PhD Thesis

Metabolomics Applications in Immunological Studies of Marine Molluscs

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Metabolomics Applications in Immunological Studies of Marine Molluscs

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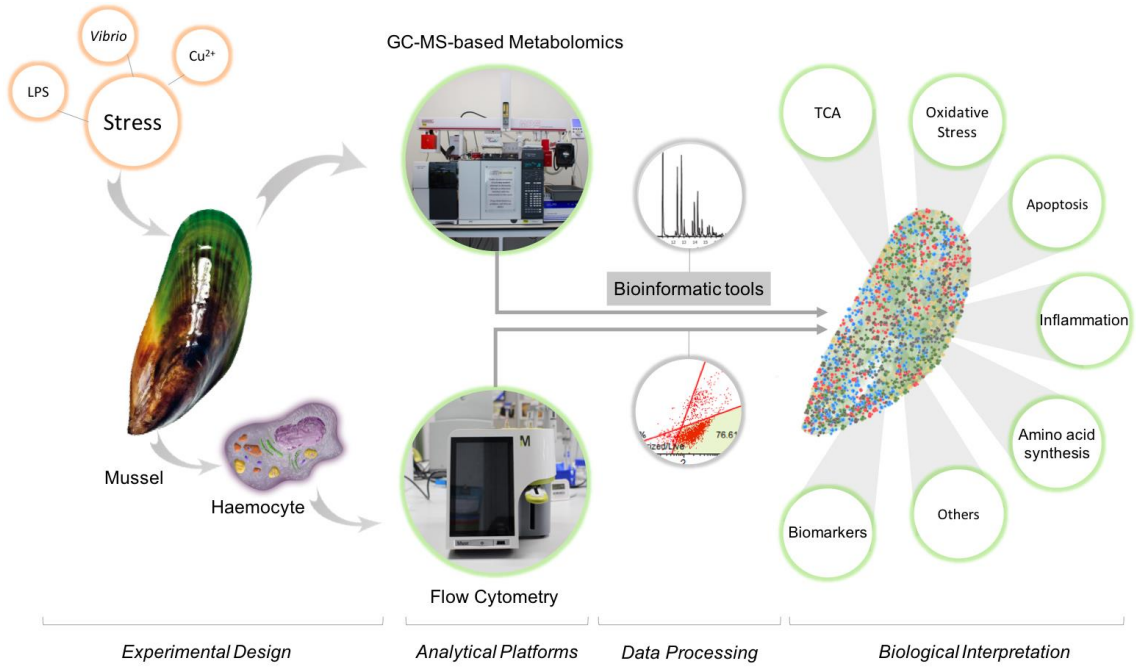
ABSTRACT



Molluscs form an important group in aquaculture as well as in coastal wild systems. However, high mortalities in molluscan species, specifically marine bivalves, have been encountered in the wild during summer times (summer mortality) as well as in aquaculture settings, which present a major economical challenge in many parts of the world. The complex interactions between host, environment and pathogens during these mortality events require new diagnostic tools and integrated approaches. Metabolomics is one of the newest and fastest growing omics. The sensitivity and specificity of metabolomics approaches make this a powerful tool for immunological studies, where it can provide insights into disease processes as well as the identification of metabolite biomarkers for early warning systems. This thesis was designed to provide, for the first time, a comprehensive understanding of the metabolic responses of mussel haemocytes and other tissues (e.g., gills, hepatopancreas, mantle) to external stimuli (*Vibrio* sp., lipopolysaccharides [LPS], Cu^{2+}) using gas chromatography-mass spectrometry (GC-MS)-based metabolomics approach. Along with the core metabolomics tool, novel flow

cytometry (FCM) protocols were developed in order to assess immunological parameters of the host upon stimulation. The combined method allows characterization of the mussel immune responses at both cellular and molecular levels and expands the number of biomarkers used to understand the animal's response.

Initially, tissue-specific metabolic responses of gill, haemolymph and hepatopancreas were observed in mussels challenged with *Vibrio* sp. Then, haemolymph was chosen as the target tissue/organ for the rest of the experiments in the thesis (Chapter 4). FCM revealed sex-based differences in immune responses of mussels to *Vibrio* sp. challenge. In this case, female mussels had lower haemocyte mortality, production of reactive oxygen species (ROS) and apoptotic cells after pathogen exposure compared to male mussels (Chapter 5). This suggests that female mussels have more efficient defence system than male mussels. However, metabolite profiles of haemolymph showed no significant difference between males and females. Subsequently, metabolic profiles of mussel haemolymph were intensively investigated in response to *Vibrio* sp. challenge, LPS and copper exposure (Chapter 6, 7 & 8). The alterations of metabolite profiles along with changes in immune characteristics due to stimulation provided insights into a number of pathways involved in immune responses of the host to *Vibrio* sp. infection and copper exposure.

The study also identified a number of candidate biomarkers involved in mussel immune processes. Among these metabolites, the presence of itaconic acid (ITA) and its accumulation were observed in different tissues of mussels following *Vibrio* sp. challenges, suggesting the important role of this metabolite as an antimicrobial compound in the innate immune system of bivalves (Chapter 4, 5 & 6). In fact, the challenge experiment (Chapter 9) revealed the complete inhibition of ITA on *Vibiro* sp. growth at

6 mM, and *Vibrio* growth was partially inhibited at 3 mM ITA. This confirmed, for the first time, the antibacterial activity of ITA against marine *Vibiro* sp. and suggests that ITA could be used as an antimicrobial compound for antibiotic resistant bacteria in aquaculture. Subsequently, the ITA concentrations in different tissues of mussels challenged with *Vibrio* sp. were quantitatively measured (Chapter 10). Interestingly, the results revealed that mussels are able to produce an effective amount of ITA to support the internal defence system, suggesting that ITA could be a valuable biomarker for health assessment of bivalves. In addition, ITA may also involve in anti-inflammation activities and other unknown functions in the bivalve innate immune system, which need further studies to reveal.

In conclusion, this thesis has successfully demonstrated the use of novel metabolomics approaches for aquaculture and marine science, which contribute new information regarding the molluscan immune system. It is envisaged that metabolomics will continue to grow as a tool of choice in studies of marine molluscs, as well as the broader field of marine science.

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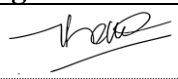
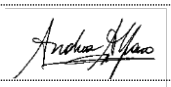
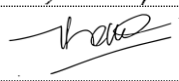
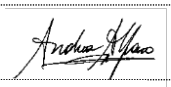
ATTESTATION OF AUTHORSHIP

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.

Van Thao Nguyen

Date: 10.12.2019

CO-AUTHORED WORKS

Chapter	Authors	Contribution	Total	Signature
2 – Review 1	Van Thao Nguyen	- Concept & structure - Writing	90%	
	Andrea C. Alfaro	- Review/edit	10%	
3 – Review 2	Van Thao Nguyen	- Concept & structure - Writing	90%	
	Andrea C. Alfaro	- Review/edit	10%	
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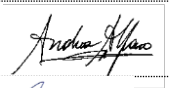
	Andrea C. Alfaro	- Experimental design - Review/edit	4%	
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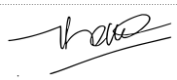
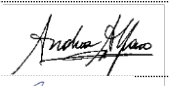
5 – Case study 2	Van Thao Nguyen	- Experimental design - Sample analysis - Data processing and analysis - Writing	90%	
	Andrea C. Alfaro	- Experimental design - Review/edit	4%	
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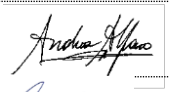
	Andrea C. Alfaro	- Experimental design - Review/edit	4%	
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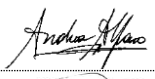
	Andrea C. Alfaro	- Review/edit	5%	
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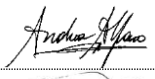
	Andrea C. Alfaro	- Experimental design - Review/edit	4%	
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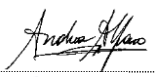
	Andrea C. Alfaro	- Experimental design - Review/edit	4%	
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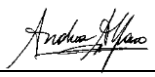
	Andrea C. Alfaro	- Experimental design - Review/edit	10%	

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Van Thao Nguyen

Section I

Introduction and Literature Review

New Zealand Greenshell™ mussel (*Perna canaliculus*) – The model organism of this thesis.

In this section:

Chapter 1: Introduction.

Chapter 2: Application of omics to investigate responses of bivalve haemocytes to pathogen infections and environmental stress.

Chapter 3: Applications of flow cytometry in molluscan immunology: current status and trends.

Chapter 1

Introduction and thesis framework

1.1 GENERAL INTRODUCTION

1.1.1 Molluscan aquaculture and New Zealand aquaculture

Molluscan aquaculture

Molluscs constitute the second largest aquaculture group after farmed fish. In 2016, the global aquaculture production of molluscs was 17.1 million tonnes (mt), worth USD 29.2 billion (FAO, 2018). There are 109 different molluscan species recorded from a total of 598 "species items" ever farmed in the world (FAO, 2018). Bivalves represent the most cultivated group within molluscs, with production increasing steadily since the 1990s from 3.3 to a record of 15.3 million metric tonnes (mt) in 2016 (FAO, 2018). Within bivalves, clams and cockles are the largest commercial group (38 %), followed by oysters (36 %), mussels (13 %) and scallops (13 %). China is the single largest bivalve producer, with a production of 13.0 million mt in 2016, which accounted for almost 85 % of the global harvest in that year. In the same year, Japan was the second largest producer, with 0.37 million mt, followed by South Korea (0.3 million mt) and Chile (0.31 million mt). Other main bivalve producing countries, included Spain, Thailand, USA, France and Italy generated more than 0.10 million mt in 2016.

New Zealand aquaculture and Greenshell^{TM} mussels

Aquaculture is an important industry in the economy of New Zealand which aims to become a NZD $1 billion primary industry by 2025 (www.aquaculture.org.nz). In 2017, aquaculture production in New Zealand reached 116,530 tonnes (FAO, 2019b). This harvest mostly comes from three main aquaculture species, including Greenshell^{TM} mussels (*Perna canaliculus*), King salmon (*Oncorhynchus tshawytscha*) and Pacific oysters (*Crassostrea gigas*) (Table 1.1). Other cultivated species with limited production include abalone (*Haliotis iris*) and flat oysters (*Ostrea chilensis*). New Zealand is committed to develop other potential species for aquaculture, such as geoduck clams

(*Panopea zelandica*) (Le, 2016), yellowtail kingfish (*Seriola lalandi lalandi*), gropers (*Polyprion oxygeneios*) and eels (*Anguilla australis*, *Anguilla dieffenbachia*) (www.niwa.co.nz).

New Zealand is considered to be a major producer of marine molluscs worldwide (FAO, 2018). Molluscan species make up most of the cultivated seafood in New Zealand. With a production of 101,5 thousand tonnes, two bivalve molluscs, *P. canaliculus* and *C. gigas*, contribute 88 % of the national aquaculture industry (Table 1.1). Among these, *P. canaliculus* alone accounts for 85.6 % of total aquaculture production.

Greenshell™ mussels (*P. canaliculus*) are endemic to New Zealand and are naturally distributed throughout the country. The first attempt in farming of *P. canaliculus* was conducted in the mid-1960s and the first commercial harvest was reported in 1971 (FAO, 2019a). Since then, the production has risen from 1.2 thousand tonnes in 1971 to 101 thousand tonnes in 2008, and has fluctuated around that level until now (FAO, 2019b). Farming of *P. canaliculus* is now carried out on longlines, in major farming areas in Coromandel, Marlborough Sounds and Stewart Island of New Zealand (FAO, 2019a). Mussels normally reach the market size of 90-100 mm in 18-24 months.

Table 1.1 Production of New Zealand Aquaculture in 2017. Data were obtained from FAO FishStat (FAO, 2019b).

Cultivated sspecies	**Production (000 tonnes)**	**Percentage (%)**
Greenshell™ mussels (*Perna canaliculus*)	99,7	85.6
King salmon (*Oncorhynchus tshawytscha*)	14,9	12.8
Pacific oysters (*Crassostrea gigas*)	1,8	1.5
Others	0.1	0.1
Total	116,5	100

1.1.2 Infectious diseases in marine molluscs and bivalve immunity

Infectious diseases in marine molluscs

Infectious diseases have been a primary concern for the growth and sustainability of molluscan aquaculture which have negative impacts on production of both hatcheries and commercial farms, as well as quality of seafood. The rapid development of aquaculture together with the international trade of seafood, transfers and introduction of molluscan seeds and stocks have increased the risk of spreading disease causative agents worldwide. Molluscan disease outbreaks are often associated with viruses, bacteria and parasites. The common pathogens and parasites affecting mollusc listed in manual of diagnostic tests for aquatic animals by the Office International des Epizooties (OIE) include two herpesviruses (abalone herpesvirus and ostreid herpesvirus 1 microvariants), six parasites (*Bonamia exitiosa*, *Bonamia ostreae*, *Marteilia refringens, Mikrocytos mackini*, *Perkinsus marinus* and *Perkinsus olseni*) and a bacterium (*Xenohaliotis californiensis*) (OIE, 2018). In addition, diverse bacteria in genus *Vibrio* have been associated with mortality outbreaks of bivalves in hatcheries, commercial farms and natural habitats (Travers et al., 2015). Examples of common *Vibrio* species include *V. splendidus*, *V. harveyi*, and *V. tubiashii*/*coralliilyticus*, *V. aestuarianus,* and *V. crassostreae*. Members of the generus *Nocardia* and *Roseovarius* are also considered important pathogenic bacteria in bivalve aquaculture (Travers et al., 2015).

In New Zealand, high mortality events associated with detection of pathogens have significantly affected three important molluscan species of the country, including Pacific oysters (*C. gigas*) flat oysters (*Ostrea chilensis*) and Greenshell^{TM} mussels (*P. canaliculus*). These mortality outbreaks often occurred during the summer months when there were elevated temperatures. The summer mortality events in *C. gigas* are often associated with detection of OsHV-1 µvar and a range of bacterial species, such as *Vibrio*

spp. (Alfaro et al., 2018, Nguyen et al., 2018). The mortality outbreaks of *O. chilensis* have been caused by the pathogen *Bonamia* sp. (Castinel et al., 2014) which led to removal of all the farmed oysters in New Zealand in 2017. The causes of mortalities in *P. canaliculus* mussels are currently not clear but are thought to be associated with pathogens (e.g., *Vibrio* bacteria, parasites, virus) and environmental factors (e.g., ocean acidification, increased temperatures).

It is generally agreed that diseases in aquaculture are caused by multiple factors, including pathogens infections, environmental stressors and immune system dysregulations. Disease dynamics are heavily influenced by environmental factors (e.g., temperature, salinity, hydrodynamic forces and water quality) and farming practices (e.g., rearing history, farming sites, rearing systems) (Alfaro et al., 2018). Host factors (e.g., sexes, life stages, genetic make-up and physiological status) also contribute to the susceptibilities and survival capacities of the host to the disease. Hence, it is crucial to take into account the complex host-pathogen-environment interactions in immune studies and disease management of molluscs.

Molluscan immune studies

To adapt with the pathogen-rich environment of marine water, marine molluscs have evolved both external and internal defence systems. The external defence is made up of several layers of physical or mechanical barriers, including the shell and the mucosal layer, while the internal defence mostly relies on the innate immune system. This system is driven by haemocytes and active molecules secreted by haemocytes and released into the haemolymph (Allam and Raftos, 2015). Hence, haemocytes are the key players in the internal defence of molluscs. The details of the roles of haemocytes and current cellular

and molecular tools applied for studies of mollusc haemocytes are presented in Chapter 2 & 3.

Due to the frequency of mortality events in molluscs, especially bivalves, and the economic importance of many bivalve species in aquaculture and coastal ecosystems, there is a growing number of studies on immunology and diseases of these species (Allam and Raftos, 2015, Guo and Ford, 2016, Nguyen et al., 2018, Travers et al., 2015). Significant progress has been made in the understanding of immunological mechanisms of molluscs at both cellular and molecular level (reviewed by Gerdol et al., 2018). Different diagnostic methods for molluscs have been developed, from traditional and immunodiagnostic methods to applications of clinical medicine for multiplex assays (Adams and Thompson, 2012, OIE, 2018). However, there are many knowledge gaps in immunology of bivalves as well as molluscs, which remain a challenge for disease management and aquaculture development as well as environmental monitoring (Alfaro et al., 2018, Gerdol et al., 2018, Green and Speck, 2018, Nguyen et al., 2018, Pernet et al., 2016). Nevertheless, the complex host-pathogen-environment interactions during mortality events require new molecular approaches, such as omics (1.1.2) along with routine cellular tools, such as flow cytometry (1.1.3).

For *P. canaliculus* which is the most important cultivated species in New Zealand, the number of immune investigations is very limited. Little is known about the immunity of *P. canaliculus* and its cellular and molecular pathways in responses to pathogens. Currently, there are only two immune-related scientific reports on this species, including isolation of *Vibrio* pathogens from larvae (Kesarcodi-Watson et al., 2009a) and adult challenge experiment with *Vibrio* sp. (Kesarcodi-Watson et al., 2009b). Since high mortalities of *P. canaliculus* in both hatcheries and commercial farms have become a

frequent phenomenon in recent years, this knowledge gap remains a big challenge for understanding the disease process and consequently the development of management strategies. Hence, this is an urgent need for future investigations.

1.1.3 Overview of omics and metabolomics

Omics approaches are based on the holistic view that organic molecules, cells, tissues and organs provide powerful information about the organism's make up, how it functions and how it responds to endogenous and exogenous forces (Kim, 2016). Therefore, an important application of omics is the identification of key biomarkers and signaling molecules, cellular pathways associated with cell processes, infection mechanisms and host responses to pathogens (Debnath et al., 2010). Omics technologies are also suitable to investigate the complex interactions between genotypes, phenotypes and the environment surrounding them (Gómez-Chiarri et al., 2015). Different omics fields focus on different portions of the entire life of an organism within its surrounding environment. Thus, genomics is the study of an organism's genome, transcriptomics is the study of the transcriptome, proteomics is the large-scale study of proteins, and metabolomics investigates metabolites present in cells, tissues and body fluids of an organism. In addition to common omics such as genomics, transcriptomics, proteomics, metabolomic, there are other omics fields that are continuously being created to focus on specific areas and applications within biological sciences (e.g., epigenomics, lipidomics, foodomics, nutritional genomics, pharmacogenomics, toxicogenomics). Details of these omics and their applications in studies of molluscan haemocytes are presented in Chapter 2.

1.1.4 Metabolomics applications in aquaculture research

Metabolomics has been incorporated in life sciences research in recent years (Fig. 1.1). However, metabolomics is relatively new in aquaculture and its applications in the field

remain limited. During the last few years, metabolomics has been increasingly applied to several aspects of aquaculture research, from larval production to nutrition and diet, disease and immunology, environmental stress and post-harvesting quality control (reviewed by Alfaro and Young 2018).

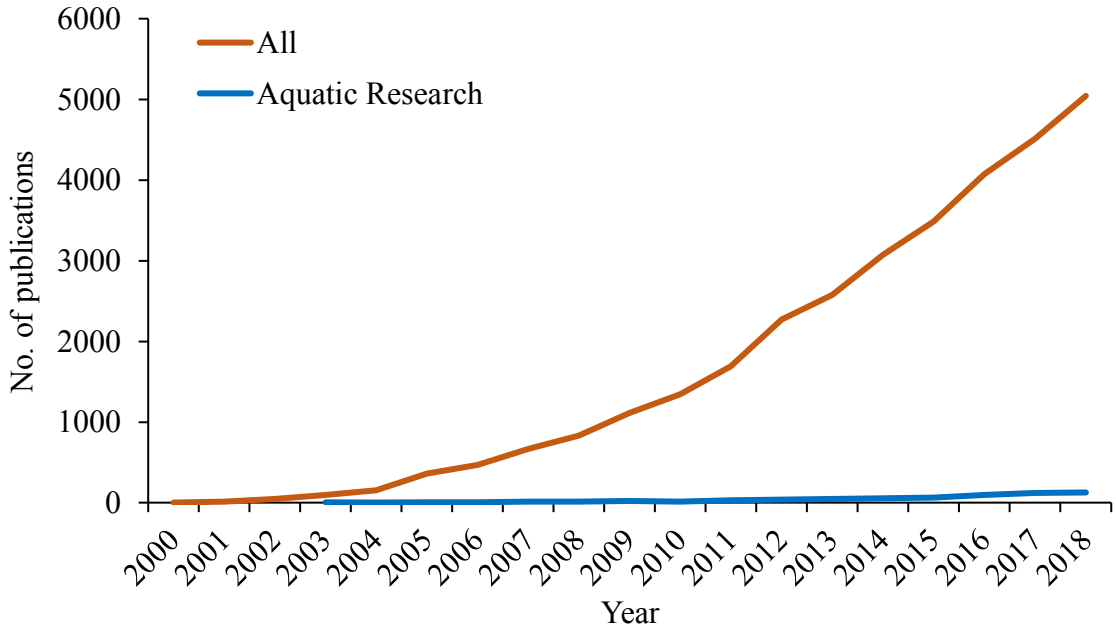

Figure 1.1 Number of metabolomics publications per year in all fields of science and in aquatic research. Data were obtained from Scopus database (28/04/2019). "metabolom*" was used as the key word of searching all publications in metabolomics field. The number of metabolomics publications in aquatic research were searched using key word "metabolom*" and either "aquaculture", "marine science", "aquatic science", "mollusc*", "shellfish", "bivalves", "fish" and "shellfish".

