status; it may be that such an interaction between exists in immune-active haemocytes. Further studies should be conducted to elucidate the role of probiotics in the regulation of immune-related mechanisms in other tissues and cell types.

5.5. Conclusions

Although probiotics are not currently used commercially in the New Zealand abalone industry, our research herein and previous publications shows that there are significant growth and health advantages in the application of species-specific probiotics. In conclusion, this is the first report that simultaneously observed the effect of a *V. splendidus* challenge and probiotic feeding on immune parameters (THC, viability, production of ROS and apoptosis), combined with a metabolomics approach, in *H. iris*. The results indicate that haemocytes participate in the innate immune system of this species by stimulating the oxidative burst and apoptotic process in response to bacterial

infection. Probiotic feeding suggest that the Abalone haemocyte parameters shown improved immune capacity post-bacterial challenge. Metabolomics studies indicated the difference between probiotic feeding and bacterial challenged animals however further analysis was suggested using advanced molecular and omics tools that may reveal insights on the specific mechanisms by which probiotics improve abalone fitness, as well as providing optimization of probiotics quality and functional properties, which are likely to benefit the growing New Zealand abalone industry.

Acknowledgement

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CHAPTER 6

GENERAL DISCUSSION

6.1. Thesis Background

The current development of the New Zealand black-footed abalone (*H. iris*) aquaculture is very promising and has achieved beneficial growth to contribute towards increasing the New Zealand seafood export value. Further growth is certainly expected in order to support New Zealand's aim of NZD \$1 billion aquaculture export value by 2025 (Carter, 2012). However, the growth of abalone aquaculture is faced by the limited knowledge regarding the immunological aspects of the New Zealand Black-footed abalone (*H. iris*).

It is well known that the impacts of diseases upon the abalone aquaculture may affect environmental, social and economic sectors. Direct effects, such as massive mortality are apparent, but indirect costs, such as lower value of end-products, difficulties accessing markets, and the costs of disease control (e.g. eradication and laboratory testing) could potentially be greater. Thus, knowledge of immunology and pathophysiology of the *H. iris* is needed to assist future development of abalone aquaculture in New Zealand.

Threats affecting the abalone aquaculture industry include infectious diseases which will always be an inherent component during intensification and commercialization of this species (Olafsen, 2001). Indeed, recent surveys upon *H. iris* wild populations in New Zealand found pathogens such as fungal hyphae which causes yellow-to-brown blisters with mucoid discharge affecting the underside of the paua tissue and the inside of the shell (Taylor, 2017). Furthermore, reports from the largest abalone farm in New Zealand (Moana Abalone Farm, previously known as OceaNZ Blue), suspected a low-level infection of the *Perkinsus olseni* pathogen in cultured abalone during the 2015 production batch (Suvalko, Personal Communication, March 2015). Low level mortality inside the

abalone farm was also observed and moribund abalone were occasionally found with withered foot. However, no scientific report has been issued regarding the specific pathogen involved. Fortunately, a recent survey of the *H. iris* wild populations found no exotic pathogens or diseases that have been associated with shellfish overseas (Taylor, 2017).

Based on the limited abalone health information and the potential economic losses to the industry, this study presents investigations on the biology, immunology and pathophysiology of the New Zealand black-footed abalone (*H. iris*). The approach to study immunology begins with the characterization of the haemocyte as the most important component of the non-specific immune system by applying a combination of classical and newer techniques. Instruments, such as the Sysmex XN-1000 haematology analyser and MUSE® analyser which utilize flow cytometry principles were used successfully on the abalone haemocyte characterization. Since these tools were normally used for the analysis of human blood and only used here for the first time as a new platform in aquaculture research, optimization of procedures were needed. The procedures included methods to identify haemocyte cells with the Sysmex XN-1000 and the methods to quantify haemocyte cell counts and viability, apoptosis and oxidative stress of haemocyte using the MUSE® analyser. The optimization also involved testing different antiaggregants to find the optimal cell antiaggregant to support an effective working procedure. Once these parameters were established, a detailed immunological study of *H. iris* was conducted under detailed experimental settings, including observation of the immune response of the animals during the application of a multi-strain probiotics diet (formulated at AUT University) feeding experiment. Observations of the non-specific immune parameters were conducted each month, during a four-month feeding trial. The final part of the research project was to observe the pathophysiology of abalone. A bacterial challenge experiment with a *Vibrio* pathogen was designed and

further observations of clinical signs of abalone were recorded. The possible presence of immune enhancement was then observed by comparing the probiotic-fed against non-probiotic-fed abalone by analysing the parameters of the haemocyte with the MUSE® analyser and comparing metabolomes of the foot tissue sample using the metabolomics approach.

Finally, this study provides a better understanding of the *H. iris* immune system and the effects of a *Vibrio* sp. pathogens during an incidence of a bacterial disease. In addition, this study established the growth and health benefits of using the probiotic treatment, which enhanced the survival of abalone in bacterial challenges. It is envisaged that the information from this study will contribute to a solid foundation and further growth of a successful New Zealand abalone aquaculture industry.