A few authors employed metabolomics to characterize the metabolic responses of molluscs to pathogen infections (Liu et al., 2013a, Liu et al., 2013b, Liu et al., 2014, Lu et al., 2017) or other environmental stresses (Digilio et al., 2016, Lu et al., 2016). These studies mainly used hepatopancreases (digestive gland) (Liu et al., 2013a, Liu et al., 2013b, Liu et al., 2014, Lu et al., 2017), gills (Liu et al., 2014, Lu et al., 2017, Lu et al., 2016) and muscle (Lu et al., 2016) as the target tissues for metabolomics analyses. The only report on haemolymph was conducted by Digilio et al. (2016) who characterized the responses of *Mytilus galloprovincialis* haemolymph to copper and temperature

challenges. Most of these studies employed nuclear magnetic resonance (NMR)-based metabolomics which is less throughput than gas chromatography–mass spectrometry (GC-MS) or liquid chromatography–mass spectrometry (LC-MS) approaches. No GC-MS or LC-MS-based metabolomics has been reported for immunological studies of molluscs, except for the study by Young et al. (2017) who investigated GC-MS-based metabolomics to characterize the metabolite profile of oyster larvae challenged with a virulent strain of OsHV-1. To this end, there is a need for GC-MS-based metabolomics approach to characterize the metabolomic profiles of molluscan haemolymph together with other tissues in response to pathogens and other environmental stressors. The sensitivity and specificity of GC-MS techniques together with specific roles of haemocytes in the innate immune system, make this approach a powerful tool for understanding the endogenous metabolic changes in host organisms during infections and also interactions between hosts, pathogens and environment. Furthermore, it can be considered to be a valuable mean for biomarker discovery which could be used in early diagnosis of diseases.

1.1.5 Flow cytometry in immunological studies of molluscs

Flow cytometry (FCM) is a laser-based technique that is used to analyse the physical and chemical characteristics of cells or particles in a heterogeneous fluid mixture. There is no doubt that FCM is a powerful technique and a routinely used tool in marine science. It allows the simultaneous analysis of multiple immunological parameters of organisms on an individual cell basis. The FCM is commonly used in immunological studies of molluscs for cell sorting, cell count, cell viability, cell cycle analysis, phagocytosis, oxidative stress and apoptosis. In addition, FCM is an upstream phenotype tool which could be combined with other omics techniques for downstream applications. Details of

this tool, its current applications and trends in molluscan immunology are presented in Chapter 3.

1.2 THESIS AIMS

This thesis aims to apply GC-MS-based metabolomics approaches to characterise the metabolic and biological responses of *P. canaliculus* mussels, as a model organism, to pathogen infections and water contaminants. The findings provide insights into the immune mechanisms of bivalves and allow for the identification of relevant biomarkers for these processes. In addition to metabolomics, different FCM assays were developed to provide fast and accurate characterization of immune responses of mussel haemocytes to external stimuli at the cellular level. Such combined approaches allow for a more detailed picture of the host's responses to external stressors at both cellular and molecular levels and generate accurate biomarker signatures for management strategies.

Overall, the thesis includes three main aims:

1. Identification of tisue-specific and sex-specific immune and metabolic responses of mussels to *Vibrio* infection.
2. Characterization of metabolite profiles and immune responses of mussel haemocytes under different stimulus conditions.
3. Identification of the antimicrobial activity of itaconic acid against *Vibrio* sp. and its role in the bivalve immune system.

1.3 THESIS STRUCTURE

In order to achieve the overall aims of this thesis, seven experiments were conducted. The results from these studies were published as peer reviewed articles and are presented in this thesis as seven experimental case studies in Chapters 4-10. These case studies, in combination with two review papers, introduction, discussion and conclusion comprise the 12 chapters of the thesis, which are divided into five main sections, as follows:

- Section 1 - Introduction and literature review: Chapter 1, 2 & 3.

- Section 2 - Tissue-specific and sex-specific responses of bivalves to pathogen infections: Chapter 4 & 5.
- Section 3 - Metabolomics profile of *Perna canaliculus* haemolymph: Chapter 6, 7 & 8.
- Section 4 - Application of metabolomics to study the antimicrobial role of itaconic acid: Chapter 9 & 10.
- Section 5 - Overall discussion and conclusions: Chapter 11 & 12.

Section 1 includes an introduction chapter (Chapter 1), and two review chapters about current applications of omics (Chapter 2) and flow cytometry (Chapter 3) to provide insights into molluscan haemocytes and immune system. Tissue-specific (Chapter 4) and sex-specific (Chapter 5) metabolic responses of *P. canaliculus* to *Vibrio* sp. presented in Section 2 were conducted to determine the target tissues for the next experiments and to identify metabolic variations due to sex differences. Based on results from Section 2, haemolymph was chosen as the tissue of interest to investigate metabolomics profiles of mussel haemolymph in response to different external stimuli, including *Vibrio* sp. (Chapter 6), LPS (Chapter 7) and copper (Chapter 8). Experiments from Chapter 4 to Chapter 8 (Section 3) identified a number of candidate biomarkers of interest (e.g., alanine, glutamic acid, glutathione, itaconic acid). Among them, itaconic acid was accumulated during pathogen infections, suggesting the special role of this metabolite in the molluscan immune system. Hence, the antimicrobial role of itaconic acid against *Vibrio* sp. and its role in the molluscan immune system was investigated in Section 4. The final section (Section 5) comprises of an overall discussion (Chapter 11) and conclusions (Chapter 12).

In addition, there are many other studies that are not included in this thesis but may directly relate to the thesis. These are mentioned in 1.4.

1.4 ACHIEVEMENTS FROM THIS THESIS

This section includes publications and presentations that directly or indirectly related to this thesis and were conducted during the period of 2016-2019.

1.4.1 Peer-reviewed papers

First or main author

1. **Nguyen, T.V**., Alfaro, A.C., Young, T., Ravi, S., Merien, F., 2018. Metabolomics study of immune responses of New Zealand greenshell™ mussels (*Perna canaliculus*) infected with pathogenic *Vibrio* sp. *Marine Biotechnology*. 20, 396-409. https://doi.org/10.1007/s10126-018-9804-x
2. **Nguyen, T.V**., Alfaro, A.C., Merien, F., Lulijwa, R., Young, T., 2018. Copper-induced immunomodulation in mussel (*Perna canaliculus*) haemocytes. *Metallomics*. 10, 965-978. https://doi.org/10.1039/c8mt00092a
3. **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*) during *Vibrio* sp. infection. *Journal of Invertebrate Pathology*. 157, 80-89. https://doi.org/10.1016/j.jip.2018.08.008
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7. Alfaro, A.C., **Nguyen, T.V**., Mellow, D., 2019. A metabolomics approach to assess the effect of storage conditions on metabolic processes of New Zealand surf clam (*Crassula aequilatera*). *Aquaculture*. 498, 315-321. https://doi.org/10.1016/j.aquaculture.2018.08.065
8. **Nguyen, T. V.**, Alfaro, A. C., Merien, F. & Young, T. 2019. In vitro study of apoptosis in mussel (*Perna canaliculus*) haemocytes induced by lipopolysaccharide. *Aquaculture,* 503, 8-15. https://doi.org/10.1016/j.aquaculture.2018.12.086
9. **Nguyen, V. T.**, Alfaro, A. C., Young, T., Green, S., Zarate, E. & Merien, F. 2019. Itaconic acid inhibits growth of a pathogenic marine *Vibrio* strain: A metabolomics approach. *Scientific Reports*. https://doi.org/10.1038/s41598-019-42315-6
10. **Nguyen, T. V**. & Alfaro, A. C. 2019. Targeted metabolomics to investigate antimicrobial activity of itaconic acid in marine molluscs. *Metabolomics,* 15, 97. https://doi.org/10.1007/s11306-019-1556-8

11. **Nguyen, T. V**. & Alfaro, A. 2019. Application of omics to investigate responses of bivalve haemocytes to pathogen infections and environmental stress. *Aquaculture,* https://doi.org/10.1016/j.aquaculture.2019.734488
12. **Nguyen, T. V**. & Alfaro, A. 2019. Applications of flow cytometry in mollusc immunology: current status and trends. *Fish & Shellfish Immunology,* 94, 239-248. https://doi.org/10.1016/j.fsi.2019.09.008

Co-author

13. Young, T., Kesarcodi-Watson, A., Alfaro, A.C., Merien, F., **Nguyen, T.V**., Mae, H., Le, D.V., Villas-Bôas, S., 2017. Differential expression of novel metabolic and immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. *Developmental & Comparative Immunology*. 73, 229-245
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15. Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Young, T., Meyer, J., **Nguyen, T. V.** & Trembath, C. 2019. Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) blood and validation of flow cytometry cell count and viability assay kit. *Fish & Shellfish Immunology,* 88, 179-188.

Book chapters

1. **Nguyen, V. T**. & Alfaro, A. 2019. GC-MS-based metabolomics to investigate summer mortality events of Greenshell mussels (*Perna canaliculus*) in New Zealand. *In:* Beale, D. J., Jones, O. a. H. & Warden, A. C. (eds.) *Environmental Metabolomics: Approaches, Challenges and Future Perspectives.* Elsevier. Submitted.

1.4.2 Presentations at conferences, symposia or workshops

Oral presentations (first or main author)

1. **Nguyen, T.V.**, Alfaro, A.C., Young, T., Merien, F., 2017. Metabolic and immunological responses of New Zealand green-lipped mussel (*Perna canaliculus*) to *Vibrio* sp. infection. The 13th Annual Conference of the Metabolomics Society, Brisbane, Australia. https://doi.org/10.13140/RG.2.2.36166.93769
2. **Nguyen, T.V.**, Alfaro, A.C., Young, T., Ravi, S., Merien, F., 2017. A metabolomics approach to understand immune responses of New Zealand Greenshell™ mussels (*Perna canaliculus*) infected with *Vibrio* corallilyticus. AUT Postgraduate Research Symposium, Auckland, New Zealand. https://doi.org/10.1007/s10126-018-9804-x
3. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Lulijwa, R., Young, T., 2018. Metabolic and immunological responses of male and female New Zealand Greenshell™ mussels (*Perna canaliculus*) during *Vibrio* sp. infection. AUT Sience Showcase, Auckland, New Zealand. https://www.doi.org/10.13140/RG.2.2.34069.78568

4. **Nguyen, T.V.**, Alfaro, A.C., Young, T., Merien, F., 2018. Showcases of Metabolomics Applications in Studies of Bivalve Immunity. NZ Marine Sciences Society Conference, Napier, New Zealand. https://doi.org/10.13140/RG.2.2.35436.21129
5. **Nguyen, T.V.**, Alfaro, A.C., 2018. Showcases of GC-MS-based metabolomics applications in aquaculture. The Australian and New Zealand Metabolomics Conference, Auckland, New Zealand.
6. **Nguyen, T. V.**, Alfaro, A. C., Young, T. & Merien, F. 2019. Metabolomics applications in characterization of pathogen infection and pollutant-induced stress in marine bivalves. *Aquaculture 2019*. Louisiana, USA: WAS.

Oral presentations (invited speaker)

7. **Nguyen, T. V**. & Alfaro, A. C. 2019. Metabolomics applications to characterize pathogen infections and environmental stress in marine molluscs. *Ecuadorian Aquaculture Congress (CEA)*, Guayaquil, Ecuador.

Poster presentations (first or main author)

8. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Young, T., 2016. Metabolomics: A novel approach for disease and immunological studies in bivalves. New Zealand Aquaculture Conference, Nelson, New Zealand. https://doi.org/10.13140/RG.2.2.12012.77448
9. **Nguyen, T.V**., Alfaro, A.C., Young, T., Merien, F., Grandiosa, R., 2017. Metabolic and immunological responses of male and female New Zealand Greenshell™ mussels (*Perna canaliculus*) to pathogenic Vibriosis. AUT Postgraduate Research Symposium, Auckland, New Zealand.
10. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Lulijwa, R., Young, T., 2018. Copper-induced immunomodulation in New Zealand Greenshell™ mussel (*Perna canaliculus*) haemocytes. AUT Sience Showcase, Auckland, New Zealand. https://www.doi.org/10.13140/RG.2.2.13973.01769
11. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Young, T., 2018. Metabolomics and flow cytometry insights into apoptosis in mussel (*Perna canaliculus*) haemocytes induced by lipopolysaccharides. 2018 Postgraduate Research Symposium, Auckland, New Zealand.
12. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Lulijwa, R., Young, T., 2018. Metabolomics application in immunotoxicological studies of mussel (*Perna canaliculus*) haemocytes exposed to copper. The 14th Annual Conference of the Metabolomics Society, Seattle, Washington, USA. https://www.doi.org/10.13140/RG.2.2.26225.79201/1
13. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Young, T., Grandiosa, R., Li, M., 2018. Sex-base differences in metabolic and immunological responses of mussel haemocytes during *Vibrio* sp. infection. The 14th Annual Conference of the Metabolomics Society, Seattle, Washington, USA. https://www.doi.org/10.13140/RG.2.2.21875.17447
14. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Young, T., Grandiosa, R., Li, M., 2018. GC-MS-based metabolomics in- sights into the immune responses of mussel haemocytes during *Vibrio* sp. infection: toward the identification of immune bio-markers. New Zealand Marine Sciences Society Conference, Napier, New Zealand. https://www.doi.org/10.13140/RG.2.2.15164.28801

15. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Lulijwa, R., Young, T., 2018. GC-MS based metabolomics and flow cytometric study of the toxic effects of copper on mussel (*Perna canaliculus*) haemolymph. New Zealand Marine Sciences Society Conference, Napier, New Zealand. https://www.doi.org/10.13140/RG.2.2.30714.34248
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17. **Nguyen, T.V.**, Alfaro, A.C., Merien, F., Lulijwa, R., Young, T., 2018. A combined approach of metabolomics and flow cytometry to investigate oxidative stress and apoptosis in mussel haemocytes exposed to copper. The Australian and New Zealand Metabolomics Conference, Auckland, New Zealand. https://doi.org/10.1039/c8mt00092a
18. **Nguyen, T. V.**, Alfaro, A. C., Young, T. & Merien, F. 2019. Untargeted metabolomics to investigate the metabolic and immunological responses of different mussel tissues to *Vibrio* sp., infection. *Aquaculture 2019.* Louisiana, USA: WAS.

Other oral or poster presentations represent by co-authors

19. Alfaro, A.C., Young, T., **Nguyen, T.V**., 2016. Novel diagnostics tool to boost aquaculture production. *New Zealand Aquaculture Conference*, Nelson, New Zealand.
20. Young, T., Kesarcodi-Watson, A., Alfaro, A., Mae, H., **Nguyen, T.V.**, Le, D.V., Villas-Bôas, S.G., Merien, F., 2016. OsHV-1 hijacks host metabolism in oyster larvae. *New Zealand Aquaculture Conference*, Nelson, New Zealand.
21. Grandiosa, R., Young, T., **Nguyen, T.V.**, Guiterrez, 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*). *New Zealand Marine Sciences Society (NZMSS) Annual Conference*, Canterbury University, Christchurch, New Zealand.
22. Lulijwa, R., Alfaro, A.C., Merien, F., Burdass, M., Young, T., Meyer, J., **Nguyen, T.V.**, Trembath, C., 2018. Characterisation of New Zealand King Salmon Whole Blood for Immunological Assays. *The 2018 NZ Marine Sciences Society Conference*, Napier, New Zealand.
23. Alfaro, A. C., **Nguyen, T. V**. & Yong, T. 2018. Novel health assessment tools for shellfish aquaculture. *Molluscs 2018.* The Museum of New Zealand, Wellington, New Zealand: The Malacological Society of Australasia (MSA).
24. Alfaro, A. C., **Nguyen, T. V**. & Young, T. 2019. Mussel health assessment: how to understand the language of phisiology. *Aquaculture 2019*, New Orleans, Louisiana, USA. WAS.

1.4.3 Awards/ research grants/ travel grants

1. January 2016: PhD Scholarship by New Zealand Ministry of Foreign Affairs and Trade.
2. January 2017: Student research grant by New Zealand Marine Sciences Society (NZMSS).
3. June 2017: Travel grant by Metabolomics Society to attend the metabolomics conference 2017 in Brisbane, Australia.

4. August 2017: Best poster award on Postgraduate Research Symposium at Auckland University of Technology.
5. June 2019: Early-career Members Network Travel Grant from Metabolomics Society.

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Chapter 2

Application of omics to investigate responses of bivalve haemocytes to pathogen infections and environmental stress

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Nguyen, T. V. & Alfaro, A. 2019. Application of omics to investigate responses of bivalve haemocytes to pathogen infections and environmental stress. *Aquaculture,* https://doi.org/10.1016/j.aquaculture.2019.734488

ABSTRACT

Recent advances in high-throughput technologies for omics analyses and bioinformatics for data interpretation have led to the application of omics approaches across all fields of life sciences. There has also been an expansion of omics research in immune studies of marine invertebrates, such as bivalves to gain insights into pathogenic infections and disease progression. Many of these omics research have been conducted on haemocytes and haemolymph, which are the most important components of the bivalve immune system. Characterization of transcriptomes, proteomes and metabolomes of bivalve haemocytes in response to pathogenic infections and other environmental stressors have revealed valuable information regarding the mechanisms that drive the innate immune system in response to stress challenges, as well as insights regarding complex host-pathogen-environment interactions across bivalve species. For instance, detailed analysis of haemocyte transcriptomes has resulted in the discovery of a number of coding and non-coding transcripts involved in immune and stress responses. In addition, comprehensive examination of the proteome and metabolome of bivalve haemocytes following stress exposure has helped identify changes in the physiological status of the organism, including specific molecular pathways involved in these processes. Furthermore, the differently expressed molecules that have been identified through these omics studies can be used as candidate biomarkers with applications in breeding selection programs, disease diagnosis and environmental monitoring. However, despite these significant biotechnological advances, the application of omics tools for bivalve haemocyte research is currently hindered by several challenges and bottlenecks. In this contribution, we aim to review the major advances, current perspectives and future directions of three main omics (transcriptomics, proteomics and metabolomics) with regards to their application in bivalve haemocyte and aquaculture research.

2.1 INTRODUCTION

Bivalves, such as mussels, oysters, clams and scallops are important in aquaculture. The world aquaculture production of bivalves reached 15.7 million tonnes in 2017, worth USD 26.6 billions (FAO, 2019). In addition to this economic value, bivalves sustain many marine ecosystems by filtering xenobiotic compounds and pathogenic microorganisms (Board and Council, 2010, Gosling, 2015). Pathogen loads can be extremely high in some regions and seasons, and these may cause massive mortalities, especially during summer months. For example, massive mortality events in Pacific oysters (*Crassostrea gigas*) (Alfaro et al., 2018) and flat oysters (*Ostrea chilensis*) (Lane et al., 2016) have resulted in significant economic losses. These summer mortality events are often associated with different type of pathogens, such as viruses (e.g., *Ostreid herpesvirus 1*) (Alfaro et al., 2018), bacteria (e.g., *Vibrio* spp.) (Allam and Raftos, 2015), and parasites (e.g., protozoans) (Lane et al., 2016). Lacking the adaptive immune system that vertebrates have, bivalves mostly rely on their innate immune system to defend against these pathogens (Fletcher and Cooper-Willis, 1982). The non-specific innate defence system of bivalves is composed of cellular components, which include haemocytes and epithelial cells, and soluble components, which are active molecules secreted by haemocytes and released into the haemolymph (Allam and Raftos, 2015). In addition, haemocytes participate in a range of non-immune related processes (e.g., wound healing, shell production, excretion and digestion) which have not been well studied (Gosling, 2015, Mount et al., 2004).

Circulating haemocytes and the haemolymph are the main components of the immune system and have been the focus of most immunological studies in bivalves. In the past, haematological investigations have been limited to various methods of cytochemical staining and microscopic observation for morphological characterization of haemocytes.

Examples of these studies include many bivalve species, such as *Mya arenaria* (Beckmann et al., 1992), *Tapes philippinarum* (Cima et al., 2000), *Ruditapes decussatus* (López et al., 1997), *Saccostrea glomerata* (Aladaileh et al., 2007), *Meretrix lusoria* and *C. gigas* (Chang et al., 2005a). The development of flow cytometry has facilitated a more extensive characterization of haemocytes, including a range of quantitative parameters such as total cell count and cell viability (Nguyen et al., 2019a), sorting of cell sub-populations (Goedken and De Guise, 2004), DNA content (Benabdelmouna and Ledu, 2016), phagocytosis (Gagnaire et al., 2006), oxidative stress and apoptosis (Nguyen and Alfaro, 2019). Furthermore, different omics approaches, such as transcriptomics, proteomics and metabolomics have been applied to characterize the complex interactions between haemocytes and environmental stressors, such as pathogens and water contaminants (Table 2.1, 2.2 & 2.3). Omics approaches aim to characterize the structure, function and dynamics of genes (genomics), expressed genes (transcriptomics), proteins (proteomics), and low molecular weight metabolites (metabolomics) in a biological sample using high-throughput analytical technologies (Fig. 2.1). The sensitivity and specificity of most omics makes them powerful tools in immune studies. Hence, major omics investigations on haemocytes have significantly contributed to a better understanding of bivalve immunity. In this contribution, we provide a review of the use of omics approaches to investigate responses of haemocytes to pathogen infections and environmental stressors, as well as applications for disease management and environmental monitoring within aquaculture settings.

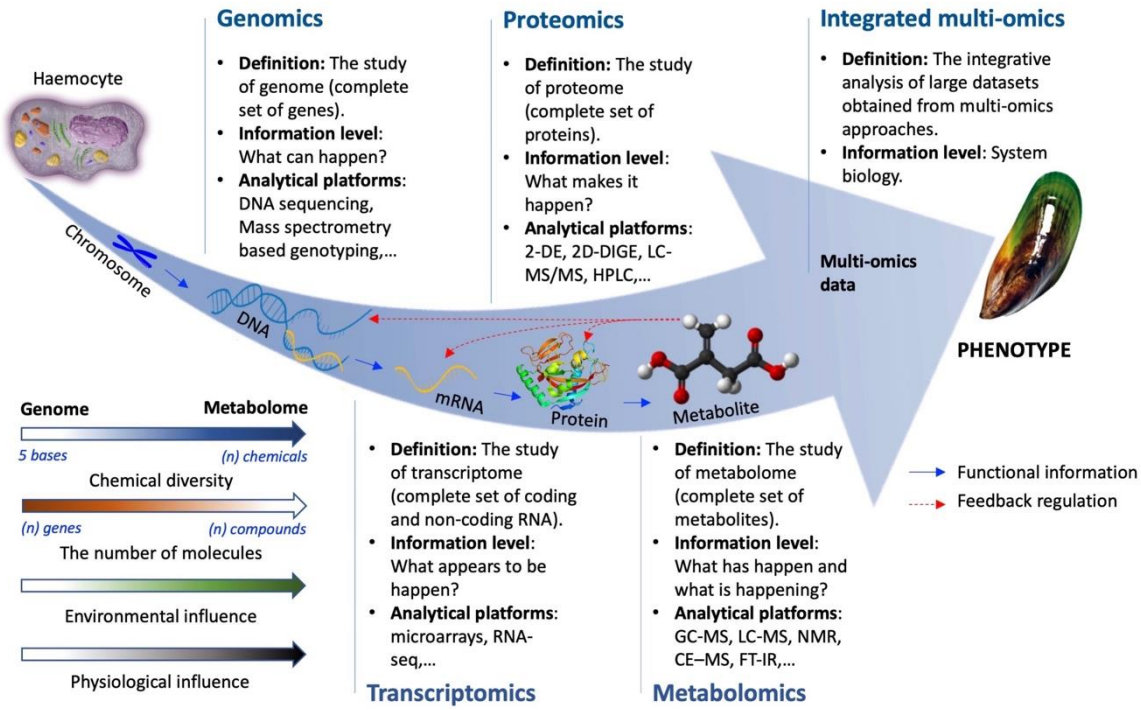

Figure 2.1 Schematic representation of omics approaches, their molecules of interest, level of information that can be obtained from each approach, analytical platforms, and the relationship between genes, RNAs, proteins and metabolites. The arrows (bottom left) demonstrate the relationships of each omics to the chemical diversity (number of chemicals), environmental and physiological influence, and the number of molecules (genes, transcripts, proteins and metabolites). The stronger colors denote higher diversity, more molecules or stronger influence. *2-DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis; LC-MS/MS, liquid chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; CE-MS, capillary electrophoresis–mass spectrometry; FT-IR, fourier-transform infrared spectroscopy.*

2.2 OVERVIEW OF HAEMOLYMPH AND HAEMOCYTES

2.2.1 Haemolymph components and roles

The haemolymph contains haemocytes and various humoral defence factors secreted by haemocytes that float in the colourless plasma (Gosling, 2015). The haemolymph usually lacks respiratory pigment because its oxygen concentration is similar to or slightly greater than that of seawater (Bayne et al., 1979). However, a few bivalves in the Families Arcidae (e.g., *Tegillarca granosa*), Astartidae (e.g., *Astarte castanea*), Carditidae (e.g., *Cardita borealis*) and Limidae (e.g., *Ctenoides ales*) are known to have haemoglobin pigment in their haemolymph (Bao et al., 2016, Klein, 2017, Terwilliger et al., 1978, Weber and Vinogradov, 2001, Yager et al., 1982).

The haemolymph is a circulatory fluid which plays multiple important roles in bivalve physiology, including internal defence, gas exchange, osmoregulation, nutrient distribution, waste elimination and hydrostatic pressure for structural support of organs, such as labial palps, foot and mantle edges (Gosling, 2015). The haemolymph pressure, in combination with muscle action in the foot is used by some bivalves, such as clams, to burrow into the substrate (Gosling, 2015). In this review chapter, we have limited the focus to the immune role of circulating haemocytes and the haemolymph.

2.2.2 Haemocyte classification

Bivalve haemocytes vary greatly from species to species, and there is currently no unified nomenclature that applies to all bivalve haemocytes. Generally, bivalve haemocytes are divided into two broad categories based on the presence of cytoplasmic granules, including granulocytes and agranulocytes. Granulocytes are about 10–20 mm diameter and are characterised by cytoplasmic granules, while agranulocytes are smaller (4–6 mm diameter) and have no or few cytoplasmic granules (Gosling, 2015).

Granulocytes usually account for the majority of the haemocytes in the haemolymph of most bivalves (Cima et al., 2000, Gosling, 2015), with the exceptions of Pacific oysters (*C. gigas*) (Chang et al., 2005b) and Sydney rock oysters (*S. glomerata*) (Aladaileh et al., 2007). Granulocytes are further sub-classified into different categories based on granular affinity to specific dyes (acidophilic/ eosinophilic, basophilic and neutrophilic granulocytes) (Bayne et al., 1979, Carballal et al., 1997, Chang et al., 2005b). In some bivalves, such as California mussels (*Mytilus californianus*) (Bayne et al., 1979), blue mussels (*Mytilus edulis*) (Carballal et al., 1997) and hard clams (*M. lusoria*) (Chang et al., 2005a), granulocytes are divided into two subclasses: acidophilic and basophilic granulocytes. In addition to basophils and acidophils, neutrophils have been observed in some bivalves, such as Manila clams (*Ruditapes philippinarum*) (Cima et al., 2000) and Pacific oysters (*C. gigas*) (Chang et al., 2005b). Granulocytes are sometimes sub-divided based on size (small, medium and large), as can be found in Zhikong scallops (*Chlamys farreri*) (Zhang et al., 2007).

Bivalve agranulocytes are often sub-divided into three subclasses: blast-like cells, basophilic macrophage-like cells and hyalinocytes (Hine, 1999). However, in some bivalves, such as in hard clams (*M. lusoria*), only two types of agranulocytes (hyalinocytes and blast-like cells) have been identified (Aladaileh et al., 2007, Chang et al., 2005b).

Granulocytes are phagocytic cells, and they play a prominent role in phagocytosis of pathogens (e.g., bacteria, protozoan parasites) and other particles (e.g., algae, cellular debris) (Gosling, 2015). High levels of intracellular enzymes associated with immunological activity in granulocytes also enable them to kill pathogens after

phagocytosis. In addition, granulocytes are involved in encapsulation of pathogens or particles that are too big to be phagocytosed (Gosling, 2015).

Unlike granulocytes, agranulocytes are less important for phagocytosis (Aladaileh et al., 2007, Gosling, 2015). In addition, hyalinocytes have been shown to assist in the aggregation processes associated with wound healing (Aladaileh et al., 2007, Gosling, 2015). Haemoblast-like cells lack the common intracellular enzyme systems associated with host defence, and they do not participate in defensive responses (Aladaileh et al., 2007).

2.2.3 Haemocyte functions in the internal defence system

Like other invertebrates, bivalves mostly rely on their non-specific defence mechanisms, which is composed of cellular and humoral components (Allam and Raftos, 2015, Song et al., 2010). As the backbone of the internal innate immune system, haemocytes act as mediators of cellular defences whereas various active molecules in the haemolymph secreted by haemocytes are major components of humoral defences (Allam and Raftos, 2015, Gerdol et al., 2018, Zannella et al., 2017). This section aims to highlight the roles of haemocytes in the cellular and humoral defence mechanisms. More details of bivalve immunity can be found in a number of recent review papers (Allam and Raftos, 2015, Gerdol et al., 2018, Zannella et al., 2017).

Cellular defence mechanisms

When bivalves are invaded by pathogens or foreign particles, the host haemocytes respond with different types of cellular defence mechanisms, which mainly include haemocytosis, phagocytosis, encapsulation, apoptosis and autophagy.

Haemocytosis is a cell-mediated immune response which involves an increase in circulating haemocytes (Garmendia et al., 2011, Jones et al., 1996, Renault, 1996). Such responses have been shown in bivalves during pathogen infections or diseases (Allam et al., 2000a, Jones et al., 1996, Mateo et al., 2009, Nguyen et al., 2018a) and environmental stresses (Couch, 1985, Hauton et al., 2000, Wedderburn et al., 2000).

Perhaps the most important mechanism of pathogen elimination in bivalves is phagocytosis, which is the engulfment of foreign particles (e.g., bacteria, algae, cellular debris, protozoan parasites) by haemocytes. The process of phagocytosis is complex and has been well described in several publications (Canesi et al., 2002, Fletcher and Cooper-Willis, 1982, Gosling, 2015). Phagocytosis includes four steps: chemotaxis, recognition and attachment, internalization and intracellular degradation (Gosling, 2015). Chemotaxis is the direct migration of haemocytes towards foreign materials through chemo-attractants produced by foreign materials, such as peptides (Fawcett and Tripp, 1994). Although chemotaxis has been shown in several bivalves, such as *Mercenaria mercenaria* challenged with *Escherichia coli* (Fawcett and Tripp, 1994) and *M. edulis* stimulated by lipopolysaccharide (LPS) (Schneeweiß and Renwrantz, 1993), the mechanism is still poorly understood in bivalves. After non-self-recognition, attachment of foreign bodies into haemocytes is carried out *via* cell surface receptors, such as integrin- and lectin-like proteins (Humphries and Yoshino, 2003). Internalization of foreign material into the cell membrane is conducted *via* endocytosis to form a primary phagosome (Gosling, 2015). The phagosome is then fused with lysosomes to form a phagolysosome (secondary phagosome) where the foreign material is degraded by numerous enzymes (intra-cellular degradation).

Encapsulation is another major mechanism used by haemocytes to eliminate foreign particles that are too large to be phagocytosed, such as multicellular parasites (e.g., nematodes, cestodes) or experimentally introduced tissues. In general, a capsule of haemocytes encloses the foreign body, and then haemocytes release cytotoxic products (e.g., degradative enzymes and free radicals) into the invader to destroy it (Jayaraj et al., 2009). Although encapsulation has been extensively studied in insects, this immune response is poorly characterized in marine bivalves. There have been some reports of encapsulation of the trematode *Aspidogaster conchicola* (Huehner and Etges, 1981), metacercarial cysts of the trematode *Himasthla* spp. (Wootton et al., 2006) and chromatography beads (Jayaraj et al., 2009, Meena et al., 2010, Wootton et al., 2006).

In addition, haemocytes also contribute to the defensive role of mucosal surfaces (Allam and Raftos, 2015). Since bivalves have an open circulatory system, haemocytes are freely circulated and abundant haemocytes have been found in peripheral compartments (Allam and Raftos, 2015). These peripheral haemocytes are known to have the ability to phagocytise foreign particles and secrete hydrolytic and antimicrobial compounds that contribute to the immune protection of these compartments (reviewed by (Allam and Raftos, 2015).

Another important cellular defence mechanism of haemocytes is apoptosis which is a type of programmed cell dead. Details of apoptosis in bivalves have been extensively described by Romero et al. (2011), Romero et al. (2015b) who showed that apoptosis in molluscs is highly evolutionarily conserved to that of vertebrates with some unique features. Autophagy is another type of programmed cell death that involves the innate immunity against intracellular pathogens. However, not much is known about the role of this process in bivalves. Autophagy have been described in oyster haemocytes (reviewed

by (Wang et al., 2018), suggesting that autophagy is involved in the defence system of Pacific oysters against pathogens. Hence, there is a need for future investigations to reveal insights into the mechanisms of pathogen clearance by autophagy in bivalves and other molluscs.

Humoral defence mechanisms

Haemocytes are able to secrete a diverse range of antimicrobial peptides (AMPs) and other cytotoxic substances into the haemolymph (Gerdol et al., 2018, Zannella et al., 2017). These active molecules, together with other nonspecific humoral defense molecules (e.g., lectins, lysosomal enzymes) form the humoral components of bivalve innate immunity (Gerdol et al., 2018).

AMPs are small (less than 10 kDa), gene-encoded cationic peptides, which constitute the innate immune effectors found among all forms of life. In bivalves, first pioneer characterizations of AMPs were reported in haemolymph of the mussel, *M. edulis* (Charlet et al., 1996) and *Mytilus galloprovincialis* (Mitta et al., 1999). To date, the most comprehensive characterization of AMPs in the animal kingdom has been described in bivalves (Allam and Raftos, 2015) where the majority are cysteine-containing peptides (Zannella et al., 2017).

Other types of antimicrobial molecules have been described in bivalve plasma, such as lysozymes, proteases, protease inhibitors and phenoloxidase. Lysozymes are antimicrobial enzymes that are commonly thought to protect bivalves from pathogen invasions (Allam and Raftos, 2015). Different types of lysozymes have been described in bivalves (Gerdol et al., 2018) and lysozyme activity has been shown to increase in bivalve plasma in response to pathogen exposure (Allam et al., 2000a, Allam et al., 2000b, Chu and La Peyre, 1993, Itoh and Takahashi, 2009) and environmental cues (Chu and La

Peyre, 1989, Paillard et al., 2004). Similarly, the upregulated levels of proteases (enzymes that degrade proteins) and protease inhibitors have been observed in bivalve plasma (Allam and Raftos, 2015, Gerdol et al., 2018). For example, Ertl et al. (2016) observed the expression of cathepsin transcripts (e.g., cathepsin B and L) in haemolymph of the Sydney rock oysters (*S. glomerate*) exposed to different environmental stressors. The up-regulation of Kazal-type serine protease inhibitors have been reported in haemolymph of the bay scallops (*Argopecten irradians*) following tissue injury and bacterial challenges (Zhu et al., 2006). Phenoloxidase (PO) is a key enzyme in the melanization cascade that also serves as an innate defence mechanism in several bivalve species (Gerdol et al., 2018, Vaillant, 2001). Strong activity of PO and its zymogen form, prophenoloxidase (ProPO), have been observed in haemolymph of different bivalve species (reviewed by (Gerdol et al., 2018).

Pattern recognition receptors (PRRs) are another group of proteins expressed by cells in the innate immune system and commonly associated with immune responses of bivalves against infections. A number of homologue genes of PRRs were found in the genome of *C. gigas*, including C-type lectins, fibrinogen-related proteins (FREPs), complement homologues, scavenger receptors (SRCR), toll-like receptors (TLRs), lipopolysaccharide and β-1,3-glucan-binding proteins (LGBP) and peptidoglycan-recognition proteins (PGRPs) (Zhang et al., 2012). Genes related to PRRs have also been reported in the genome of other bivalves, such as *M. galloprovincialis* (Murgarella et al., 2016) and *Bathymodiolus platifrons* (Sun et al., 2017). Transcriptomics studies have revealed the altered expression of PRRs-related genes in haemolymph of many bivalve species challenged with pathogens, suggesting the importance of PRRs in bivalve immune responses (reviewed by (Allam and Raftos, 2015).

2.3 TRANSCRIPTOMICS

2.3.1 General overview of transcriptomics

Transcriptomics is the study of transcriptomes, which are the sum of all coding and non-coding RNA transcripts produced by the genome during development or under specific circumstances. Hence, a transcriptome provides a snapshot of the total transcripts present in a cell at a given time, which reflects the genes that are actively expressed (Lowe et al., 2017). Transcriptomics uses several different techniques, including serial/cap analysis of gene expression (SAGE/CAGE), expressed sequence tag (EST), suppression subtractive hybridization (SSH), microarrays and RNA sequencing (RNA-Seq) to measure the expression of genes in distinct cell populations that are affected by different treatments, diseases or environmental factors at different time points. Some of these techniques, such as SAGE, CAGE, and EST (based on Sanger sequencing of cDNA or EST) are no longer used for transcriptomics analyses.

SSH is a polymerase chain reaction (PCR-based approach) which amplifies differentially expressed cDNAs (complementary DNAs) fragments. SSH offers many advantages in profiling gene transcripts, so it is still a widely used method for identification of gene expression (Rusaini, 2018). DNA microarrays are a set of microscopic DNA spots which are arrayed on a solid substrate (Romanov et al., 2014). Since microarrays can only detect sequences homologous to what is on the array, prior genomic knowledge of the organism of interest (e.g., annotated genome sequence, ESTs) is required to generate the probes of the array (Lowe et al., 2017). Due to the relatively low cost and high throughput, microarrays remain a reliable tool in model organisms, where highly standardized platforms have been developed over the years. RNA-Seq is a revolutionary tool that uses recently developed deep-sequencing technologies combined with computational methods for both mapping and quantifying transcriptomes (Lowe et al., 2017, Wang et al., 2009).

RNA-Seq methodologies have several advantages (e.g., higher throughput, sensitivity, accuracy, long read lengths, no need of prior knowledge of the organism's genome) and have been rapidly adopted over hybridisation-based approaches as the dominant transcriptomics technique in the last two decades (Su et al., 2014).

2.3.2 Transcriptomics for bivalve haemolymph analyses

The recent identification of genome sequences and genomes for many bivalve species (Gómez-Chiarri et al., 2015) has revealed a large number of immune genes. This information has allowed for targeted transcriptomics studies to identify gene expression and biochemical pathways in different tissues and organs of bivalves in response to biotic and abiotic environmental stresses *in vivo* or *in vitro* (Gómez-Chiarri et al., 2015). Furthermore, transcriptomics studies on bivalve haemocytes have contributed to the discovery of previously unknown coding (and non-coding) transcripts. Different transcriptomics techniques (from medium to high throughput) have been used for analysis of haemocyte transcriptomes, such as ESTs, SSH, microarrays and high-throughput RNA-seq (Table 2.1).