6.2. Discussion

“There is only one constant element in immunity, whether innate or acquired, and that is phagocytosis. The extension and importance of this factor can no longer be denied.”

Elie Metchnikoff – Father of Natural Immunity (1845-1916) – source
Metchnikoff and the Origins of Immunology: From Metaphor to Theory

Since Metchnikoff discovered the macrophage cell in his observations regarding the immune system of frogs in 1883, significant advances have been made in the understanding of immunity. Currently, the field of immunology is based on the notion that haemocytes are unique cells that perform phagocytosis as a first line of defence to protect themselves against pathogens (Bayne, 1990).

My interest in the New Zealand black-footed abalone immunology led me to previous studies that have highlighted the presence of immune cells, known as the haemocyte, which exhibit phagocytic activity in molluscan invertebrates (Sahaphong *et al.*, 2001; Martello and Tjeerdema, 2001; Travers *et al.*, 2008 a; Donaghy *et al.*, 2010). Observations of haemocyte cells, especially in abalone, have been conducted previously in *H. asinina* (Sahaphong *et al.*, 2001), *H. cracherodii*, *H. rufescens* (Martello and Tjeerdema, 2001),

H. iris (Nollens *et al.*, 2004), *H. tuberculata* (Travers *et al.*, 2008) and *H. discus discus* (Donaghy *et al.*, 2010). However, different terminology have been used for the haemocytes observed in the aforementioned studies which promoted confusion in the literature.

This study provided results to indicate that *H. iris* has two cell types recognized by flow cytometry using the XN-1000 haematology analyser (Sysmex Japan) and observation through classical staining methods. The XN-1000 is a relatively new technology for the determination of white blood cell (WBC) differential, using white cell differential (WDF), white cell nucleated (WNR), and white cell precursor (WPC) channels. The advantages of this analyser have been documented in adult humans (Briggs *et al.*, 2012; Seo *et al.*, 2015) and pediatric human population (Becker *et al.*, 2016). This study is the first to apply the technology on invertebrate species.

Two haemocyte categories of *H. iris* identified by the XN-1000 in this study were suggested to be type I (monocyte-like) and type II (lymphocyte-like) cells because these cells were based on similarities of shape and function to monocyte and lymphocyte white blood cells described in vertebrates (Abbas *et al.*, 2010). These results corroborate previous studies that have indicated similar haemocyte categories found in the *Oncomelania* sp. (Sasaki *et al.*, 2003) and the *H. diversicolor* (Chen *et al.*, 1996).

Further analysis of the haemocyte types was done by observing the phagocytic activity of *H. iris* haemocytes. Using the Zymosan particles to test the phagocytic capability, it was determined that both type I and type II haemocyte cells showed phagocytic activity, but type I cells (monocyte-like cells) had a higher number of cells participating in phagocytosis. Phagocytosis activity was known to be the front line of cellular defence in molluscs since the role of haemocytes in immune response relies on the capacity of haemocytes to engulf and degrade foreign material through phagocytosis (Hooper *et al.*, 2007).

A concern from previous studies has been that in other *Haliotis* species, haemocyte populations may result from a gradient of potential maturation stages (Travers *et al.*, 2008). My study may not fully support the statement since two different cell types were found with the capability of phagocytosis. Although this study was limited to describing the specific functions of these cells types, the information presented should be useful as a fundamental basis for studying further the haemocyte functions and stepping further towards formulating an effective tool for the evaluation of the immune response of abalone exposed to pathological and environmental stressors.

This study of the immune status of abalone focused on the analysis of haemocytes since these “*blood*” cells were able to be measured from the total haemocyte cell count, migratory activity, phagocytic capacity and the respiratory burst responses of haemocytes (Malham *et al.*, 2003). It was understood that the abalone haemocyte produce mediators of stress (Ottaviani *et al.*, 2007). This assumption was based on previous studies which have attempted to discover the relationship of external stress on the immune parameters by applying stressors such as different salinities, shaking, reduction of dissolved oxygen, high temperature and increasing the ammonia and nitrate concentration on the abalone (Malham *et al.*, 2003; Cheng *et al.*, 2004; Martello and Tjeerdema, 2001; Cheng *et al.*, 2004 a, b, c, d).

“You know my method. It is founded upon the observation of trifles.”

Arthur Conan Doyle, *The Boscombe Valley Mystery*

The evaluation of haemocyte functions through *in vitro* observations of *H. iris* haemocytes requires careful consideration since rapid morphological changes, such as aggregation of cells, cell adhesion and development of pseudopods, occur after the collection of haemolymph. Aggregation of haemocytes with formation of clumps was mainly attributed to homeostasis and wound healing (Sminia, 1981), while the extension of pseudopods by haemocytes and their adhesion capacity, facilitate cellular migration

and the immune defence (Letullier *et al.*, 2014). In Chapter 3 recommendations were given to the extent that an antiaggregant solution must be applied to reduce these effects.