Traditional transcriptomics approaches (ESTs and SHH) have been used in many bivalve studies to generate ESTs and SSH libraries of haemocytes (Table 2.1). For example, Gueguen et al. (2003) used ESTs generated from a haemocyte cDNA library of bacteria-challenged oysters (*C. gigas*) for immune gene discovery. This approach allowed for the characterization of 20 genes among the 1142 ESTs generated, which may be involved in immune responses. Among them, six genes related to Rel/NF-kB pathway were characterized for the first time in a bivalve species. Gestal et al. (2007) obtained 253 clones from SSH libraries of haemocytes of *R. decussatus* stimulated with a mixture of dead bacteria. Among these, 184 ESTs were identified and were probably involved in 8

Table 2.1 Transcriptomics applications on bivalve haemocytes/ haemolymph

Techniques/ platforms	Bivalve species	Stimulants	Key Findings	References
Expressed sequence tag (EST)	*Crassostrea gigas*	A mixture of four pathogenic *Vibrio* strains	- 1142 ESTs were generated from 1248 randomly selected clones. - 20 genes may be implicated in immunity with 6 genes related to NF-kB signalling pathway.	Gueguen et al. (2003)
Suppression subtractive hybridization (SSH)	*Ruditapes decussatus*	A mixture of dead bacterial strains	- A total of 253 clones was obtained from haemocyte SSH libraries. - 3.16% of the total identified genes were possibly related to immune functions. - This is the first report on myticin isoforms 1, 2 and 3 and mytilin in clam.	Gestal et al. (2007)
SSH	*Mya arenaria*	*Vibrio splendidus*	- Approximately 8000 reads were obtained from each subtracted cDNA pool. - Transcripts were clustered into cellular functions including structural proteins, immunity, stress proteins, apoptosis, cell process, metabolism and signal transduction.	Araya et al. (2010)
SSH	*Ruditapes philippinarum*	*V. tapetis*	- 9098 sequences were obtained with 235 ESTs from two cDNA libraries. - 60% of these ESTs are identical to genes involved in different physiological functions (e.g., immunity, apoptosis and cytoskeleton organization).	Brulle et al. (2012)
DNA microarrays	*R. philippinarum*	*V. alginolyticus*	- A 8 ×15 k oligo-microarray was constructed from a total of 12,156 annotated sequences. - 579 differentially expressed transcripts include numerous immune-related sequences, through a time course of infection.	Moreira et al. (2014)
DNA microarrays	*Venerupis philippinarum*	*Perkinsus olseni*	A total of 1264 genes were differentially expressed in the control and infected groups.	Romero et al. (2015a)
RNA-Seq/ 454-pyrosequencing	*R. philippinarum*	PAMPs	- 974,976 reads were sequenced, consisting of 51,265 contigs. - The 35 most frequently found contigs included a large number of immune-related genes.	Moreira et al. (2012).
RNA-Seq/ illumina sequencing	*R. philippinarum*	*Perkinsus olseni*	- 123,195,758 PE reads were sequenced, consisting of 33,079 transcripts with 7300 annotated transcripts. - Many transcripts were functionally linked to signalling, cell proliferation, cell adhesion, immune system and response to stress and stimuli.	Hasanuzzaman et al. (2017)
RNA-Seq/ 454 pyrosequencing	*M. edulis*	*V. splendidus*	- 1,024,708 nucleotide reads were sequenced and assembled into 19,622 annotated sequences. - Expression of defensin, lysozyme and proteasome 26S was observed in haemocytes exposed to *V. splendidus*.	Tanguy et al. (2013)
RNA-Seq/ illumina sequencing	*M. edulis*	Cadmium	- 341,718,828 reads consist of 352,976 contigs. - 1112 transcripts were differentially regulated by cadmium exposure.	Granger Joly de Boissel et al. (2017)

RNA-Seq/ Illumina HiSeq-2500	*Mytilus coruscus*	*Vibrio alginnolyficus*	- A total of 68,924,728 and 61,106,355 clean reads were obtained from the control and *V. alginolyticus* infected libraries respectively. - 27,345 unigenes (42.77%) were annotated from 63,942 unigenes. - Bacterial challenge led to differential expression of 1526 genes associated with 122 pathways.	Dong et al. (2017)
RNA-Seq/ Illumina HiSeq-2000	*Mytilus chilensis*	No stimulation	- 799,899,594 reads were assembled into 225,336 contigs. - 20,306 single nucleotide polymorphisms were identified in immune-related transcripts in *Mytilus chilensis*.	Núñez-Acuña and Gallardo-Escárate (2013)
RNA-Seq/ Illumina HiSeq-2000	*Mytilus chilensis*	Saxitoxin	- 800 million reads consistof 138,883 contigs. - There were upregulation of PRRs and other immune effectors.	Detree et al. (2016)
RNA-Seq/ Illumina HiSeq-2000	*Mytilus galloprovincialis*	PAMPs and *Vibrio anguillarum,* heat-inactivated *Vibrio anguillarum*	- 393,316 million raw RNA-Seq reads were obtained from different tissues and assembled into 151,320 non-redundant transcripts. - Haemocyte and gill transcriptomes shared 60 % of the transcripts. - Stimulated hemocytes showed abundant defense and immune-related proteins.	Moreira et al. (2015)
RNA-Seq/ 454 pyrosequencing	*M. galloprovincialis, M. edulis* and *R. decussatus*	PAMPs	- 400,000 reads were obtained for each transcriptome. - There is a high abundance of sequences directly related to immune responses in the three studied species.	Moreira et al. (2018)
RNA-Seq/ Illumina HiSeq-2000	*Mya arenaria*	Disseminated neoplasia disease	- 95,399,159 reads consist of 73,732 contigs. - The majority of the cell cycle pathways described in vertebrates were identified in *M. arenaria* haemocytes and the majority of the transcripts identified in cell cycle were highly expressed during the development of the disease.	Siah et al. (2013)
RNA-Seq/ Illumina HiSeq-2000	*Pecten maximus*	Inactivated-*Vibrio anguillarum,* PAMPs	- 216,444,674 sequence reads consist of 73,732 contigs. - 934 contigs encode proteins with a putative role in immune response.	Pauletto et al. (2014)
RNA-Seq/ Illumina Genome Analyzer II	*Crassostrea virginica*	No stimulation	- 52,857,842 pair-end reads consist of 66,229 contigs. - Analysis of the gene set yielded a diverse set of 657 genes related to innate immunity.	Zhang et al. (2014)
RNA-Seq/ 454 pyrosequencing and 8 × 15 K oligo-microarray	*O. edulis*	*Bonamia ostreae*	- 2,075,254 sequence reads include 1,042,177 reads from genomic DNA and 1,033,077 reads from haemocytes. - 984 annotated sequences are directly or indirectly related to innate immunity.	Pardo et al. (2016)
RNA-Seq/ Illumina HiSeq-2000	*Saccostrea glomerata*	Different environmental stressors	- 484,121,702 paired-end reads were sequenced. - There is a wide range of genes potentially involved in innate immunity with some haemocyte-specific expressed transcripts.	Ertl et al. (2016)

RNA-Seq/ Illumina Hiseq-2000	*Pinctada fucata*	*Vibrio alginolyticus*	- 56,345,139 reads were generated with 33,548,634 raw reads from the control group and 36,859,244 from the bacterial challenged group. - 74,007 unigenes with an average length of 680 bp were generated after assembly. - 636 genes were significantly differentially expressed after bacterial challenge.	Wang et al. (2016)

functional categories, including immune defence, cell signalling and adhesion, cytoskeleton structure, cell cycle, cell metabolism and respiration chain, ribosomal proteins and other unknown functions. For *M. arenaria* clams, Araya et al. (2010) identified large numbers of homologous genes in a SSH library of haemocytes challenged with *Vibrio splendidus*. These were clustered into cellular functional groups, including structural proteins, immunity, stress proteins, apoptosis, cell process, metabolism and signal transduction. Similarly, SSH analyses of haemocytes of Manila clams (*R. philippinarum*) subjected to *Vibrio tapetis* revealed 235 ESTs from two cDNA libraries (Brulle et al., 2012). Around 60% of these ESTs were identical to genes involved in different physiological functions (e.g., immunity, apoptosis and cytoskeleton organization). Overall, these initial transcriptomics studies have led to a significant increase in the number of ESTs in databases of bivalve haemocytes, as well as bivalves which constitute the basis for DNA microarray technology.

A few authors have used DNA microarrays to measure the expression levels of large numbers of genes from bivalve haemocytes (Moreira et al., 2014, Romero et al., 2015a). For example, an immune-enriched DNA microarray was employed to characterize gene expression profiles of haemocytes from *Venerupis philippinarum* triggered by *Perkinsus olseni* at different infection stages (Romero et al., 2015a). The increased expression of genes associated with pathogen recognition, production of nitrogen radicals, antimicrobial activity and cellular processes (e.g., inhibition of serine proteases and

proliferation) were observed at an early phase of infection. At the intermediate stage, there were over-expressions of many genes related to cell movement. Metabolic pathway genes were the most affected at the later stage of infection (thirty days after infection) and apoptosis-related genes mostly expressed during pathogenesis. Similarly, a DNA microarray experiment was designed to study the gene transcription profiles of haemocytes from *R. philippinarum* clams infected with *V. alginolyticus* (Moreira et al., 2014). The microarray experiment revealed a total of 579 differentially expressed transcripts, including numerous immune-related sequences, through a time course of *V. alginolyticus* infection. Functional analysis of these expressed genes revealed insights into haemocyte functions in response to *Vibrio* infection. For example, the main functions of haemocytes in early responses against the *Vibrio* challenge involved cellular component organization, biogenesis, cell migration, signalling and death. Expression of many cytoskeleton genes suggests a possible chemotactic response in hemocytes. Together, these microarray approaches have significantly contributed to our understanding of haemocyte immune functions and their responses to pathogens at different infection stages.

High throughput RNA-Seq methods have recently been applied to characterize whole transcriptome profiles of haemocytes from many bivalve species (Table 2.1). Transcriptomes of bivalve haemocytes range from 400,000 reads in *M. galloprovincialis* and *R. decussatus* (Moreira et al. 2018) to 800 million sequence reads in *Mytilus chilensis* (Detree et al. 2016). These sequence reads have been assembled into unigenes assigned into sub-categories within three major categories: biological processes, cellular components and molecular functions (Dong et al., 2017, Pauletto et al., 2014, Siah et al., 2013, Tanguy et al., 2013). Functional analyses of these haemocyte transcriptomes have revealed a large number of transcripts that functionally clustered in common, well-

recognized immune pathways, such as signal transduction, complement cascades, PRRs (e.g., lectin, b-glucan recognition proteins, peptidoglycan recognition proteins, toll-like receptors), apoptosis (e.g., IAP, BAX, BAC-2), antimicrobial molecules (e.g., AMPs, lysozyme, protease and protease inhibitors) and others (Dong et al., 2017, Hasanuzzaman et al., 2017, Moreira et al., 2012, Pauletto et al., 2014, Tanguy et al., 2013, Zhang et al., 2014). Interestingly, this approach resulted in the discovery of many sequences from molecules never before described in bivalves (Moreira et al., 2012). In addition to identification of immune related genes and pathways, RNA-Seq transcriptomics approaches have been used to investigate specific molecular mechanisms involved in the cell cycle, firstly investigate with regard to the development of hemic neoplasia in *M. arenaria* (Siah et al., 2013). The results of that study showed that most cell cycle pathways described in vertebrates were also identified in *M. arenaria* haemocytes, and the majority of the transcripts identified in the cell cycle were highly expressed during the development of the disease.

In addition to pathogens, transcriptomic responses of bivalve haemocytes to environmental stressors have been conducted by several authors (Detree et al., 2016, Ertl et al., 2016, Granger Joly de Boissel et al., 2017). For example, the transcriptome of haemolymph and other tissues of *S. glomerata* exposed to different environmental stressors show a low level of similarity at the nucleotide level, but a relatively high similarity at the protein level to the *C. gigas* genome (Ertl et al., 2016). Interestingly, some transcripts coding for cathepsins, heat shock proteins, peroxiredoxin and superoxide dismutase were found to be expressed only in the hemolymph, suggesting their important roles in haemocyte functioning and innate immunity. RNA-seq was used to identify the transcriptomic response of *M. chilensis* haemocytes to saxitoxin (a principal phycotoxin) (Detree et al., 2016). This study identified immune receptors and pathways potentially

involved in the recognition and defence mechanisms in *M. chilensis* haemocytes against STX's toxicity. For instance, the upregulation of PRRs in the haemocyte transcriptome after saxitoxin exposure, such as toll-like receptors, tumor necrosis factor receptors and scavenger-like receptors suggests the involvement of PRRs in response to saxitoxin. Similarly, expressions of genes related to TLRs were reported in the transcriptome of *M. edulis* haemocytes exposed to cadmium (Granger Joly de Boissel et al., 2017). In addition, cadmium exposure led to induction of superoxide dismutase (SOD), glutathion-s-transferase (GST), cytochrome P450 2C8 (CYP2C8), a multidrug resistance protein (MRP1) and heat shock protein 70 (HSP70), as well as genes involved in phagocytosis (actin and CDC42) and apoptosis (caspase 8 and XIAP/IAP), suggesting that cadmium can regulate key molecular mechanisms.

Overall, transcriptomics approaches have led to the extensive characterization of large sets of immune-related transcripts in bivalve haemocytes and have significantly improved our understanding of haemocyte roles in defence mechanisms. These genes could be good candidates to develop genetic markers associated with pathogen susceptibility, which could be used in breeding selection programs through marker assisted selection (MAS). Haemocyte transcriptomes provide a valuable resource for further studies to elucidate the host defence mechanisms of bivalves against pathogens and environmental stressors which could provide novel strategies for management of diseases in bivalve aquaculture and environmental monitoring.

2.3.3 Challenges of transcriptomics applications in aquaculture

The advancement in high-throughput DNA/RNA sequencing technologies has significantly enriched genomic databases for many aquatic species. However, applications of transcriptomics in immunological studies of bivalves in aquaculture are

currently facing some challenges. While the complete genomes of many bivalves have been sequenced (Li et al., 2017, Mun et al., 2017), the lack of genomic databases for other commercial bivalve species represents a bottleneck for transcriptomic applications in bivalve species. In addition, the high heterozygosity and overall complexity of bivalve genomes make them difficult to assemble. Furthermore, the lack of specific knowledge regarding the function of most bivalve genes remains a difficulty in data interpretation. The cost for RNA-seq in many labs is around 300 USD per sample, which is a challenge for large scale studies.

2.4 PROTEOMICS

2.4.1 General overview of proteomics

Proteomics is the large-scale study of the proteome, which is the entire sum of proteins in cells, tissues, biofluids or organisms. Similarly to the transcriptome, the proteome is highly unstable over time and variable between cell types and changeable in response to external stimuli (Fliser et al., 2007). Hence, examination of proteomics captures a snapshot of the protein environment at a given time (Graves and Haystead, 2002). Proteomics provides not only information on the expression level of relevant genes, but also post-translational modifications (Rodrigues et al., 2012).

Several proteomics techniques have been developed, and are widely used in aquaculture, including gel-based and gel-free approaches (Rodrigues et al., 2012). Two-dimensional gel electrophoresis (2-DE) is based on two steps of separation of proteins according to their isoelectric points, using isoelectric focusing (IEF) followed by molecular weights in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This is the most commonly used proteomics methodology due to its simplicity, reliability and affordability (Rodrigues et al., 2017). However, the limitation of this traditional method is its low sensitivity. Therefore, a gel-free approach based on mass spectrometry (MS) coupled with liquid chromatography (LC) has recently emerged and represents the most common approach today. Due to its high throughput, LC-MS-based approaches are able to analyze thousands of peptides in a single sample, thus making it highly effective in protein quantitation and post-translational modification analysis (Rodrigues et al., 2017).

2.4.2 Proteomics for bivalve haemolymph analyses

With recent advances in genomic sequencing and analytical technologies, proteomics has been increasingly applied in diverse research aspects of marine science (Debnath et al.,

2010, Rodrigues et al., 2012). Using bivalves as model organisms, several studies have employed proteomics to understand the underlying host response mechanisms to pathogens (Corporeau et al., 2014, Ji et al., 2013, Vaibhav et al., 2017) and environmental stress (e.g., heavy metals, temperature, organic pollutants) (Campos et al., 2012, Tomanek, 2014).

Plasma proteins have been characterized in several bivalves (Abebe et al., 2007, Hattan et al., 2001, Li and Flemming, 1967, Renwrantz et al., 1998), and are known to play multifunctional roles in immune responses, heavy metal transport, antioxidation, wound repair and shell mineralization (Itoh et al., 2011, Koutsogiannaki and Kaloyianni, 2010, Xue et al., 2012). This suggests that haemolymph could be a useful targeted organ for proteomics. Consequently, alterations in haemolymph proteomes due to exposure to stress and diseases have been reported in several bivalves (Table 2.2) which provide several novel insights into the role of haemocytes in bivalve innate immunity.

Proteomics approaches have been investigated to characterize haemolymph proteomes in response to diseases (e.g., QX, bonamiosis) (Cao et al., 2009, Simonian et al., 2009), pathogen infections (Chen et al., 2011, Novoa et al., 2016) and immunostimulant (e.g., Poly I:C) (Green et al., 2016, Jiang et al., 2018). These studies have mainly focused on identification of proteome maps, protein biomarkers, and characterization of specific functions of proteins or specific immune processes of haemocytes (e.g., phagocytosis). For example, haemolymph proteomics has been used to identify biomarkers of QX disease-resistance in selectively bred Sydney rock oysters (*S. glomerata*) caused by protozoan *Marteilia sydneyi* infection (Simonian et al., 2009). Six proteins in proteome maps were clearly associated with QX disease resistance and two proteins (p9 and p11) were identified as potential markers for selective breeding of QX disease-resistant

Table 2.2 Proteomics applications on bivalve haemocytes/ haemolymph

Techniques/ platforms	Bivalve species	Stimulants	Key Findings	References
2-DE	*Ostrea edulis* and *C. gigas*	Bonamiosis caused by the protozoan *Bonamia ostreae*	Protein spots exclusive to healthy and infected groups were detected.	Cao et al. (2009)
2-DE	*S. glomerata*	QX disease by protozoan parasite	Proteome maps were developed for QX disease-resistant and -susceptible oysters	Simonian et al. (2009)
2-DE	*S. glomerata*	Metals (copper, lead or zinc)	Eighteen of the 25 spots were significantly affected by just one of the three metals.	Thompson et al. (2011)
2-DE and MALDI-TOF-MS	*Chlamys farreri*	Acute viral necrosis virus (AVNV) infection	42 proteins were identified which were classified into eight categories.	Chen et al. (2011)
2-DE and LC–MS/MS	*S. glomerata*	Metals (cadmium, copper, lead and zinc)	- The identified proteins include some that are commonly associated with environmental monitoring, such as HSP 70, and other novel proteins. - The most common biological functions of proteins were associated with stress response, cytoskeletal activity and protein synthesis.	Thompson et al. (2012a)
2-DE	*S. glomerata*	Metal contamination in the field	- An average of 514 spots were identified per oyster proteome. - There were unique protein expression profiles for each field trial that mostly associated with cytoskeletal activity and stress responses.	Thompson et al. (2012b)
LC-MS/MS	*S. glomerata*	Heavy metals ($PbCl_2$, $CuCl_2$, or $ZnCl_2$)	84 proteins were identified, and statistical analysis revealed 56 potential biomarker proteins.	Muralidharan et al. (2012)
2-DE and ESI-Q-TOF MS/MS	*M. galloprovincialis*	Males vs Females	No differences were observed in the profiles obtained for male and female serum proteins.	Oliveri et al. (2014)
2-DE and LC-MS/MS	*S. glomerata*	Elevated carbon dioxide	- An average of 320 protein spots were detected among each of the 27 2-DE gels analysed. - Proteins that differed significantly in concentration between pCO_2 treatments fell into five broad functional categories: energy metabolism, cellular stress responses, the cytoskeleton, protein synthesis and the extracellular matrix.	Thompson et al. (2016)
LC-MS/MS	*M. galloprovincialis*	Herpesviruses	- Myticin C peptides were identified in both mussel haemolymph and haemocytes. - Modified myticin C peptides showed antiviral activity against human herpes simplex virus types 1 (HSV-1) and 2 (HSV-2).	Novoa et al. (2016)
2-DE and LC-MS/MS	*C. gigas*	Poly(I:C)	- A total of 110 and 77 protein spots were identified in the two separate batches of *C. gigas* hemolymph, respectively. - Poly(I:C) injection increased the relative in- tensity of four protein spots, including a small heat shock protein (sHSP),	Green et al. (2016)

			poly(I:C)-inducible protein 1 (PIP1) and two isoforms of C1q- domain containing protein (C1qDC).	
nano-HPLC-ESI-MS/MS	*M. galloprovincialis*	*Vibrio cholerae* and *Escherichia coli*	The extrapallial protein present in serum of *M. galloprovincialis* enhanced adhesion to and killing by hemocytes of *V. cholerae*, expressing the MS hemagglutin (MSHA), as well as of *E. coli*, carrying type 1 fimbriae.	Canesi et al. (2016)
LC-ESI-MS/MS	*C. gigas*	FITC-labeled latex beads	352 significantly high expressed proteins were identified within the phagocyte proteome.	Jiang et al. (2018)
2D-PAGE and LC-MS/MS	*M. galloprovincialis*	Injury	6 proteins were identified different between control and experimental mussels, including myosin, tropomyosin, CuZn superoxide dismutase (SOD), triosephosphate isomerase, EP protein and small heat shock protein.	Franco-Martínez et al. (2018a)

oysters. Similarly, a proteomics approach was developed to analyze the bases of tolerance/resistance to bonamiosis in the European flat oysters (*Ostrea edulis*) and Pacific oysters (*C. gigas*) (Cao et al., 2009). This study demonstrated that the expression of haemolymph proteins could be used to understand the interaction between oysters and *Bonamia ostreae*, and to find the bases of tolerance/resistance to bonamiosis. A 2-DE approach was used to analyse protein expression profiles from the haemocytes of *C. farreri* infected with acute viral necrosis virus (AVNV) (Chen et al., 2011). A total of 42 proteins were identified in haemolymph profiles which were classified into different biological and molecular categories (e.g., metabolism, immunity, transcriptional regulation, transduction). Using a proteomic approach, Novoa et al. (2016) identified myticin C peptides and observed the expression of myticins and antimicrobial peptides in mussel haemocytes exposed to OsHV-1, suggesting antiviral activity against OsHV-1. In addition to pathogens, injection with poly(I:C) led to increases of four proteins in haemolymph of *C. gigas* compared to relative seawater-injected controls (Green et al., 2016). Furthermore, proteomics has been combined with transcriptomics to provide insights into the phagocytic killing of *C. gigas* haemocytes (Jiang et al., 2018). The results

showed 352 significantly highly expressed proteins within the phagocyte proteome and 262 correspondingly highly expressed genes in the transcriptome. The pathway analysis of these significantly expressed proteins revealed a number of antimicrobial-related biological processes of phagocytes, including oxidation-reduction and lysosomal proteolysis processes.

Many authors employed proteomics to analyse different expressions of haemolymph proteins in response to different environmental stressors (Table 2.2). Overall, stress stimulation has led to expressions of haemolymph proteins that belong to broad functional categories. Many of these expressed proteins can be considered to be single biomarkers or combinational biomarkers of stress responses, such as metal contamination in Sydney Rock oysters (Thompson et al., 2012a). Metal exposure (e.g., cadmium, copper, lead and zinc) was also reported to lead to different expressions of proteins of *S. glomerata* haemolymph in both laboratory (Muralidharan et al., 2012, Thompson et al., 2011, 2012a) and in field studies (Thompson et al., 2012b). These haemolymph proteins were mostly associated with stress responses, cytoskeletal activity and protein synthesis. Similarly, expressed proteins in haemolymph proteomes of *S. glomerata* oysters exposed to ambient and elevated carbon dioxide (pCO_2) belong to five broad functional categories, including energy metabolism, cellular stress responses, the cytoskeleton, protein synthesis and the extracellular matrix (Thompson et al., 2016). In addition, the induced muscle injury by puncture of adductor muscles for three consecutive days was reported to lead to alterations in the haemolymph proteome of *M. galloprovincialis* mussels (Franco-Martínez et al., 2018b). Many of these proteins were related to muscle damage (troponin, creatine kinase and aspartate aminotransferase) and oxidative stress (SOD, trolox equivalent antioxidant capacity and esterase activity).

Overall, these initial proteomics applications for the characterization of bivalve haemolymph demonstrate the applicability and reliability of using proteomics for insights into the role of bivalve haemolymph in response to external stressors. Further development of proteomics to understand the molecular mechanisms underlying immune responses and identification of protein biomarkers for environmental pollution and disease resistance in marine bivalves would be extremely useful for biomonitoring and stock assessment in aquaculture.

2.4.3 Challenges of proteomics applications in aquaculture

Although proteomics appears to be a powerful tool in aquaculture, its application in this field is currently facing several bottlenecks. The first challenge is the limited information at the genomic level (DNA and expressed RNAs) of aquaculture species, which is needed for the interpretation of proteomic data and protein identification (Rodrigues et al., 2012). However, present advances in genome sequencing technologies and the decrease in costs for full genome sequencing suggest that more genomes and transcriptomes of aquaculture species will be generated in the near future, which may clear this bottleneck for aquaculture proteomics research (Rodrigues et al., 2012). However, the high costs of proteomics analyses (approximately 300 – 400 USD per sample) remains a limitation for many large-scale projects. To this end, there is a need for cost-effective high-throughput proteomics workflows to make this approach more accessible to researchers.

2.5 METABOLOMICS

2.5.1 General overview of metabolomics

Metabolomics is the scientific study of the set of metabolites within biological samples (e.g., tissues, body fluids, entire organisms), which is called the metabolome. Metabolomics is one of the newest omics and has been rapidly growing in the last decade. Since metabolites are end-product of gene expression and cell activity, they are highly sensitive to environmental change. Thus, metabolomics can represent a physical snapshot of what is actually happening in the organism at a given time (Alfaro and Young, 2018). To this end, metabolomics is applied to characterize endogenous metabolic changes in biological samples within different environmental conditions and biomarkers involved in these processes.

Metabolomics uses various analytical platforms, such as infrared spectroscopy (IR), raman spectroscopy, nuclear magnetic resonance (NMR) and many mass spectrometry (MS) techniques, including direct-infusion mass spectrometry (DI-MS), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-IMS), capillary electrophoresis–mass spectrometry (CE-MS), gas chromatography–mass spectrometry (GC-MS), and liquid chromatography–mass spectrometry (LC-MS). With sufficient high throughput and resolution capabilities, NMR and MS are the most widely applied analytical tools (Young and Alfaro, 2016).

2.5.2 Metabolomics for bivalve haemolymph analyses

The low molecular weight of compounds found in bivalve haemolymph has resulted in fewer metabolomics studies on this biofluid compared to other tissues. However, several recent metabolomics approaches have revealed the important biological functions of

metabolites in bivalve haemolymph in response to environmental stresses or pathogen infections (Table 2.3).

Table 2.3 Metabolomics applications on bivalve haemocytes/ haemolymph

Techniques/ platforms	Bivalve species	Stimulants	Key Findings	References
Untargeted GC-MS	*Perna canaliculus*	*Vibrio* sp.	- Metabolite profiles of haemolymph, gills and hepatopancreas were characterized with a large number of metabolite different between control and infected tissues - There were tissue specific metabolic responses between these tissues against *Vibrio* infection.	Nguyen et al. (2018d)
Untargeted GC-MS	*Perna canaliculus*	*Vibrio* sp.	- A large number of metabolites different between control and infected mussel haemolymph. - No difference in metabolite profiles of haemolymph from male and female mussels exposed to *Vibrio* sp.	Nguyen et al. (2018c), Nguyen et al. (2018e)
Untargeted GC-MS	*Perna canaliculus*	Copper	- There were alterations of 25 metabolites within the metabolite profile of copper exposed haemolymph (125 µM) compared to those of control samples. - Changes in levels of these metabolites may be considered important signatures of oxidative stress (e.g., glutathione) and apoptosis processes (e.g., alanine, glutamic acid).	Nguyen et al. (2018b)
Untargeted GC-MS	*Perna canaliculus*	LPS	11 metabolites were elevated in metabolite profiles of LPS-exposed mussel haemocytes.	Nguyen et al. (2019a)
Untargeted GC-MS	*Perna canaliculus*	Storage conditions	- There were changes of 11 metabolites between the different temperatures and sampling times. - Among them, lactic acid, succinic acid, malic acid, fumaric acid and glutamic acid were identified as significantly affected by both storage condition and period.	Alfaro et al. (2019)
NMR	*M. galloprovincialis*	Copper and temperature	- 27 metabolites were identified unambiguously in spectrum of haemolymph. - Alanine, lysine, serine, glutamine, glycogen, glucose and protein aliphatics were strongly affected by copper exposition while high temperature (24 °C) and high copper levels caused a coherent increase glucose, serine, and lysine.	Digilio et al. (2016)
Targeted GC-MS	*Perna canaliculus*	*Vibrio* sp.	- The concentrations of itaconic acid were successfully measured in haemolymph and different tissues of mussels after *Vibrio* exposure. Identification the role of itaconic acid in bivalve immune system.	Nguyen and Alfaro (2019)

For example, Nguyen et al. (2018e) compared the metabolite profiles of mussel *Perna canaliculus* haemolymph exposed to *Vibrio* sp. and non-exposed mussel haemolymph, using a GC-MS-based metabolomics approach. The authors identified 22 metabolites that differed between the two groups with 10 up-regulated metabolites and 12 down-regulated in infected mussel haemolymph samples. Alterations of these metabolites were suggested to be involved in several perturbations of the host innate immune system following infection. For instance, altered glutathione metabolism and the transsulfuration pathway (cysteine and methionine metabolism) were shown after a decrease of free methionine content, and increased levels of glutamic and succinic acids, which were thought be related to oxidative stress. The elevated levels of succinic acid, fumaric acid and malic acid were suggested to be a consequence of the interruption of the tricarboxylic acid cycle. Accumulation of succinic acid, ITA and decreases of gamma-aminobutyric acid (GABA) may suggest inflammatory responses in infected mussels. Finally, decreases in several amino acids in infected mussel haemolymph suggested diverse disruptions of amino acid metabolism and protein biosynthesis.

Similarly, GC-MS-based metabolomics was employed to characterise the metabolic profile of mussel (*P. canaliculus*) haemolymph exposed to copper *in vitro* (Nguyen et al., 2018b). Results showed alterations of 25 metabolites within metabolite profiles of Cu^{2+}-exposed haemolymph (125 μM) compared to those of control samples. Changes in levels of these metabolites may be considered important signatures of oxidative stress (e.g., glutathione) and apoptosis (e.g., alanine, glutamic acid). Along with metabolomics, flow cytometric analyses showed significant increases in haemocyte mortality, production of reactive oxygen species (ROS) and apoptosis (*via* alteration of caspase 3/7 activation and mitochondrial membrane potential) of Cu^{2+}-exposed haemocytes which further

confirmed the oxidative stress and apoptosis induced by copper exposure at the cellular level.

In addition to GC-MS, NMR was employed to characterise the metabolic responses of mussel (*M. galloprovincialis*) haemolymph to copper and temperature challenges (Digilio et al., 2016). The results showed that the metabolic disturbances in mussel haemolymph due to elevated temperature was increased by the presence of copper. In fact, copper exposure at low temperatures (16 °C) led to metabolic changes in alanine, lysine, serine, glutamine, glycogen, glucose and protein aliphatics, which strongly contributed to the classification model. At high temperatures (24 °C), copper exposure caused a coherent increase of glucose, serine and lysine in mussel haemolymph.

Metabolomics is also a powerful tool for biomarker discovery. In fact, studies of the metabolomic profiles of bivalve haemolymph have revealed a number of candidate metabolite biomarkers involved in different biological processes (Alfaro et al., 2019, Nguyen et al., 2018b, Nguyen et al., 2018c). As an example, the accumulation of itaconic acid has been reported in haemolymph and others tissues of *P. canaliculus* mussels following pathogen exposure to *Vibrio* sp., suggesting the antimicrobial role of this metabolite in the bivalve innate immune system (Nguyen et al., 2018a, Nguyen et al., 2018c, Nguyen et al., 2018d). Subsequently, itaconic acid concentrations were quantitively measured in haemolymph and different tissues of mussels following *Vibrio* exposure (Nguyen and Alfaro, 2019). Interestingly, a challenge experiment of *Vibrio* sp., with itaconic acid (6 mM) led to the complete inhibition of bacterial growth (Nguyen et al., 2019b). These metabolomics studies indicate that marine bivalves are able to produce itaconic acid to inhibit *Vibrio* bacteria upon infection, and itaconic acid could be use as metabolite biomarker for bacterial infection and health status of marine bivalves.

Overall, these studies demonstrate that haemolymph serum is suitable for metabolomics studies and could be used to detect detailed and rapid changes in the physiological status of bivalves. These original investigations of haemolymph metabolomics also open the doors for using haemolymph metabolites as biomarkers for early diagnosis of pathogen infection and pollutant-induced stress syndrome in marine bivalves.

2.5.3 Challenges of metabolomics applications in aquaculture

There is no doubt that metabolomics will play an increasing and more important role in all aspects of aquatic research. However, applications of metabolomics in aquaculture are facing several challenges. Although several different platforms are being applied in aquaculture, each technique has some specific limitations (Young and Alfaro, 2016). Since metabolomics is a relatively new omics, metabolite databases are not completely available. Hence, unknown metabolites are sometimes encountered, and their analysis is limited, which represents an obstacle for biological interpretation. However, as larger databases are constructed for a range of marine organisms, this problem should be overcome in the near future. For targeted metabolomics, which is a quantitative approach of metabolomics, there are a number of challenges related to identification of unknown metabolites, especially small molecules, validation of biomarkers and inter-laboratory reproducibility (Nagana Gowda and Raftery, 2013, Roberts et al., 2012). Like proteomics, the limited databases for genomics and transcriptomics makes it difficult for integrated omics to link gene expression with altered proteins and physiological states of the host under a variety of perturbations. Metabolomics is relatively cheap compared to other omics approach (e.g., transcriptomics, proteomics), which may be around 30-40 USD per sample. However, a large-scale metabolomics study is an expensive effort, especially if high quality instruments are required.

2.6 CONCLUSIONS AND FUTURE PERSPECTIVES

The growing fields of omics and their applications to bivalve haemocyte studies have significantly improved our understanding of the functions of haemocytes in response to pathogen infections and environmental stress. These studies also demonstrated that bivalve haemocytes are a good model system for omics studies of innate immune responses. However, future development of omics applications for bivalves and bivalve haemocytes face several challenges, especially for emerging omics technologies which lack extensive libraries and databases (e.g., genomics databases across a range of species) and/or require complex data processing and interpretation of results. Identification of unknown proteins and metabolites still presents a problem for data interpretation, as well as the relative high costs of some analyses and instrument accessibility.

Despite these challenges, there is no doubt that omics applications will continue to expand in bivalve haemocytes, as well as other tissues. Future investigations of omics in bivalve haemocytes should also consider the following directions. Firstly, the integration of different omics approaches can greatly expand our knowledge of bivalve haemocytes regarding identification, characterisation and functional aspects across biological processes. However, such deep integrated approach is still at the development stage and will require complex methodologies that involve large-scale data sets and data structures from different omics platforms (Nguyen et al., 2018e). In addition, the applications of other omics (e.g., metagenomes, epigenomes) are still unexplored for bivalve haemocytes, which presents opportunities for future investigations to enhance our knowledge of haemocyte–pathogen interactions in bivalves. As downstream tools, omics could be combined with upstream phenotyping tools, such as flow cytometry to characterize the omics profiles of different cell subpopulations. The novel knowledge generated from emerging approaches will no doubt improve our understanding of bivalve

physiology, especially with regards to immune defences to pathogens and environmental stresses, and is likely to provide a much needed highly informative tool for managers within aquaculture settings in the near future.

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Chapter 3

Applications of flow cytometry in mollusc immunology: current status and trends

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ABSTRACT

Flow cytometry (FCM) is routinely used in fundamental and applied research, clinical practice, and clinical trials. In the last three decades, this technique has also become a routine tool used in immunological studies of molluscs to analyse physical and chemical characteristics of haemocytes. Here, we briefly review the current implementation of FCM in the field of molluscan immunology. These applications cover a diverse range of practices from straightforward total cell counts and cell viability to characterization of cell subpopulations, and further extend to analyses of DNA content, phagocytosis, oxidative stress and apoptosis. The challenges and prospects of FCM applications in immunological studies of molluscs are also discussed.

3.1 INTRODUCTION

FCM is an extremely powerful method for cell analysis that is fast, accurate, simple to use and can achieve simultaneous measurement of multiple cellular parameters. Modern flow cytometers have been around for over 70 years since the first impedance-based flow cytometry device with an electronic cell volume calculator patented in 1953 (Coulter, 1956). Recent advances in instrumentation, software and fluorochrome chemistry have led to the emergence of FCM applications in a number of fields, including haematology (Brown and Wittwer, 2000), food industry (Comas-Riu and Rius, 2009), virology (Zamora and Aguilar, 2018), pathology (Quirke and Dyson, 1986), plant biology (Doležel et al., 2007), marine biology (Darevsky et al., 1997), molecular biology (Boeck, 2001) and immunology (Cordier, 1986, Fleisher and Oliveira, 2019).

In immunological studies, FCM is a standard laboratory tool used for both fundamental and applied research, especially for clinal studies (Cordier, 1986, Cossarizza et al., 2017, Fleisher and Oliveira, 2019). Immunologists use FCM to enumerate specific cell subpopulations and measure a diverse number of cytometric parameters, such as membrane surface and intracellular characteristics, cell death, phagocytosis, autophagy, mRNA, transcription factors, signal transduction pathways, lymphocyte metabolism (Cossarizza et al., 2017, Fleisher and Oliveira, 2019). In molluscan immunological research, FCM has been applied to investigate haemocytes since the 1990s, but mostly in bivalve species (Ashton-Alcox and Ford, 1998, Brousseau et al., 1999, Fisher and Ford, 1988, Ford et al., 1994, Friedl et al., 1988). The applications of FCM for gastropods mainly emerged in the 2000s (Russo and Madec, 2007, Travers et al., 2008). In these early applications, FCM was primarily used for identification of cell populations and viability (Ashton-Alcox and Ford, 1998, Ford et al., 1994, Friedl et al., 1988). Currently, the use of FCM has been expanded to multiple parameters of molluscan immunology,

including cell count and viability, cell types, phagocytosis, oxidative stress, apoptosis, DNA and protein content. However, the number of FCM parameters used in immunological studies of molluscan species are limited compared to those used in vertebrate immunology.

This contribution highlights the emerging applications of FCM in immunological studies of molluscs for morphological and functional analyses of haemocytes. In addition, we highlight the challenges of using FCM and perspectives for the future development and application of this tool in molluscan immunology.

3.2 OVERVIEW OF FLOW CYTOMETRY

FCM is a laser-based technique that is used to analyse the physical and chemical characteristics of cells or particles in a heterogeneous fluid mixture as they pass through a light source. In principle, cell components are stained with fluorescently labelled dyes, so that the quantity of a particular cell component is calculated based on the fluorescence intensity. When the suspensions of cells are injected into the flow cytometer, cell components are excited by the laser, which emits light in a band of wavelengths. Hence, the fluorescence intensity is measured for each particular cell at the rate of thousands of particles per second (Fig. 3.1). This allows rapid and quantitative analysis of cells in a heterogeneous fluid mixture.

FCM analyses are conducted with different flow cytometric instruments that normally consist of four core components: an illumination source, a fluidic system, an optical bench, electronics and a computer control system (Jaroszeski and Radcliff, 1999, Shapiro, 2003). In brief, the fluidic system transports stained cells from a suspension into the laser intercept (light beam) in a single file for laser interrogation by one or more light sources. These light sources generate light signals at a specific frequency which are collected, filtered and directed by the optical system to photodetectors. The photodetectors, in turn, measure scatter light signals and convert them into electronic signals which are converted by the electronics system to data for storage, visualization and subsequent analysis by software (Jaroszeski and Radcliff, 1999) (Fig. 3.1). There are two measurements of the light scatter by two optical detectors, including forward scatter (FSC) and side scatter (SSC). FSC scatters along the path of the laser which allows identification of cells by size. The measurement of SSC is at a 90°C angle relative to the laser which is helpful for identification of the internal complexity (e.g., granularity) of a cell. The combination of FSC and SSC allows physical sorting a heterogeneous cell mixture into different

populations in specialized flow cytometers with sorting capabilities (Shapiro, 2005). Another type of light used in FCM for cell sorting is fluorescent light. It is emitted by fluorophores that have been stained with a specific structure on the cell.