One of the focuses of this research was the selection of antiaggregant, since this directly affects the observation of the haemocyte. It was known that different antiaggregants affected the haemocyte responsiveness (Torreilles *et al.*, 1999; Hinzmann *et al.*, 2013), therefore a preparation protocol to assess the potential antiaggregants was conducted to assess abalone (*H. iris*) haemocytes. Rather than using classical methods such as cell staining to assess parameters such as haemocyte viability, I explored the application of a portable MUSE® flow cytometer as a method to provide rapid results of total haemocyte counts and viability provided its effective preparation and simple application for *in situ* or *ex situ* experiments. The results indicate that the MUSE® flow cytometer was suitable for analysing a wide range of abalone haemocyte cell features, such as apoptosis detection, oxidative stress, changes in mitochondrial potential and cell cycle distribution.

The comparison of potential antiaggregants, such as K₂EDTA, Alsever's Solution and Heparin was conducted by observing the total cell count and viability through the MUSE® flow cytometer. Results showed that factors such as different components of the antiaggregant, preparation methods and the effect of temperature may affect the suitability of the antiaggregant media and their ability to maintain cell integrity. The study confirms that the K₂EDTA microtainers and Alsever's Solution were suitable antiaggregant media since they contain chelating agents that sequester Ca²⁺ and Mg²⁺ ions from the haemolymph of molluscs (Chen and Bayne, 1995). Furthermore, the use of heparin was found to not be an effective solution since it was known to act primarily through an anti-thrombin mechanism which is not present in abalone (Yip *et al.*, 1995).

The use of AS and K₂EDTA were useful for haemocyte handling compared to Heparin since AS and K₂EDTA provided a high number of viable cells and total cell counts. In

addition, these antiaggregants did not affect the haemocyte morphology. The K₂EDTA antiaggregant was more advantageous since it was available in a micro-container format. The use of AS on the other hand, may not be practical in field studies since it requires solution preparation before mixing with haemolymph. Although the K₂EDTA antiaggregant has an advantage, the haemolymph must be mixed before further use. The study in Chapter 3 resulted in the identification of various methods of haemolymph mixing which could affect the viability and cell counts measured by the MUSE® flow cytometer. It was shown that the simple mixing procedure by inverting K₂EDTA tubes as much as 20-30 times provided homogenous mixture of the haemolymph (i.e., high amount of viable cells), whereas tubes without mixing showed significantly lower amounts of viable cells. Other alternative mixing methods were explored, such as the use of a vortex and mixing using a pipette.

The influence from external factors, such as the temperature, length of storage, pH of the antiaggregants (Vargas-Albores and Ochoa, 1992), osmolarity (Hinzmann *et al.*, 2013) and components of the antiaggregant may affect the haemocyte measurement results. In this study, I found that temperature and time of storage affected the haemocyte parameters of *H. iris*, and that 24 hours storage of haemocytes in K₂EDTA and AS at room temperature (21°C) were able to preserve viability while low temperature (4°C) storage reduced the viability of *H. iris* haemocytes.

Further studies on the abalone haemocytes should consider the pH of the antiaggregant solution, since a lower antiaggregant pH compared to the haemolymph pH may induce protein precipitation (Vargas-Albores and Ochoa, 1992). The osmolarity of the antiaggregant was also recommended to be higher than the osmolarity of the haemolymph fluid since it balances between the concentration of the active compound to be effective and to have the osmotic power to maintain the viability of haemocytes (Hinzmann *et al.*, 2013).

Further investigation and experimentation into the effects of antiaggregants on haemocytes is strongly recommended. Although the recommended antiaggregants (AS and K₂EDTA) were useful for haemocyte handling, these antiaggregants should be used carefully for quantitative measurements since previous studies have suggested that EDTA-based antiaggregants were able to interfere with the immune capacity of invertebrate haemocytes by inhibiting release of oxygen radicals.

The findings of this research have shown that the sampling technique may affect haemocyte measurements and it is important that the haemolymph withdrawal follows a precise protocol. Sampling should cause minimal injury to experimental animals, while repeated sampling of haemolymph from an abalone should be carefully evaluated since it may affect parameters such as haemocyte density, protein concentration, and enzyme activities (Ford and Paillard, 2007).

An important outcome of this research was the innovative use of the Muse® Cell Analyser as an accurate and quick method to measure quantitative parameters of the haemocyte. I found that assays, such as total cell counts, viability, apoptosis and oxidative stress using the automated Muse® Cell Analyser were successfully applied in this research and should be extremely valuable in future studies of shellfish haemocytes. Through this study it was demonstrated from initial experiments that the results of THC (total haemocyte count) and viability analysis were comparable with results from Trypan blue exclusion tests and cell counts through a haemocytometer.