In FCM, cells need to be labelled with fluorescent reagents which include a wide range of commercially available dyes, stains, monoclonal antibodies (mAbs) and quantum dots (QDs). A fluorochrome (fluorophore or simply flours) is a fluorescent chemical compound that absorbs and re-emits light of different wavelengths upon excitation. Fluorophores are typically directly coupled to antibodies to create labelled antibody regents (fluorophore-conjugated antibodies). There are a number of commonly available fluorochromes in the market, such as fluorescein isothiocyanate (FITC), peridin chlorophyll protein (PerCP), allophycocyanin (APC) and phycoerythrin (PE) (Fleisher and Oliveira, 2019). There are other specific dyes for cell functional studies, such as DNA dyes (e.g., ethidium bromide [EtBr], propidium iodide [PI]), glutathione-sensitive dyes (e.g., 7-amino-4-chloromethylcoumarin, monochlorobimane), calcium-sensitive dyes (e.g., fluo-4, fura red) and hydrogen peroxide (H_2O_2)–responsive dyes (e.g., dihydrorhodamine 123, 2',7'-dichlorofluorescein) (Darzynkiewicz et al., 2010, Fleisher and Oliveira, 2019, Rabinovitch et al., 1993, Vowells et al., 1995). In addition, quantum dots (QDs), a new class of inorganic fluorochromes produced from semiconductor materials, is now emerging in polychromatic flow cytometry (Chattopadhyay et al., 2010).

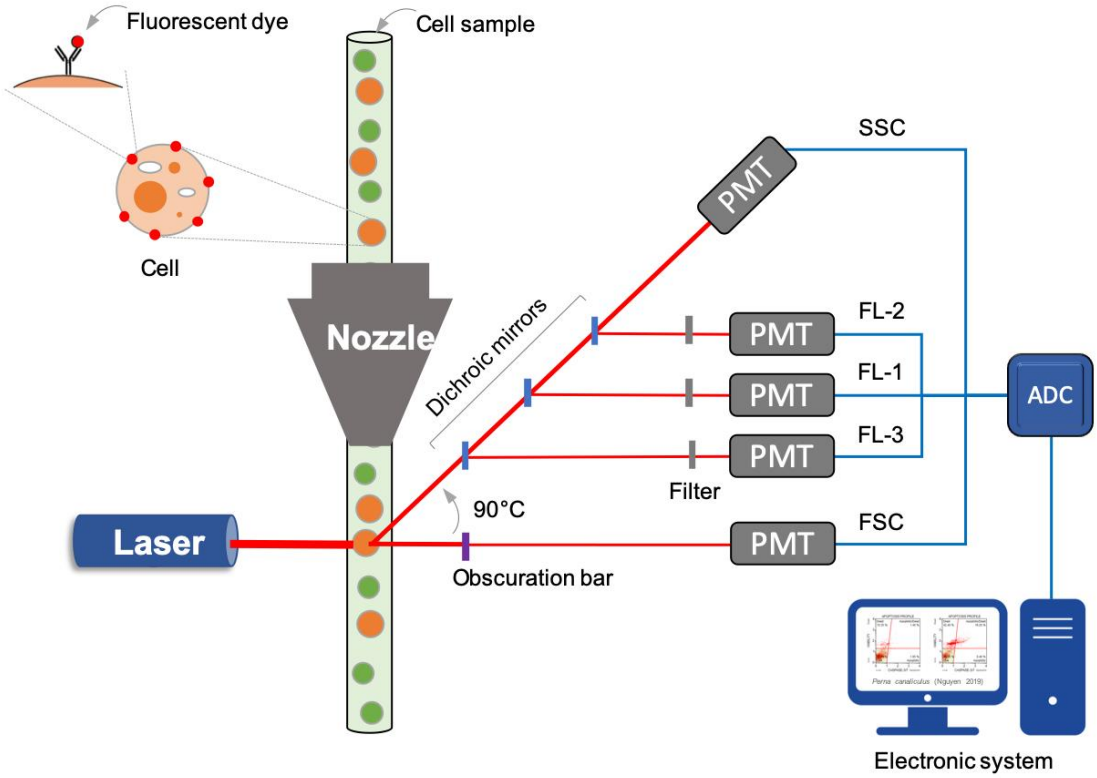

Figure 3.1 Schematic diagram of a flow cytometer. Abbreviations: *SSC, side scatter; FSC, forward scatter; PMT, photomultiplier; ADC, analog-to-digital converter; FL, fluorescence.*

3.3 CURRENT APPLICATIONS OF FCM IN IMMUNOLOGICAL STUDIES OF MOLLUSCS

3.3.1 Total haemocyte counting

As haemocytes are chief immuno-effector cells that play a critical role in the innate immune system of molluscs, alternations of total haemocyte counts (THC) in haemolymph are an important immunological parameter to assess the health state of the host. In fact, changes in circulating haemocytes represent a common response of molluscs to infections or diseases (Allam et al., 2000, Jones et al., 1996, Mateo et al., 2009, Nguyen et al., 2018a) and environmental stresses (Couch, 1985, Hauton et al., 2000, Wedderburn et al., 2000). Traditionally, cell counting was carried out by microscopic methods (Ford et al., 1994, Fournier et al., 2001, Strober, 2001) which are time-consuming and relatively less accurate. In this regard, FCM provides a fast, easy, convenient and affordable alternative for counting mollusc haemocytes. In principle, various DNA-binding dyes (e.g., SYTO 9, SYTO 13 and SYBR Green) are used to stain all cells with a nucleus (nucleated cells) which can be discriminated with debris and non-nucleated cells.

FCM has been intensively used to quantify circulating haemocytes of molluscs under normal conditions (Donaghy et al., 2010, Donaghy et al., 2009) and in response to environmental stress, such as bacterial infections (Ashton-Alcox and Ford, 1998, Nguyen et al., 2018a, Nguyen et al., 2018b, Parisi et al., 2008) and temperature variations (Chen et al., 2007). For example, THC variations of *Mytilus galloprovincialis* mussels have been reported when injected with living or heat-killed *Vibrio anguillarum* and *Micrococcus lysodeikticus* (Ashton-Alcox and Ford, 1998). Nguyen et al. (2018a) observed a significant increase in THC in mussels at 6 h post-infection with *Vibrio* sp. (8.01×10^6 cfu·ml^{-1}) compared to controlled mussels (2.68×10^6 cfu·ml^{-1}).

THC is normally performed along with other parameters in multiparameter assays, such as viability (Nguyen et al., 2018b), ROS (Nguyen et al., 2018c) and apoptosis (Nguyen et al., 2018c). Some studies have used fixed haemocytes (Chen et al., 2007, Donaghy et al., 2010, Donaghy et al., 2009) and others have used fresh cells (Alfaro et al., 2019, Grandiosa et al., 2018, Nguyen et al., 2019).

3.3.2 Cell types

Different types of mollusc haemocytes have different functions (Gosling, 2015). Hence, identification of cell subpopulations is important to understand the immune function of each cell type. In specialized flow cytometers with sorting capabilities, cell populations can be separated into subpopulations typically based on size, morphology and expression of surface proteins (Cossarizza et al., 2017). In immunological studies of molluscs, identification of haemocyte subtypes have been mostly based on relative flow-cytometric morphological parameters: FSC and SSC. These studies have led to the characterization of different haemocyte subpopulations of different molluscan species such as disk abalones (*Haliotis discus discus)*, spiny top shells (*Turbo cornutus*) (Donaghy et al., 2010), Eastern oysters (*Crassostrea virginica*) (Goedken and De Guise, 2004), Mediterranean mussels (*M. galloprovincialis*) (Parisi et al., 2008) and European flat oysters (*Ostrea edulis*) (Xue et al., 2001). For example, there are two types of haemocytes (blast-like cells and hyalinocytes) in *H. discus discus* and four main haemocyte types (blast-like cells, type I and II hyalinocytes and granulocytes) in *T. cornutus* (Donaghy et al., 2010). Interestingly, differences in immune-related activities were observed among the cell types (Donaghy et al., 2010). *C. virginica* haemocytes were grouped into three morphologically different sub-populations, including hyalinocytes, granulocytes and intermediate cells (Goedken and De Guise, 2004). Similarly, haemocytes of *O. edulis* (Xue et al., 2001) and *M. galloprovincialis* (Parisi et al., 2008) were classified into three

subclasses, including small hyalinocytes, large hyalinocytes and granulocytes. Characterization of molluscan haemocyte subpopulation is often combined with other multiple parameter assays to determine differences between cell types in immune-related activities, such as phagocytosis (Aladaileh et al., 2007, Donaghy et al., 2009), oxidative stress (Labreuche et al., 2006a, Labreuche et al., 2006b, Wang et al., 2012) and apoptosis (Xue et al., 2001).

3.3.3 Viability

A cell viability assay is the quantification of the amount of live or death cells in a population. The cell viability or cell death is an important parameter for health assessment of the host. In addition, it is often necessary to detect and exclude dead cells which could unwantedly uptake of fluorescent probes, resulting in artefacts. The Trypan blue staining technique is a routine method of viability determination (Cossarizza et al., 2017, Strober, 2001). In principle, live cells have intact cell membranes that exclude certain dyes (e.g., trypan blue, eosin, or propidium) whereas dead cells with damaged and permeable membranes do not. Stained cells are visually exampled with a hemocytometer under a conventional microscope to determine whether cells take up (blue cytoplasm) or exclude dyes (clear cytoplasm) (Strober, 2001). However, this technique is time consuming and has many other limitations (Strober, 2001). Alternatively, FCM is a rapid and reliable method which allows the analysis of not only cell viability, but a variety of parameters at the same time. In FCM, cells are stained with intercalating dyes that bind to DNA, such as PI, EtBr, 7-aminoactinomycin D (7-AAD). Hence, non-viable cells (dead cells) are characterized with high fluorescence whereas viable cells are non-fluorescent (Cossarizza et al., 2017).

The viability is often assessed via viability assays which have been extensively applied to measure the viability of molluscan haemocytes in response to different stress conditions, such as pathogen infections or diseases (Nguyen et al., 2018b), water contaminants (Nguyen et al., 2018c), thermal stress (Hégaret et al., 2003) or other stress conditions (Alfaro et al., 2019, Ashton-Alcox and Ford, 1998, Nguyen et al., 2019). The assay provides a quantitative number/percentage of live and dead cells in the population (Fig. 3.2). For example, decreases of haemocyte viability were reported in *P. canaliculus* exposed to *Vibrio* sp. (Nguyen et al., 2018b) and copper (Nguyen et al., 2018c). Similarly, significantly slower percentages of live haemocytes were found in *C. virginica* oysters after the temperature increase (Hégaret et al., 2003).

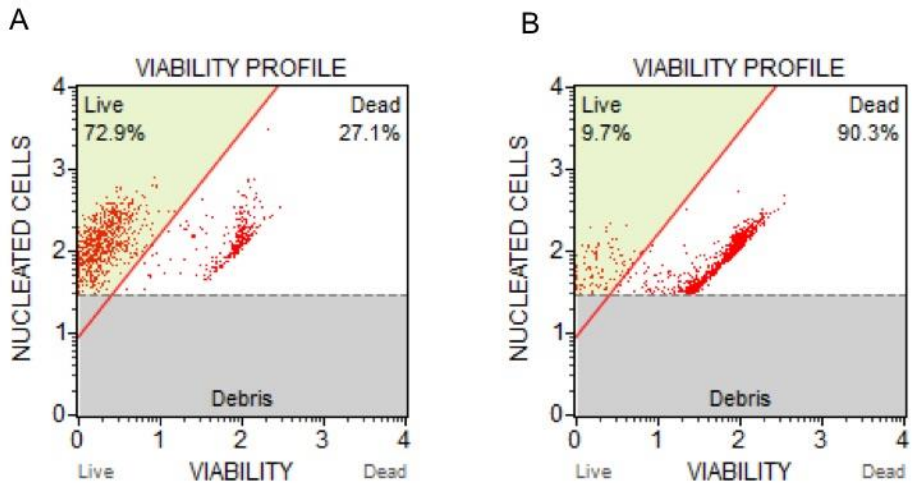

Figure 3.2 Viability profile of *Perna canaliculus* haemocytes under normal condition (control) (A) and *Vibrio* sp. infection (B). Viability of haemocytes was accessed by viability assay using a Muse™ Cell Analyzer. The red lines were used to separate live cells (left) and dead cells (right).

The viability determination of mollusc cells is often combined with other parameters in multiple cellular parameter assays, using fluorescent dyes with very different absorptions and wavelengths. For instance, live/dead cell distinction with 7-AAD was used in combination with Annexin V apoptosis measurements to determine percentage of late apoptosis in *P. canaliculus* haemocytes *in vivo* challenged with *Vibrio* sp. (Nguyen et al.,

2018d) or *Haliotis iris* haemocytes under different probiotic-supplemented diets (Grandiosa et al., 2018). Similarly, 7-AAD was combined with dyes for caspase-3/7 and mitochondrial potential to identify early and late apoptosis in *P. canaliculus* haemocytes *in vitro* exposed to copper (Nguyen et al., 2018c).

Alternatively, cell viability can be accessed via light-scattering measurement without any staining. Dead cells or cells with damaged and permeable membranes possess a reduction of FSC signals and an increase in SSC signals. Hence, the combination of FSC and SSC parameters could be used to distinguish live cells and dead cells (Cossarizza et al., 2017).

3.3.4 Phagocytosis

The internal defences of molluscs rely only on the innate immune system, and phagocytosis is the most important line of defense in this system (Naik and Harrison, 2013). Phagocytosis is the engulfment of foreign particles (bacteria, algae, cellular debris and protozoan parasites) by phagocytes. Granulocytes are phagocytic cells, and they play a prominent role in phagocytosis of molluscs while agranulocytes are less important for phagocytosis (Aladaileh et al., 2007, Gosling, 2015).

FCM provides unique integration of functional and phenotypic information for phagocytosis studies (Cossarizza et al., 2017). FCM has been used to identify the phagocytotic activity in haemocytes of molluscs under different stress conditions, such as salinity (Gagnaire et al., 2006a), temperature (Gagnaire et al., 2006a), heavy metals (Brousseau et al., 1999) and different contaminants (Ladhar-Chaabouni and Hamza-Chaffai, 2016). In phagocytosis assays, molluscan haemocytes are often incubated with fluorescent target particles, such as fluorescent beads (Brousseau et al., 1999, Donaghy et al., 2010, Donaghy et al., 2009, Xue et al., 2001) or zymozan particles (Lambert et al., 2003, Travers et al., 2008). Phagocytosis is then measured as phagocytosis index which

is determined by the percentage of phagocytic cells that had ingested beads. However, determination of phagocytic cells is different from study to study. Some authors defined phagocytic cells with at least one fluorescent bead (Brousseau et al., 1999, Donaghy et al., 2010), while others used at least two (Araya et al., 2009) or three fluorescent beads (Delaporte et al., 2003, Hégaret et al., 2003). This makes it difficult to compare phagocytic activity levels across studies. Along with the phagocytosis index, Donaghy et al. (2010) also used the mean number of beads per phagocytic haemocytes to identify phagocytic activities in haemocytes from *H. discus discus* and *T. cornutus* stimulated by latex beads. Although the phagocytosis index was similar between these two species, the mean number of engulfed beads was slower in *T. cornutus* than in *H. discus discus*.

Many authors compared phagocytotic activity between different cell types (Aladaileh et al., 2007, Donaghy et al., 2009). For example, Donaghy et al. (2009) used FCM to identify different subpopulations of *Crassostrea ariakensis* haemocytes and phagocytic activities of each cell type. The results showed that granulocytes were the main phagocytic cells, while hyalinocytes also showed a certain level of phagocytosis, and no phagocytic activity was observed in the blast-like cells. Similarly, Aladaileh et al. (2007) observed the ingestion of yeast cells in both granulocytes and hyalinocytes of Sydney rock oysters (*Saccostrea glomerata*), but granulocytes were more efficient phagocytes than hyalinocytes.

Upon phagocytosis of pathogen, phagocytic cells release ROS which triggers apoptosis in molluscan haemocytes (Terahara and Takahashi, 2008, Torreilles et al., 1996). On the other hand, phagocytosis of apoptotic cells helps to clean of unwanted cells or cell components that might trigger inflammatory response (Savill, 1997). Hence, assessment of phagocytosis via FCM is often combined with simultaneous measurement of other

functional parameters, typically oxidative stress (Donaghy et al., 2010, Donaghy et al., 2009, Goedken and DeGuise, 2002, Hégaret et al., 2003, Noël et al., 1993) or apoptosis (Goedken and DeGuise, 2002, Höher et al., 2012, Liu et al., 2016).

3.3.5 Oxidative stress

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) are naturally produced as by-products of normal metabolism of oxygen and in responses to endogenous and exogenous stimuli (Sies et al., 2017). ROS generation plays an important role in different biological processes, cell signalling, homeostasis and protective mechanisms (Jabs 1999; Torreilles et al. 1996). However, excess ROS production during endogenous and exogenous stimuli leads to an imbalance between ROS and antioxidants in favour of ROS which is called oxidative stress (Sies et al., 2017). The excessive ROS production can cause oxidative damage to cells and tissues via degradation of DNA, proteins and lipids (Jabs 1999; Torreilles et al. 1996).

Originally, ROS production of molluscs was measured using reduction of nitroblue tetrazolium (NBT) assays (Anderson et al., 1992b, Gómez-Mendikute and Cajaraville, 2003, Pipe, 1992) and luminol-dependent chemiluminescence (Anderson, 1994, López et al., 1994). With its advantages, FCM has progressively replaced these assays for identifying ROS production. FCM assays use different dyes based on auto-oxidation, photochemical reactions, mitochondrial respiration, and various enzymes (e.g., cytochrome P450, NADPH oxidase) (Cossarizza et al., 2017). Most of these dyes are photostable fluorogenic probes which are permeable DNA-specific dyes (Cossarizza et al., 2017). 2',7'-dichlorofluorescein (H2DCF) and dihydroethidium (DHE), have been used extensively to measure hydrogen peroxide (H_2O_2) and superoxide anion (O2-),

respectively, in mollusc haemocytes. DCFH-DA is a nonfluorescent fluorescein analogue which is a membrane permeable probe. The method used in molluscs studies was adapted from Bass et al. (1983). When added into haemocytes, DCFH-DA diffuses into the cell membrane where it is hydrolysed to 2′,7′-dichlorofluorescein (DCFH) by esterase enzymes. DCFH is then oxidized to DCF molecule by ROS (mostly of hydrogen peroxide and related superoxide species). DCF can be detected on the FL1 detector of the flow cytometer which results in green fluorescence, indicating haemocytes with oxidative activity.

DCFH-DA has been successfully used to determine the levels of H_2O_2 in mollusc haemocytes under normal conditions (Wang et al., 2012) or in response to pesticides (Patetsini et al., 2013), zymosan (Buggé et al., 2007, Castellanos-Martínez et al., 2014), heavy metals (Mottin et al., 2010), temperature (Donaghy and Volety, 2011), pathogens (Labreuche et al., 2006a, Lambert et al., 2003), interactive effects of metals and pathogenic organisms (Paul-Pont et al., 2010) and other physiological conditions (Park et al., 2012). In addition, the DCF green fluorescence levels were used to evaluate ROS production of haemocyte subpopulations distinguished according to their relative size (FSC) and complexity (SSC) (Labreuche et al., 2006a, Labreuche et al., 2006b, Wang et al., 2012). For instant, during the infection with *Vibrio aestuarianus* strain on *Crassostrea gigas*, Labreuche et al. (2006a) found a strong enhancement of ROS production in hyalinocytes and granulocytes which was higher in granulocytes than in hyalinocytes. In contrast, very low ROS production was observed in small agranulocytes. Similarly, Wang et al. (2012) found higher ROS production in granulocytes than in hyalinocytes of *Perna viridis* mussels.

Another common dye used in detection of superoxide production in mollusc studies is dihydroethidium (DHE). DHE is a cell permeable dye which freely permeate cell membranes and react with superoxide anions to form 2-hydroxyethidium which intercalates with DNA resulting in red fluorescence (Carter et al., 1994, Rothe and Valet, 1990, Zhao et al., 2005). This dye has been used to measure ROS production in mollusc haemocytes under different external stimuli, such as pathogen infections (Nguyen et al., 2018b, Nguyen et al., 2018d), LPS (Nguyen et al., 2019), heavy metal (Koutsogiannaki et al., 2014, Nguyen et al., 2018c), pesticide (Patetsini et al., 2013) and storage conditions (Alfaro et al., 2019). As an example, Nguyen et al. (2018b) used Muse® Oxidative Stress kit (EMD Millipore) based on DHE staining to measure ROS production in *Perna canaliculus* haemocytes challenged with *Vibrio* sp. This assay requires no wash and takes 30 min to incubate 20 µl of haemocytes (1×10^6 cell·ml^{-1}) in 180 µl of working solution containing DHE dye at 37 °C. The measurement was performed via Muse® Cell Analyzer which normally takes less than 1 minute per sample, depending on cell concentration and desired number of events to acquire. The assays provide total cell count and percentage of cells produced ROS in the population (Fig. 3.3).

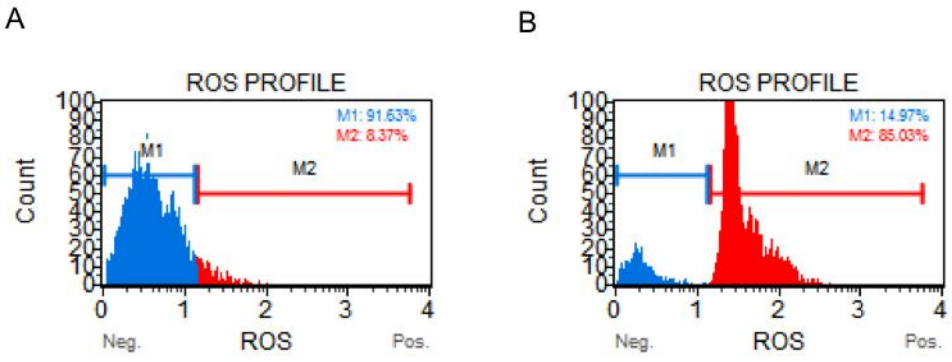

Figure 3.3 ROS profile of *Perna canaliculus* haemocytes under normal condition (control) (A) and *Vibrio* sp. infection (B). ROS percentage was determined by oxidative stress assay with DHE dye using a Muse® Cell Analyzer. M1 indicates cell population without ROS (ROS–) while M2 is cell population with ROS (ROS+).

3.3.6 Apoptosis

Apoptosis is a form of programmed cell death that occurs in multicellular organisms during various important cellular processes, ranging from embryonic development to host defense against pathogen infections (Elmore, 2007). Apoptosis is generally characterized by distinct morphological and biochemically characteristics (Elmore, 2007) which are regulated via two major pathways: intrinsic and extrinsic pathways (Estevez-Calvar et al., 2013, Kiss, 2010, Sokolova, 2009, Terahara and Takahashi, 2008, Zhang et al., 2011). The intrinsic pathway (mitochondrial-mediated apoptotic pathway) is stimulated by various types of intracellular stressors, while the extrinsic pathway (death receptor-mediated apoptotic pathway) is activated by external stimuli. Apoptosis is a conserved mechanism across taxa and components of apoptotic pathways in molluscs seem to be similar to those of vertebrates with some unique features (Romero et al., 2011, Romero et al., 2015). Apoptosis is an important internal defence mechanisms in molluscs in response to environmental changes, pollutants and pathogens (Romero et al., 2015).

FCM has become the most widely used method of choice for multiparametric analysis of apoptosis (Telford et al., 2011). Many distinct characteristics of an apoptotic cell can be measured using FCM, including plasma membrane changes, changes in mitochondrial transmembrane potential, caspase activation and DNA cleavage (Allen and Davies, 2007, Fleisher and Oliveira, 2019). In immunological studies of molluscs, annexin V, mitochondrial transmembrane potential ($\Delta\psi m$), capsase activation are common biomarkers used in characterization of apoptosis.

Annexin V

Annexin V is a cellular protein in the annexin group that has the ability to bind to phosphatidylserine (PS) of cells. Translocation of PS from the cytosolic side of the intact

plasma membrane to the extra-cellular surface is a hallmark of early apoptosis (Telford et al., 2011). The Annexin V assays utilize annexin V as a useful marker for detection of PS on the external membrane of apoptotic cells during early apoptosis (Van Engeland et al., 1998). Annexin V staining must always be used in conjunction with a cell dead marker (e.g., PI, 7-AAD) as an indicator of the integrity of the cell membrane to rule out "leaky" necrotic cells as well as distinguish between live, dead and apoptotic cells (Cossarizza et al., 2017, Telford et al., 2011). Annexin V assays have been successfully used to characterize early apoptotic haemocytes of molluscs under different experiments, such as responses of *P. canaliculus* to *Vibrio* sp. infection (Nguyen et al., 2018d), *Haliotis iris* to multi-strain probiotics (Grandiosa et al., 2018), and cadmium-induced apoptosis in oyster haemocytes (Sokolova et al., 2004). As an example, Nguyen et al. (2018d) used a Muse® Annexin V and Dead Cell assay with annexin V for apoptosis and 7-AAD for dead cells

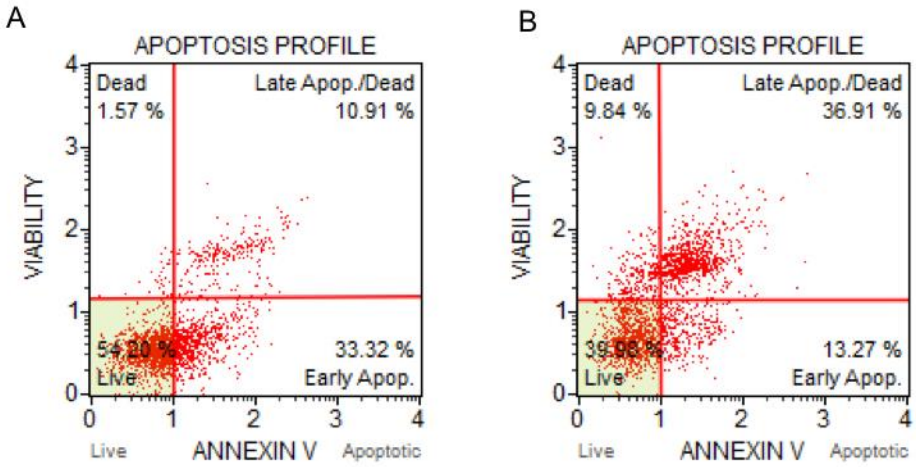

Figure 3.4 Apoptosis profile of *Perna canaliculus* haemocytes under normal conditions (A) and *Vibrio* sp. infection (B). Detection of apoptosis by concurrent staining with Annexin V and 7-AAD. Bivariate analysis of Annexin V/7-AAD staining distinguishes four populations of cells, including non-apoptotic cells (Annexin V^-, 7-AAD^-), early-stage apoptotic cells (Annexin V^+, 7-AAD^-), late-stage apoptotic cells (Annexin V^+, 7-AAD^+) and dead cells (Annexin V^-, 7-AAD^+) (Nguyen et al., 2018d).

to characterize the apoptosis in *P. canaliculus* following *Vibrio* sp. infection. They observed the increase of apoptotic cells and dead cells along with the decrease of live cells upon *Vibrio* sp. infection (Fig. 3.4).

Mitochondrial transmembrane potential

During the early apoptotic process, cells decrease mitochondrial transmembrane potential (Δψm) before rupture of the plasma membrane (Green and Reed, 1998, Ly et al., 2003). Thus, Δψm loss is another reliable indicator of apoptosis. Assessment of Δψm can be performed using fluorescent dyes, such as JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide), 3,3' dihexyloxacarbocyanine iodide (DIOC6), JC-10, tetramethylrhodamine methyl ester perchlorate (TMRM) (Fleisher and Oliveira, 2019). This method is based on the fact that these dyes are cationic probes that accumulate in healthy mitochondria resulting in high fluorescence. In cells with depolarized mitochondria, the dye is released, thereby resulting in a decrease in fluorescence and a downward shift. These dyes are often used with PI or 7-AAD to distinguish between apoptotic and nonapoptotic cells.

Determination of Δψm has become a method of choice for characterization of apoptosis in molluscs (Donaghy et al., 2012, Gervais et al., 2015, Nguyen et al., 2018c, Xue et al., 2001). JC-10 is a common dye which has been used to measure the MMP in *O. edulis* haemocytes exposed to UV (Gervais et al., 2015) and the parasite *Bonamia ostreae* (Gervais et al., 2016), and *Crassostrea gigas* haemocytes through the use of chemical inhibitors (Donaghy et al., 2012). Others used tetramethylrhodamine, ethyl ester perchlorate (TMRE) for monitoring changes in MMP in *M. galloprovincialis* haemocytes exposed to commercial nanosized carbon black (NCB) (Canesi et al., 2008) and nanoparticles (Canesi et al., 2015, Ciacci et al., 2012). Nguyen et al. (2018c) used a

commercial MitoPotential dye and 7-AAD to measure the Δψm and cell death in *P. canaliculus* haemocytes exposed to increasing concentrations of copper (Fig. 3.5). Furthermore, the use of DIOC6 dye for measurement of Δψm combined with morphometric parameters (FSC and SSC) were applied to compare the Δψm in different sub-populations of *O. edulis* haemocytes (Xue et al., 2001).

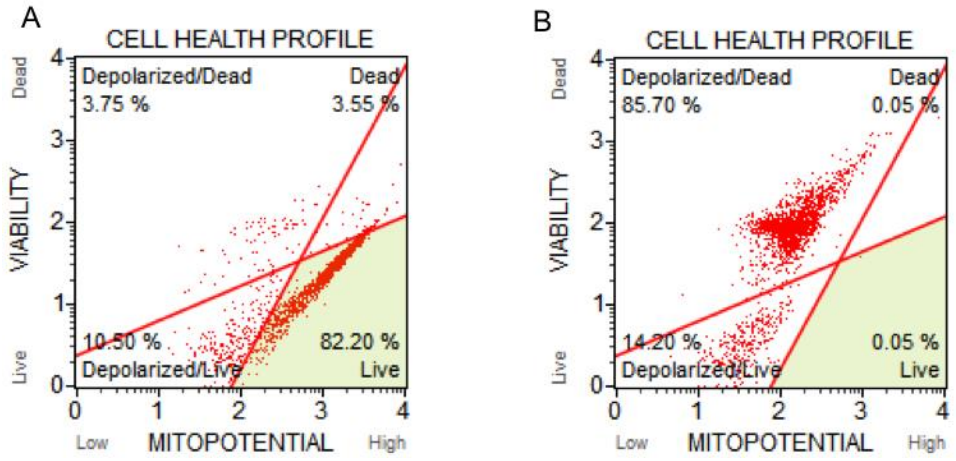

Figure 3.5 Apoptosis profile of *Perna canaliculus* haemocytes under normal conditions (A) and copper exposure (B). Detection of apoptosis was conducted by MitoPotential assay which provides four distinguishable cell populations, including live cells with depolarized mitochondrial membranes, live cells with intact mitochondrial membranes, dead cells with depolarized mitochondrial membranes and dead cells with intact mitochondrial membranes (Nguyen et al., 2018c).

Caspase activation

Caspase activation is another hallmark of apoptosis (Allen and Davies, 2007). Caspases (cysteinyl-directed aspartate-specific proteases) constitute a family of protease enzymes which are the key molecular components of both intrinsic and extrinsic pathways (Cohen, 1997, Fan et al., 2005). Functionally, caspases are categorized into three major types, including initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase- 3,-6,-7) and inflammatory caspases (caspase-1,-4,-5,-11,-12,-13) (Galluzzi et al., 2016, Riedl and Shi, 2004). Several caspases have been identified in different mollusc species, including

caspase-2 (Romero et al., 2011, Zhang et al., 2011, Zhang et al., 2014), caspase-8 (Huang et al., 2010, Romero et al., 2011, Xiang et al., 2013) and caspase-3/7 (Motta et al., 2013, Romero et al., 2011).

A number of assays have been developed for detection of apoptosis based on the pivotal and early involvement of caspases in cell death events (Cossarizza et al., 2017, Kaufmann et al., 2008). Activation of caspases can be identified by different techniques, such as immunoblotting, flow cytometry and microscopic techniques (Kaufmann et al., 2008). In FCM, cell populations are stained with the intracellular mAbs or small inhibitor peptides conjugated to a fluorophore which are assigned as affinity ligands to active site of relevant caspases (Cossarizza et al., 2017). Hence, antibodies/inhibitors-caspase complexes are detected based on the presence of the fluorescent tag (FITC or SR) inside viable cells (Pozarowski et al., 2003, Smolewski et al., 2002). Caspase dyes are often used together with dead cell stains (e.g., 7-AAD, PI) to distinguish between apoptotic, live and necrotic cells.

Caspase-3 is a frequently activated death protease in apoptotic cells of both vertebrates (Porter and Jänicke, 1999) and invertebrates (Guo et al., 2017, Lacoste et al., 2002, Motta et al., 2013, Sokolova et al., 2004). Using the FCM method, increases of caspase-3 activation have been reported in *P. canaliculus* haemocytes exposed to copper (Nguyen et al., 2018c) or LPS (Nguyen et al., 2019), and *Mytilus edulis* haemocytes exposed to copper (Höher et al., 2013). As an example, Nguyen et al. (2018c) used the Muse® Caspase-3/7 kit to quantitative measurements of apoptotic status in *P. canaliculus* haemocytes *in vitro* exposed to copper. The kit utilizes a Muse® Caspase-3/7 reagent NucView® for the detection of caspase-3/7 activity along with 7-AAD as a dead cell marker. The assay provided relative percentages of four cell subpopulations, including

live cells, early apoptotic cells, late apoptotic cells and dead cells (Fig. 3.6). They observed significant increases of dead cells and apoptotic cells in copper-exposed haemocytes compared to the control haemocytes.

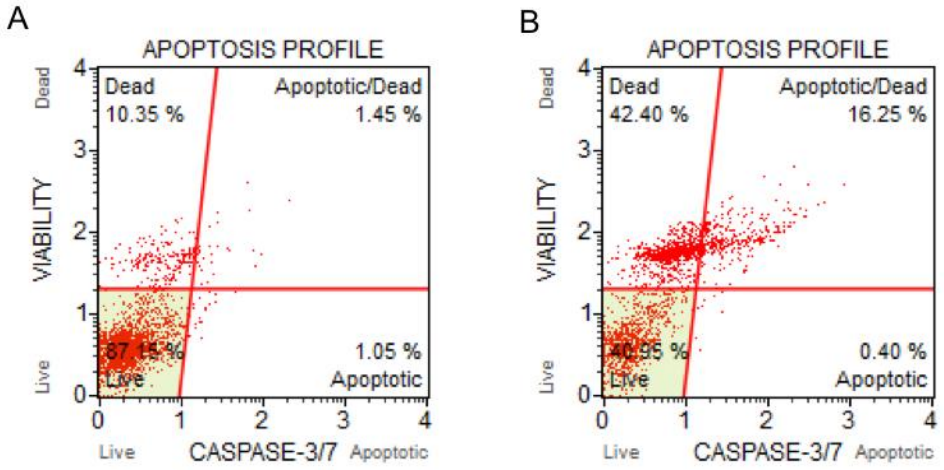

Figure 3.6 Apoptosis profile of *Perna canaliculus* haemocytes under normal conditions (A) and copper exposure (B). Apoptotic changes were detected using Muse™ Caspase-3/7 assay. The assay provides four distinguishable cell populations, including live cells, early apoptotic (live) cells, late apoptotic (dead) cells and dead cells.

3.3.7 DNA content and cell cycle characteristics of haemocytes

FCM is the most valuable technique of choice for cellular DNA content and cell cycle analysis (Benabdelmouna and Ledu, 2016). It allows for the characterization of cells in the major phases of the cell cycle (G0/G1, S and G2/M), determining frequency of apoptotic cells based on fractional DNA content, and additionally detecting DNA amounts (polyploid or aneuploid) of the cell population (Benabdelmouna and Ledu, 2016, Darzynkiewicz et al., 2017). Comparatively to other available traditional methods (e.g., histology and hemocytology), FCM is a rapid, accurate, non-subjective and cost-effective approach (Benabdelmouna and Ledu, 2016). The analysis of DNA content and cell cycle characteristics are based on the staining of DNA with fluorescent dyes (e.g. PI, 7-AAD, Hoechst stains, TO-PRO-3, SYTOX, acridine orange, pyronin Y) (Cossarizza et al., 2017).

FCM has been extensively used to characterize DNA content and cell cycle in various mollusc species (Benabdelmouna and Ledu, 2016, Da Silva et al., 2005, Delaporte et al., 2008, Grand et al., 2010, Moore et al., 1991, Reno et al., 1994, Siah et al., 2008). For example, FCM has been successful used to determine the DNA content and genomic characteristics of abnormal cells affected with hemic neoplasia in the haemolymph of many molluscan species, such as in the *Mytilus* mussels (Elston et al., 1990), the soft shell clams (*Mya arenaria*) (Delaporte et al., 2007, Reno et al., 1994) and in the common cockles (*Cerastoderma edule*) (Da Silva et al., 2005, Grand et al., 2010). These studies have successfully distinguished between negative and heavily affected bivalves using FCM. Recently, Benabdelmouna and Ledu (2016) used FCM to determine genomic abnormalities in haemocytes of blue mussels, *Mytilus* spp. associated with mortality outbreaks in from the Atlantic coast of France. Different thresholds of genomic abnormalities (GA %) based on the percentage of haemocytes in S-G2/M phase were set up at individual and populations levels. The results provide evidence of heavy genomic abnormalities and GA % was found to be significantly predictive of the final mortality. Together, these studies demonstrate that FCM is a powerful tool for accurate diagnosis of diseases in marine bivalves.

3.3.8 Protein analysis

FCM has been appears to be an important tool for future protein analysis which could be applied for routine assays to quantify the number of proteins expressed in a cell and on the cell surface (Hogg et al., 2015). This is based on the principle that intracellular proteins in the complex mixture can be specifically labelled using fluorophore-conjugated antibodies which can be quantify using FCM.

There are a few studies using FCM for protein analysis in mollusc species (Friedl et al., 1988, Rey-Campos et al., 2019). For example, Friedl et al. (1988) measured the total protein in haemocytes of the American oysters (*C. virginica*) by FCM. They observed a broad uniform distribution of proteins which was similar to that obtained for cell size. Rey-Campos et al. (2019) used FCM to measure myticin C, the most expressed antimicrobial peptides in mussels, in different haemocyte populations of *M. galloprovincialis* mussels infected with *Vibrio splendidus*, and observed the decrease in the number of myticin C positive haemocytes after infection.

3.4 CHALLENGES AND PERSPECTIVES

One of the big challenges of working with molluscan haemocytes is cell aggregation. To prevent haemocytes from clumping during bleeding, many different types of anti-aggregant solutions have been used, such as Alsever's solution, modified Alsever's solution, heparin sodium solution and modified Leibovitz L15 (Gagnaire et al., 2006b, Jiang et al., 2016, Wang et al., 2017, Zhou et al., 2017). However, the use of anticoagulant for molluscan haemocytes may inhibit haemocyte activities (Gagnaire et al., 2004). For example, the use of modified Alsever's solution was reported to decrease oxygen metabolite production in bivalves (Torreilles et al., 1999). The aggregation of molluscan haemocytes could be reduced or prevented by the maintenance of haemocytes on ice or low temperature, such as 4 °C (Anderson et al., 1992a, Auffret and Oubella, 1997). Hence, in our recent studies, we have simply used cold filtered artificial seawater to mix with haemolymph in order to prevent cell clotting (Alfaro et al., 2019, Nguyen et al., 2018b, Nguyen et al., 2018c, Nguyen et al., 2018d, Nguyen et al., 2019). This method requires a minimal sample manipulation (no lyse, no cell wash) to mimic physiological conditions.

In addition to anticoagulants, the use of staining dyes may be toxic for cells. It may cause a loss in cell viability and in certain situations even apoptosis or severe damage (Cossarizza et al., 2017). Hence, it is important to take into account the toxicity of the dyes for the haemocytes and the combination of different assays is sometimes necessary to identify the true value of the measured parameters.

Recently, there has been an emergence of metabolomics applications in molluscan immunity studies (Liu et al., 2014, Nguyen et al., 2018c, Nguyen et al., 2018e, Nguyen et al., 2019). Many authors also combined FCM and untargeted GC-MS metabolomics in

molluscan immunology (Nguyen et al., 2018b, Nguyen et al., 2018c, Nguyen et al., 2018d). For example, Nguyen et al. (2018c) employed FCM and GC-MS metabolomics to characterize effects of copper on haemocytes. Along with the increase of an oxidative stress biomarker, ROS, in copper-exposed haemocytes, they found the accumulation of glutathione and other metabolites in glutathione pathways, suggesting the role of glutathione as a metabolite maker of oxidative stress. Similarly, the increases of apoptosis hallmarks including caspase 3/7 activation and $\Delta\psi m$ loss were found to be linked with the increase in alanine and decrease of glutamic acid in the taurine metabolism, which plays a key role in apoptosis regulation (Nguyen et al., 2018c). Hence, these examples demonstrated that the combination of FCM and metabolomics could expand the number of cell and molecular markers to provide an extensive evaluation of functional markers. Such kind of integrated approach could be expanded to combinations between multiple FCM parameters and other omics (e.g., transcriptomics, proteomics) or integrated omics to create a detailed picture of immune responses within cells and generate accurate biomarker signatures of molluscan diseases.