Trials using the MUSE® flow cytometer have also been conducted by our research group to measure cell count and viability on other New Zealand invertebrate species, such as the geoduck (*Panopea zelandica*), scallops (*Pecten novaezelandiae*), New Zealand Greenshell Mussels (*Perna canaliculus*) and the southern rock lobster (*Jasus edwardsii*). Through these experiments we have shown that the MUSE® flow cytometer served as a useful and rapid tool for haemocyte observations among various invertebrate species. It

is important to note that future studies should take into account any variations in preparation of the cells as well as potential improvements in the specificity and sensitivity of these parameters. In addition, the treatment of haemocytes may differ among species especially regarding the selection of antiaggregants. Thus, the initial investigation of the antiaggregants was an integral part of the thesis research.

*“Optimal functioning of the immune system, it turns out,
is dependent upon feeling good.”*

Marcey Shapiro,
Freedom from Anxiety: A Holistic Approach to Emotional Well-Being

Studies on growth and survival of the New Zealand abalone in aquaculture settings have been well examined, with regards to nutrition (Roberts *et al.*, 1999; Stuart and Brown, 1994; Allen *et al.*, 2006; Preece and Mladenov, 1999; Kawamura *et al.*, 2001; Tung and Alfaro, 2012; Tung and Alfaro, 2011; Dyck *et al.*, 2010; Tuterangiwhiu, 2015; Tung and Alfaro, 2010) and the effect of environmental parameters (Searle *et al.*, 2006; Clarke and Creese, 1998; McShane and Naylor, 1995; Cornwall *et al.*, 2009; Cunningham *et al.*, 2016; James and Barr, 2012).

In the present study, one of the aims was to investigate the effect of probiotics on abalone growth and health. Increasing evidence has been published regarding the many benefits of probiotic microorganisms, especially on the growth and survival of abalone (Macey and Coyne, 2005; Ten Doeschate and Coyne, 2008; Iehata *et al.*, 2010; Faturrahman *et al.*, 2015; Macey, 2005; Guo *et al.*, 2017; Gao *et al.*, 2018a; Gao *et al.*, 2018b; Huddy *et al.*, 2010; Hadi *et al.*, 2014). An important advantage of the probiotic feeding strategy was the immune-prophylactic effect against bacterial pathogens (Cross *et al.*, 2004; Jiang *et al.*, 2013) and benefit in health and disease resistance in abalone

species (Jiang *et al.*, 2013; Macey and Coyne, 2005, Iehata *et al.*, 2010; Silva-Aciares *et al.*, 2013).

It is important to understand that the effect of probiotic feeding on invertebrate organisms depend highly on various factors, such as the dose, duration of trial and bacterial species used (Silva-Aciares *et al.*, 2013; Jiang *et al.*, 2013; Park *et al.*, 2016; Dias, 2016; Tseng *et al.*, 2009; Yan *et al.*, 2014; Anguiano-Beltrán, 2012). A previous study conducted by Hadi *et al.* (2014) initiated interest since the probiotics was shown to enhance performance in growth and survivability of *H. iris* abalone. Juvenile abalone fed with commercial feed enhanced with the multi-strain probiotic containing *Exiguobacterium* JHEb1, *Vibrio* JH1 and *Enterococcus* JHLDe had significantly greater growth compared to abalone fed the same feed and no probiotics over a two-month feeding period. Probiotic organisms were understood to provide additional enzymes to assist breakdown of dietary components, such as complex carbohydrates and proteins, hence making them more easily available, and reducing the energy required for digestion (Hadi *et al.*, 2014). Further results from the study by Hadi *et al.* (2014) showed that abalone had apparently brighter blue colour on the shells of probiotic-fed abalone although quantitative measurement of the colour intensity was not carried out.

The present research was the first to report the effect of probiotics in modulating various properties of the immune system in *H. iris*. Multiple aims were set out to study the effects of probiotics, including the investigation of the effect of probiotics on the immune system (Chapter 4) and how the probiotics could provide abalone “*disease resistance*” against pathogens (Chapter 5). Aside from improved growth and survival rates, the probiotics feeding activity appeared to improve the immune response of abalone, as evidenced from haemocyte counts, cell viability, progression of apoptotic processes, and production of ROS after four-months of probiotics feeding. In addition,

metabolomics analyses were conducted on tissue from foot muscle. These analyses further indicated differences in metabolite profiles which related to biochemical pathway perturbations which were mitigated to some degree by the use of probiotics.

A limitation to this study was that the contribution of each probiotic species used was not investigated. The probiotic *Exiguobacterium* sp. was shown to improve health and growth in the previous abalone study (Hadi *et al.*, 2014), and also considered as a probiotic in other invertebrates (Orozco-Medina *et al.*, 2009; Cong *et al.*, 2017), although it has been reported as a pathogen in other abalone species (Anguiano-Beltrán *et al.*, 2012). The *Enterococcus* sp. used in this study was found to be a probiotic species in a study of *H. gigantea* culture (Iehata *et al.*, 2009), while a non-pathogenic *Vibrio* species isolated from the *H. iris* was also used as part of the mixture.

This study (Chapter 4 and Chapter 5) provides an introduction to the understanding of the underlying mechanisms of how animals achieve higher survival rates when exposed to probiotics. The combination of the haemocyte studies using recent flow cytometry technology (Chapter 2 and Chapter 3) provided valuable insights. Moreover, the results from the metabolomics analyses (Chapter 4 and Chapter 5) contribute to the understanding of biochemical modifications at the cellular level, following probiotic activity.

The discussion in Chapter 4 explains how animals in the controls (without probiotic feeding) had poor survival during the experiment due to the visible stress from opportunistic pathogens. The addition of multi-strain probiotics in the commercial feed may inhibit opportunistic pathogens antibiosis or by competition for nutrients and/or space, and alteration of microbial metabolism (Irianto and Austin, 2002). Hence, the negative effects of pathogens could be reduced by the application of the probiotics in the abalone farm. Observations of haemocyte viability showed that control animals had lower

viable haemocytes, which indicates possible oxidative damage on the haemocyte (Franco *et al.*, 2009) due to a defence mechanism against pathogen (Terahara & Takahashi, 2008). The reduction of haemocyte viability observed in other studies may have been caused by environmental parameters, such as temperature (Hégaret *et al.*, 2003), pH (Wang *et al.*, 2009), air exposure (Cardinaud *et al.*, 2014) and the presence of heavy metals (Mottin *et al.*, 2010). I am confident that environmental parameters did not interfere significantly in our experimental environment, since the culture conditions were kept in check, especially through temperature monitoring and routine water changes.

The observation of ROS production in haemocytes of abalone were suggestive of an immune response, since a significantly higher percentage of ROS producing haemocytes were found in the probiotic-fed abalone compared to control animals. Probiotics may have influenced the ROS regulatory system by stimulating the homeostasis of abalone which resulted the production of oxygen-dependent and oxygen-independent molecules by haemocytes (Nappi & Ottaviani, 2000). Future studies should focus on the analysis of ROS-regulatory enzyme expression on abalone haemocytes to understand the mechanism behind this up-regulation.

The findings in this study clearly improved our understanding of the abalone's immune system, however questions about the nature of abalone haemocyte proliferation, differentiation and maturation; were left for future studies. It is well known that probiotic bacteria may introduce signalling molecules to alert the immune system of the host (De *et al.*, 2014), hence triggering a complex process of signalling pathways, receptors, cytokines, growth factors, and transcription factors which eventually lead to the production of haemocytes (Pila *et al.*, 2016).

The probiotic bacteria applied in this study, possibly carries molecular signatures, such as lipopolysaccharide (LPS) that are highly immunogenic. Thus, the probiotics may have directly affected the haemocytes, since haemocytes produce the mediators of stress,

main immune response and play the role in maintaining immune homeostasis (Ottaviani & C. Franceschi, 1997; Ottaviani *et al.*, 1997; Jiang *et al.*, 2014). Probiotic-fed abalone were thought to have more mature phagosomal haemocytes since previous studies indicated that haemocyte were found significantly exhibiting fundamental proteins, such as such as V-type proton ATPase and Ras-related protein Rab in haemocytes (Dias, 2016).

Through the disease challenge experiments (Chapter 5), it was suggested that an ‘*immunological loitering*’ effect may have been induced in the abalone fed with probiotics, since a continuous stimulation of the immune system by probiotic bacteria may have caused enhancement of the host immune system. This means that the abalone fed with probiotics were better prepared against bacterial pathogens, hence showing a ‘*disease resistant*’ effect. The ‘*immunological loitering*’ effect may be explained as an enhanced resistance of invertebrates that was a result from simple defence mechanisms sustaining their activity a period of time after induction (Wagner, 1961; McTaggart *et al.* 2012). In this situation, it was understood that the persistent production of immune related molecules may have existed in the haemolymph throughout the period of the feeding activity (Little and Kraaijeveld, 2004). In addition, this phase may also explain the presence of significantly higher amounts of haemocytes in the circulation. In other invertebrates, it is known that ‘*immunological loitering*’ can last several days or weeks depending on the species and the immune factor causing the risk of infection to be generally lower (Haine *et al.* 2008). The ‘*immunological loitering*’ is temporary and does not provide resistance for the rest of the life of the host, or across generations. It was known that invertebrate innate immune system was capable to respond faster and more effectively to a subsequent immune challenge even after longer time and apart from ‘*immunological loitering*’ (Sadd & Schmid-Hempel 2006, Tidbury *et al.* 2010). In future experiments it should be interesting to discover the existence of increased resistance through repeated immune challenges or alternatively known as ‘*immunological priming*’

(Little & Kraaijeveld 2004). Such kind of experiments may unravel the dependence of the immune defence on immune stimulation and answer whether invertebrates truly possess adaptive response.

“Challenges in life can either enrich you or poison you. You are the one who decides.”

Steve Maraboli, *Unapologetically You: Reflections on Life and the Human Experience*

The specific study conducted in Chapter 5 was an effort to further elucidate the disease resistance and immune modulatory effects of the New Zealand black-footed abalone due to dietary addition of the promising multi-strain probiotic, namely *Exiguobacterium* JHEb1, *Vibrio* JH1 and *Enterococcus* JHLDc. The study follows the consensus from previous studies (Macey and Coyne, 2005; Silva-Aciares *et al.*, 2013; Jiang *et al.*, 2013; Park *et al.*, 2016) which shows that the addition of probiotics to the feed provides a disease resistance effect on abalone against bacterial pathogens.

This study introduced a bacterial challenge with the *V. splendidus*, which was considered as a pathogen on abalone species through initial experiments conducted in Chapter 5. This bacterial strain was known to infect molluscan species that live in similar environment as *H. iris*, and has been noted as a potential risk for other cultured mollusc species in New Zealand (Webb, 2013; Kesarcodi-Watson *et al.*, 2009). During this study, the specific virulence factors and mode of action of *V. splendidus* was not known and remains a topic for future study.

In this study, a high number of opportunistic bacterial pathogen was introduced beyond the physical barriers of the abalone by means of injection. The presence of the bacteria organism may have largely been responsible for the lower haemocyte viability

in control animals and probiotic-fed challenged abalone. The possible mode or virulence of *V. splendidus* was not specifically known since the genetic characterization of this bacteria was not conducted therefore the specific strain information was also not known. In comparison, *V. splendidus* LGP32 strain, has a mode of virulence which includes infecting the intracellular part of the haemocyte (Duperthuy *et al.*, 2011). In order to clarify this, future studies should observe further effects of bacterial challenge upon the cellular features of the haemocytes.

The result from the bacterial challenge showed significant differences in haemocyte parameters (count and viability, ROS production and apoptosis) between probiotic-fed and control-fed abalone. It was previously known that the total haemocyte count from the probiotic fed animals were significantly higher compared to the control animals prior to the bacterial challenge (Grandiosa *et al.*, 2018). Post bacterial challenge, probiotic feeding may have increased the viability of the haemocyte in abalone when subjected to bacterial challenges and this event may lead to a better chance in resisting the pathogenic organism. In addition, it was assumed that more mature haemocytes were present (Dias, 2016) and may have been responsible for overcoming the bacterial pathogens by means of increased phagocytic activity.

Post-bacterial challenge, control-infected abalone had significantly larger amount of ROS producing cells after 48 hours. It was thought that the production of ROS relates to the immune defence mechanism normally found in invertebrates which is the mechanism of phagocytosis (De Zoysa *et al.*, 2008). Previous studies mentioned that during phagocytosis, the membrane bound enzymes and host NADPH-oxidase become activated, hence regulation of glycolytic reactions with high oxygen consumption occurs (Roch, 1999; Xiang, 2001). During the process, the molecular oxygen reduces to the O_2^- anion, leading subsequently to the production of other forms of ROS to kill or inactivate foreign invaders (Roch, 1999; Xiang, 2001). During this study, it was not known whether

accumulations of the ROS caused cell damage in the experimental animals since ROS were known to be toxic. Further study may be worthwhile to observe the mechanism occurring in this phase on whether the enzymatic or non-enzymatic antioxidant defences play a role in controlling ROS (Roch, 1999; Yu, 1994).

Only a small percentage of cells were detected exhibiting ROS in the probiotic-fed infected treatment. It has been known that ROS plays an important role in phagocytic cells as a part of their defence mechanisms. ROS could act as the killing agent in combination with lysosomal enzymes to eliminate viruses, bacteria, yeast, fungi and protozoa (Fingerman & Nagabhushanam, 2000). The presence of probiotics may have induced an '*immunological loitering*' effect as mentioned previously, thus reducing the negative effect of pathogenic *V. splendidus*.

Apoptosis may occur naturally without bacterial challenge since an apoptotic process is beneficial to protect the host and eliminate the infection of potential opportunistic pathogens (Böttger *et al.*, 2008). However, the occurrence of apoptosis in challenged animals was interesting since the presence of early apoptosis haemocytes in control infected animals was observed. One of the known factors to affect apoptosis is the excess of ROI (reactive oxygen intermediates) during phagocytic events (Terahara & Takahashi, 2008). In previous studies, apoptosis have been thought as a defensive mechanism against pathogen since some pathogens have strategies to proliferate in host cells and disseminate via host circulatory systems by inhibiting host cell apoptosis (Williams, 1994; Cuffs and Ruby, 1996; Moss *et al.*, 1999; Scanlon *et al.*, 1999). It is not yet clear whether bacterial pathogens induced early apoptosis of haemocyte. Further studies should be conducted to investigate apoptosis with other available methods to provide better understanding of the underlying mechanism of bacterial infection.

“Any sufficiently advanced technology is indistinguishable from magic.”

Arthur C. Clarke, *Profiles of the Future: An Inquiry Into the Limits of the Possible*

The application of metabolomics was considered to be an important tool recently applied in aquaculture (Alfaro & Young, 2016; Viant, 2007). A metabolomics approach was conducted in Chapter 4 and Chapter 5, since it was known to be a method that can be used to elucidate possible phenotypic changes caused by environmental variations or xenobiotic agents, such as diseases, diets, and drugs (Goodacre, 2007). The advantage of metabolomics is that it can be used to evaluate visually non-observable traits in an organism with high sensitivity.

The study in Chapter 4 applied the use of GC-MS metabolomics analysis of the foot tissue of abalone to find differences between control-fed and probiotics-fed individuals, while the study in Chapter 5 focused on differences between diseased challenged animals and whether the probiotics feed had an influence. To the best of our knowledge, this is the first application of metabolomics to study the effects of probiotics of abalone. Through the study in Chapter 4, the metabolite profiles of the foot tissue revealed variations in the relative abundance of 17 unique metabolites. Enriched levels of FAA's (e.g., lysine, proline, asparagine, glutamine, serine) were shown in probiotics-fed abalone which may reflect differential capacities for protein turnover. Enhanced digestibility of dietary protein may occur since the probionts used in this study possess high proteolytic abilities (Hadi *et al.*, 2014).

Many signature metabolites were identified and found to be responsible for the increase immunity of abalone. Probiotic-fed animals appear to have a better health status due to the observable higher levels of free amino acids (FAA). Other amino acids found in the probiotic-fed abalone tissue, such as proline, may play an important role in the modulation of immune responses (Zhao *et al.*, 2015). It was also observed the

upregulation of glutamine and asparagine in probiotics-fed abalone, which are known to be a signature of growth stimulation and improved immunocapacity (Hochachka *et al.*, 2003). Other differential metabolite signatures in probiotics-fed abalone tissue included increased levels of alpha-ketoglutarate, which was known to have a crucial role in cellular energy metabolism (Tannahill *et al.*, 2013) and production of ROS (Tretter *et al.*, 2016). The increase in succinate was also considered important since it plays a role in metabolic pathways (Tannahill *et al.*, 2013), including the formation and elimination of ROS (Tretter *et al.*, 2016). Higher levels of lactic acid in tissues of probiotics-fed abalone could signal differences in glycolytic metabolism. The relative increase in this metabolite may reflect inputs from the selected lactic acid producing probiont (*Enterococcus* JHLDc), which was incorporated in the treatment diet. Other differential metabolite signatures in probiotics-fed abalone tissue included increased levels of the FFA's palmitoleic acid and adrenic acid, and higher levels of oxalic acid.

The major limitation of this study was that the mechanisms involved and the relation between the metabolites were poorly understood since the metabolomics study in Chapter 4 only generated some hypotheses on potential mechanisms of metabolic changes by using biochemical pathways (Young & Alfaro, 2016). We suggest that future studies of metabolomics should combine with the study of the expression of key genes involved in particular metabolic regulation and cellular signalling pathways, since it may offer more information on the occurring changes (Castro *et al.*, 2015; Jin *et al.*, 2015). As a consequence, it would be interesting to thoroughly investigate the functional role of these metabolites in abalone immunity in the future.

Benefits of the probiotic enrichment should be studied further when using a different pellet and different environmental parameters. The components composition affects the metabolism since it was known that increasing the dietary protein level results in better

growth for the *H. iris* (Tung & Alfaro 2011 a). It was also known that a difference in temperature regime also affects the growth where lower temperature guarantees improved growth of the *H. iris* (Tung & Alfaro 2011 a).

The study in Chapter 5 focused on the observation of metabolites from abalone exposed to *V. splendidus* infection. The statistical analysis conducted was different from the analysis in Chapter 4 since interaction analyses and pathway analyses were used to investigate specific characteristic of metabolites. The use of probiotics enhanced the immune system of abalone since it was thought caused an immune resistance against *V. splendidus*. The comparison between control and infected abalone were identified from two key metabolites, glutathione and cysteine, which were involved in regulating ROS (Mailloux *et al.*, 2013; Manduzio *et al.*, 2005). In healthy abalone, it was suggested that glutathione production was needed to keep oxidative damage within tolerable limits in ROS production (Mailloux *et al.*, 2013). Reactive oxygen species (ROS) in healthy organisms are produced as a natural by-product of the normal metabolism of oxygen and have important roles in cell signalling and homeostasis (Manduzio *et al.*, 2005; Sundaresan *et al.*, 1995). The abundance of *V. splendidus* in infected abalone may have induced higher levels of oxidative stress, thus causing disruption of the cellular glutathione metabolism in the foot tissue cells, which may lead to further cellular dysfunctions.

The significant difference of cysteines in the metabolite profile of healthy versus infected abalone may indicate activation of its role in redox signalling and the chronic effects of oxidative stress (Giles *et al.*, 2004). In healthy cells, it was postulated that cysteine would be able to participate in numerous mechanistically distinct redox reactions, including thiol/disulfide exchange, oxygen transfer redox couples, and thiol/thiyl radical transfer reactions. However, depending on the oxidation level in the

cell, cysteines can be oxidized to different states (Held and Gibson, 2012). Cysteine modification may occur in infected animals since abalone may experience severe oxidative stress caused by the *V. splendidus*, thus resulting in significant different levels of cysteine in abalone foot tissue between healthy and infected animals.

The difference of the metabolite profiles between probiotic fed and control abalone may indicate that the specific probiotic diet received by the animals in the preceding four months of feeding may have affected the metabolism of the animals. Probiotic enhanced diet have been reported to supply fatty acids and vitamins, thus deficient or excess levels of nutrients may alter the immune system (Sakata, 1990). In addition, previous studies have shown that probiotic bacteria may participate in the digestion processes of molluscs by producing extracellular enzymes, such as proteases, lipases and necessary growth factors (Hadi *et al.*, 2014; Prieur *et al.*, 1990).

It was observed that all of the metabolites that were involved in the study between the treatments of probiotic and health status exhibited an increase in monounsaturated and polyunsaturated fatty acids. Furthermore, pentadecanoic acid, hexadecanoic acid, 11-eicosenoic acid, 13, 16-eicosadienoic acid, arachidonic acid and margaric acid concentrations were significantly higher ($p < 0.05$). Probiotics applied in abalone have been known to produce polyunsaturated fatty acids (PUFA) (Weiner *et al.*, 1989; Skerratt, 2002). However, the significant presence of fatty acids in the abalone tissue may be a result of a metabolic process related to the immune status of the abalone resulting from the interaction of treatments.

Fatty acids have been known to influence immune cell function through a variety of complex mechanisms (Calder, 2008). Specific fatty acids, such as eicosanoids, have roles in inflammation and regulation of T and B lymphocyte functions (Calder, 2008). However, the roles of fatty acids found in the interaction of diet and health status in

abalone are yet to be completely understood. The presence of free fatty acids in this study may relate to a signalling pathway orchestrated by the presence of fatty acid binding proteins (FABP). Previous studies applying bacterial challenge on *H. tuberculata*, have observed an up-regulation of FABP in animals that survived exposure to *Vibrio harveyi* (Travers *et al.*, 2010). FABP are known to mediate lipid biological process and systemic metabolic homeostasis by regulating a diverse array of lipid signals (Furuhashi and Hotamisligil, 2008). Further study is needed to elucidate the role of probiotics in the regulation of fatty acids, and whether it provides tissue-specific or cell-type-specific control of lipid signalling pathways and metabolic regulation with or without the presence of pathogens.

In this research, the probiotics experiments were focused on the enhancement of a formulated feed with probiotics, which was prepared based on culture-dependent methods involving the use of specific isolation media for growth and culture of live probiotics. Challenges certainly exist in translating the findings from research into effective commercial products that could be applied in a larger intensive abalone aquaculture system, since preparation of the probiotics was laborious and time-consuming. Technologies, such as encapsulation of probiotics may provide solutions for future development at a commercial scale. Indeed, this avenue is being explored by the Aquaculture Biotechnology Research Group, AUT.

6.3. Conclusions

This thesis work applied innovative approaches to study the immune system of abalone. The combination of flow-cytometry and metabolomics approaches provided useful information on a range of immunological aspects of abalone with many specific insights that can assist the abalone aquaculture industry, as well as a guide for future

research on this and other shellfish species. The main objectives of this study were met since we have (i) identified the haemocyte types of *H. iris* using a combination of classical and innovation tools; (ii) developed effective protocols for *in vitro* observation of abalone haemocytes; (iii) successfully developed an innovative procedure for an efficient and effective measurement of immune parameters (THC, viability, production of ROS and apoptosis) using the MUSE flow cytometer; and (iv) simultaneously observed the effect of probiotic feeding and bacterial challenge (*Vibrio splendidus*) on the immune parameters (THC, viability, production of ROS and apoptosis) in combination with metabolomics studies to elucidate the immune response of *H. iris*.

These findings have significant implications for the understanding of the abalone immune system and how probiotic feeding significantly affects haemocytes parameters and the metabolite profile of abalone foot tissue post-pathogen challenge. This study also confirms previous findings that probiotics increase the health and contributes with additional evidence that it could provide immune resistance on juvenile abalone. Much remains to be elucidated regarding the role of ROS and the mechanism underlying immunological responses. However, this study reinforce the fact that the immune system in invertebrates, including its inflammatory components, is fundamental to host defence against pathogenic microorganism. Further investigation and experimentation into the immune aspects should delve further in understanding such mechanism of the immune system by incorporating recent metabolomics approaches in combination with transcriptomics and genomics studies. The overall impact of this study may improve abalone farming practices and help abalone farmers' better deal with stress and diseases affecting cultured *H. iris*, ultimately contributing to the sustainability of the abalone industry in New Zealand.

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