Recent advances in technology have led to a comprehensive range of innovative flow cytometers. Many of these are small in size and simple in operation, and allow for routine sampling in the field. As an example, the Muse® Cell Analyzer has been extensively used in our lab for both field and laboratory measurement of many cell health parameters of molluscs. At the moment, only the cell count and viability assays appear to be suitable for field sampling because they are simple and fast and the dye used can just be stored at cool temperature (2-8 °C). Other assays, such as oxidative stress and apoptosis, require the dyes to be stored at –20 °C and they need long incubation times (30 minutes), which are not likely to be suitable for the field. Hence, future modifications of storage conditions of these dyes or greater molecular and cellular labelling techniques will make other assays

more applicable in the field. Furthermore, continued advances in instruments along with the decrease in the price will allow for the wider use of FLC in essentially any setting.

The use of FCM in immunological studies has advanced in vertebrates with a diverse range of measurable parameters (Cossarizza et al., 2017, Fleisher and Oliveira, 2019). However, FCM parameters used in molluscan immunological studies are limited to a few measurable parameters. This is a great opportunity for marine scientists to continue to explore the advantages of modern FCM to provide insights into molluscan immunology. However, the lack of standardized cellular reference materials for molluscan haemocytes remains a big challenge for the development and validation of new assays. Among the current parameters used, only viability and cell subpopulations has been validated (Allam et al., 2002, Ashton-Alcox and Ford, 1998, Donaghy et al., 2010, Ford et al., 1994). There are currently no guidelines for the validation of FCM methods to be used in molluscan species. Hence, there is a need to conduct appropriate validations for the numerous FCM assays used in molluscan immunological studies.

3.5 CONCLUDING REMARKS

A variety of FCM assays currently exist for immunological studies of molluscs which have enabled deep understanding of molluscan immunology. FCM analyses in molluscs have focused on characterization of cell types, response of haemocytes to a particular stress, diseases or toxic agent and diagnosis of diseases. However, applications of FCM in marine molluscs as well as in invertebrates are quite limited compared to those in vertebrates. To this end, marine scientists could take advances by applying and optimizing FCM methodologies that have been developed for mammalian cells for studies in mollusc species. As a tool, FCM can be used in combination with other techniques, such as omics approaches, which have advanced significantly in recent years. Ultimately, this will lead to a more improved understanding of molluscan immunology, and contribute to defining accurate biomarker signatures of diseases or stress conditions within wild and aquaculture settings.

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Section II

Tissue- and sex-specific responses of bivalves to pathogen infections

A female New Zealand Greenshell™ mussel (*Perna canaliculus*) with orange gonad.

In this section:

Chapter 4

Metabolomics insights into tissue-specific responses of the New Zealand Greenshell™ mussels (*Perna canaliculus*) to *Vibrio* sp. infection

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ABSTRACT

While tissue-specific immune responses are well-understood in mammals, such studies are lacking for marine bivalves. In this study, we investigated immune responses in gill, hepatopancreas and haemolymph of mussels (*Perna canaliculus* Gmelin, 1791) following experimental infection with *Vibrio* sp. DO1 (*Vibrio coralliilyticus*/*neptunius*-like isolate). Significant differences in metabolite profiles and metabolic responses between tissues were observed. Overall, haemolymph and gills shared common metabolic responses characterised by increases in itaconic acid and decreases in other amino acids (e.g., branched-chain amino acids , lysine, tryptophan) and fatty acids (e.g., DHA, EPA, palmitoleic acid). Increases in itaconic acid, decreases in fatty acids and increases in amino acids were found in hepatopancreas tissues. The alterations of these metabolites suggest osmotic stress, oxidative stress, changes in amino acid metabolism and protein synthesis in the immune system of *P. canaliculus* caused by *Vibrio* sp. infection. Interestingly, the accumulation of itaconic acid in all three tissues of infected mussels suggests that this metabolite has an important role in the mediation of bivalve antimicrobial activities and immune responses. These results indicate that careful consideration should be given to tissue sampling choices for immunological and metabolomics studies. In addition, further investigations are needed to elucidate mechanistic responses across different tissues associated with pathophysiological processes in bivalves.

4.1 INTRODUCTION

Bivalves represent one of the most important groups in aquaculture, with mussels, oysters, clams, and scallops being the most widely cultivated. Global bivalve production has increased from 3.3 million metric tonnes in the 1990s to 15.3 million metric tonnes in 2016 (FAO, 2019). In some countries, such as New Zealand, bivalve farming accounts for more than 88 % of the country's aquaculture production (FAO, 2019). In New Zealand, Greenshell™ mussels (*P. canaliculus*) and Pacific oysters (*Crassostrea gigas*), together with king salmon (*Oncorhynchus tshawytscha*), represent the three main cultivated species for this growing aquaculture sector. However, infectious diseases caused by viruses (e.g. OsHV-1), bacteria (e.g., *Vibrio* sp.) and parasites (e.g., *Bonamia* sp.) are one of the main challenges for further expansion and growth (Alfaro et al., 2018, Castinel et al., 2014). Among these pathogens, Gram-negative bacteria belonging to the genus *Vibrio* (e.g., *V. aestuarianus*, *V. coralliilyticus V. splendidus* and *V. harveyi*) have been associated with a number of infectious diseases with sometimes devastating consequences for bivalve stocks worldwide (Travers et al., 2015). *Vibrio coralliilyticus*, is a well-known coral pathogen (Ben-Haim et al., 2003, Rozenblat and Rosenberg, 2004), and has contributed to dramatic bivalve aquaculture losses, and prompted considerable research interest in recent years (Nguyen et al., 2018a). For example, *Vibrio* sp. DO1 (*V. coralliilyticus*-like isolate) was isolated from *P. canaliculus* larvae (Kesarcodi-Watson et al., 2009a) and was confirmed to be pathogenic when used in experimental challenges of mussel larvae (Kesarcodi-Watson et al., 2009b) and adults (Nguyen et al., 2018a, Nguyen et al., 2018b). Despite the fact that *V. coralliilyticus* is known to affect a range of bivalve species worldwide, there is limited information about its pathogenicity, infection mechanisms and disease mitigation.

Studies have started to emerge relating to the overall health and immune system of shellfish with commercial importance (reviewed by Guo and Ford 2016, Zannella et al. 2017). These studies have used a range of immunological and analytical tools to target diverse sets of tissues and organs. Haemocytes for example, which play a key role in the innate immune system (e.g., phagocytosis, encapsulation and nacrezation of foreign particles), are commonly used to investigate immune responses of bivalves to pathogens (Allam et al., 2000, Allam et al., 2006, Ciacci et al., 2017, Oubella et al., 1994, Pruzzo et al., 2005). Bivalve gills are also often analysed in immunological studies, since they accumulate marine pathogens and are targets of infection (Liu et al., 2014, Lu et al., 2017). The molluscan hepatopancreas is an integrated organ of immunity and metabolism (Rőszer, 2014), also making it one of the most common target tissues in immune studies (Jiang et al., 2017, Liu et al., 2014, Lu et al., 2017, Ren et al., 2017).

It is well-known that characteristics of the tissue microenvironment in mammals can shape the outcome of innate immune responses (Chieosilapatham et al., 2018, Hu and Pasare, 2013). However, such tissue-specific regulation of innate immune responses are not well-characterized in bivalves. To our knowledge, the only study thus far to investigate tissue-specific immune responses in bivalves was conducted by Liu et al. (2014), who compared metabolite profiles in gills and hepatopancreas of mussel (*Mytilus galloprovincialis*) after a *Vibrio harveyi* challenge using NMR-based metabolomics. Differential metabolic responses to the infection between the tissues were observed, with glucose synthesis and ATP/AMP turnover in hepatopancreas tissues being altered, and an elevation of phosphocholine levels being detected in gill tissues. Comparisons between immune responses of haemolymph/haemocytes and other tissues (e.g., gills, hepatopancreas) has not yet been investigated in marine bivalves.

Our recent studies have demonstrated the power of gas chromatography-mass spectrometry (GC-MS)-based metabolomic approaches to characterise changes in endogenous metabolites underlying metabolic responses of bivalve haemocytes against external stresses (e.g., pathogens, contaminants, temperature) (Alfaro et al., 2019, Nguyen and Alfaro, 2018, Nguyen et al., 2018c). However, we did not investigate such metabolic responses in other tissues (e.g., gills, hepatopancreas). In the present study, GC-MS-based metabolomics was applied to compare the endogenous metabolic changes in haemolymph, gills and hepatopancreas of the New Zealand Greenshell^{TM} mussels (*P. canaliculus*) following *Vibrio* sp. infection. Thus, the study aims to characterize tissue-specific immune responses which are crucial for understanding of bivalve immune system and pathogenesis of *Vibrio* bacteria. In addition, we also intended to identify potential biomarkers underlying these processes in mussels.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design and bacterial culture

Adult mussels (weight = 80.41 ± 18.81 g; shell length = 10.51 ± 0.93 cm) were obtained from Westpac Mussel Distributors Ltd (Auckland, New Zealand) and acclimatized for one week in a re-circulation system (5 µm filtered seawater [FSW]; temperature = 16 ± 0.5 °C; salinity = 35 ppt; pH = 8.0). A pool of 20 mussels were randomly divided into two equal groups and placed into plastic tanks containing 50 l of filtered seawater that were continuously aerated with air stones. Ten mussels in one tank were injected with 50 µl of fresh *Vibrio* sp. DO1 suspended in autoclaved phosphate-buffered saline (PBS) at a concentration of 1×10^7 cells·ml^{-1} in the posterior adductor muscle. Mussels in the control groups were only injected with 50 µl of PBS.

The bacterial strain *Vibrio* sp. DO1 (*V. coralliilyticus*/*neptunius*-like isolate, 99.5 % 16S rDNA sequence similarity with *V. corallyliticus*, Genbank: EU358784), which was previously isolated by Kesarcodi-Watson et al. (2009a) was provided by Cawthron Institute (Nelson, New Zealand). Bacterial suspensions were prepared following the method described by Kesarcodi-Watson et al. (2009b) with modifications. Briefly, the bacterial isolates, which were stored at –80 °C in 50 % glycerol were revived by thawing for one hour prior to incubation in 10 ml volumes of sterilized Marine Broth 2216 (MB, Difco) at room temperature for 12 h on a G10 Gyrotory shaker (New Brunswick Scientific Co., Edison, NJ, U.S.A) at 100 rpm. The bacterial suspension was streaked on thiosulfate citrate bile salts sucrose (TCBS) agar plates (Fort Richard Laboratories, Auckland, New Zealand) and sub-cultured three times to ensure purity. Bacterial colonies were cultured in 10 ml MB for 12 h, then transferred into 500 ml MB and incubated for 36 h at room temperature on a G10 Gyrotory shaker at 100 rpm. The final broth cultures were centrifuged on an Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) at

2423 × g for 10 min, and washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The bacterial suspension was re-suspended in autoclaved PBS to the original concentration (~10^8 CFU·ml^{-1}). A series of 10-fold dilutions in PBS was prepared and measured on a spectrophotometer (Ultrospec 2100 pro UV–Vis, Biochrom Ltd, Cambridge, UK) at 600 nm to determine the cell concentration of final broth cultures. In addition, 100 µl from each dilution was spread on TCBS agar plates to verify experimental concentrations.

Twenty-four hours after injection, mussel haemolymph was withdrawn from the posterior adductor muscle of all animals using a 23 gauge and 1/4″ needle attached to a 3-ml sterile syringe (Terumo, Belgium) (Fig. 4.1). Haemolymph samples were immediately transferred to 2 ml Eppendorf tubes kept on ice, and 300 µl were sub-aliquoted into 2 ml Cryovial (BioStor™) and flash-frozen in liquid nitrogen (LN). The remaining haemolymph was kept on ice to use for bacterial counts and flow cytometric assays. Gill and hepatopancreas tissues were quickly excised after haemolymph withdrawal and flash-frozen in LN. All samples in LN were stored at –80 °C until metabolite extractions could be undertaken.

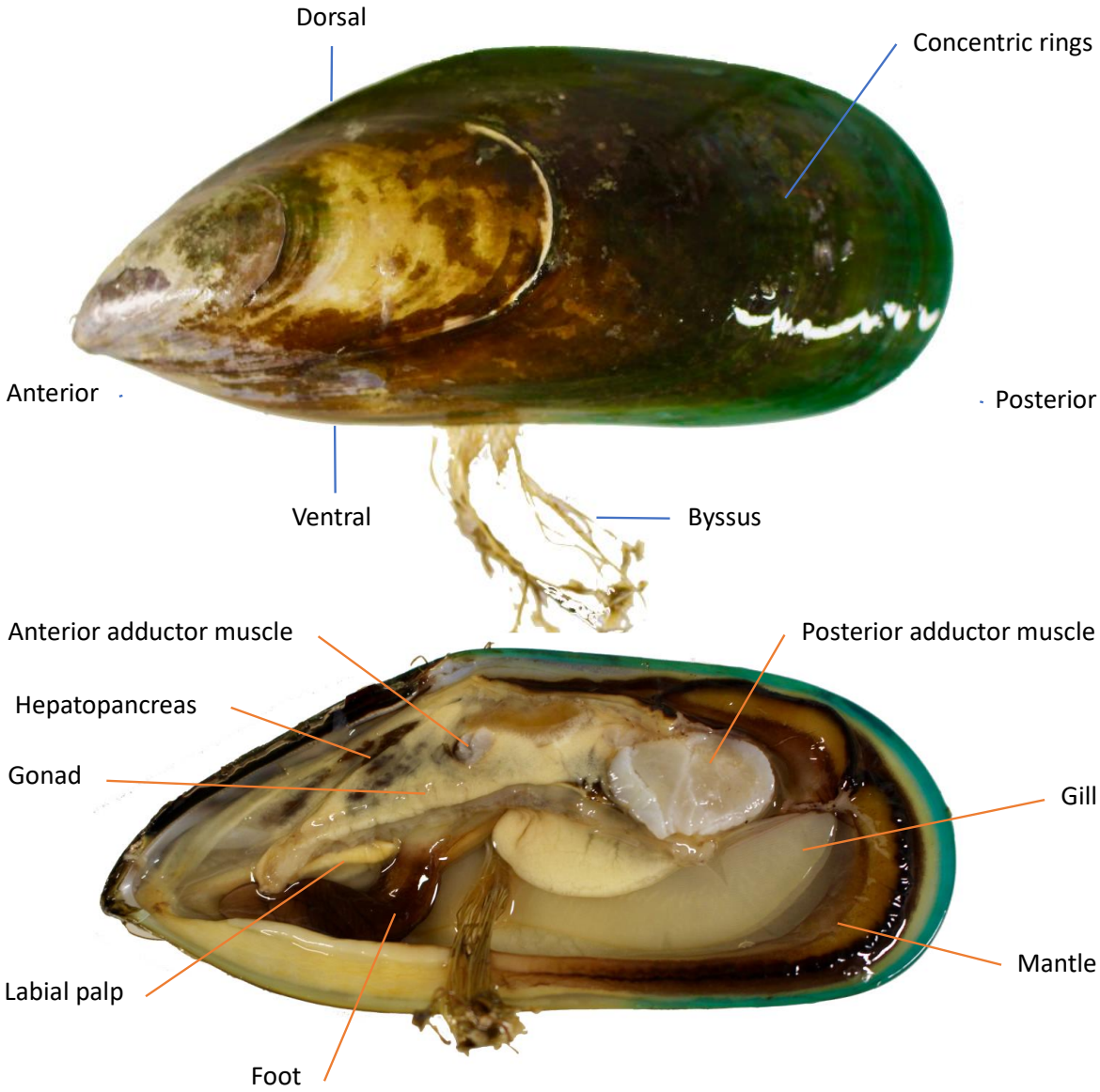

Figure 4.1 The external and internal features of the New Zealand Greenshell™ mussels (*P. canaliculus*).

4.2.2 Bacterial quantification

Bacteria were enumerated using the spread plate method with a turntable and glass spreader, according to Sanders (2012). Briefly, for non-infected control mussels, 0.1 ml of haemolymph was directly spread on thiosulfate citrate bile salts sucrose (TCBS) agar plates (Fort Richard Laboratories, Auckland, New Zealand). For infected mussel haemolymph, a series of dilutions (up to 10^{-4}) were prepared by diluting the stock haemolymph with ASW, and 0.1 ml from each diluted haemolymph was spread on TCBS

agar plates with three replicates for each. All plates were incubated at room temperature for 24 h, then plates with 30 to 300 colony forming units (cfu) were enumerated; where $cfu \cdot ml^{-1}$ = (no. of colonies x dilution factor) / volume of each sample.

4.2.3 Quantification and viability of haemocytes

After withdrawal from the mussels, 200 µl of fresh haemolymph were immediately diluted with 200 µl of cold (4 °C) filtered (0.2 µm) ASW and mixed thoroughly to avoid cell aggregation. Samples were kept on ice and transferred to the laboratory for analysis within 30 minutes. Haemocyte concentrations and viability were measured via flow cytometry on the Muse® Cell Analyzer (EMD Millipore, Hayward, CA, USA) using the Muse® Cell Count and Viability kit (Merck Millipore, Abacus dx, New Zealand), following the following the manufacturer's specifications with modifications for bivalve haemocytes. Briefly, 20 µl of diluted haemolymph were mixed with 380 µl of Muse® Count and Viability assay reagent in a 1.5 ml micro-centrifuge tube and incubated for 5 min in the dark at room temperature. Finally, the samples were mixed thoroughly and run on the Muse® Cell Analyzer (flow rate = 0.59 $\mu l \cdot sec^{-1}$; number of events = 1000; RED threshold = 11; FSC threshold = 10).

4.2.4 Metabolite extraction and derivatization

Stored gill and hepatopancreas samples were freeze-dried overnight, then ground using a mortar and pestle. Approximately 5 mg of gill and hepatopancreas tissues were weighted and placed into 1.5 ml micro-centrifuge tubes for metabolite extractions. Stored mussel haemolymph samples were dried in a SpeedVac Concentrator with a Refrigerated Vapor trap (Savant™ SC250EXP, Thermo Scientific) for 4 h (0 °C, vacuum ramp 3, 42 torr/min). All samples were co-extracted with an internal standard (L-alanine-2,3,3,3-d_4) in cold methanol-water solution (MeOH:H_2O), according to Smart et al. (2010) with

minor modifications. Briefly, each dried sample was mixed with 20 µl of d4-alanine (10 mM) and 500 µl of cold (−20 °C) 50 % MeOH:H_2O solution. The mixture was homogenized for 1 minute using a Geno/Grinder® - automated tissue homogenizer and cell lyser (SPEX CertiPrep, Metuchen, NJ, USA), re-frozen on dry ice and then thawed again on ice. Extracts were cold (−6°C) centrifuged at 587 × *g* for 10 minutes (Centrifuge 5424, Eppendorf AG, Hamburg, Germany) and the supernatants from the extractions were collected in 2 mL plastic vials placed on dry ice. The second extraction was carried out with 500 µL of cold (−20°C) methanol-water (80% v/v, MeOH:H_2O) with the same process as the first extraction. The supernatant was collected, then mixed with the first supernatant and kept on dry ice. The mixture was dried using a Savant™ SC250EXP SpeedVac Concentrator with a Savant™ RVT5105 Refrigerated Vapor Trap as specified above.

Extracted metabolites were derivatized based on the protocol described by Smart et al. (2010). Briefly, dried samples were re-suspended in 400 µl of 1 M sodium hydroxide and quantitatively transferred to silanized borosilicate glass tubes (12 × 75 mm) (Kimble™, ThermoFisher, Auckland, New Zealand) containing 334 µl of methanol and 68 µl of pyridine. This was followed by a series of reagent additions and vortexing: 40 µl of MCF reagent – 30 s, 40 µl of MCF – 30 s, 400 µl of chloroform – 10 s, and 800 µl of 50 mM sodium bicarbonate – 10 s. The mixture was centrifuged at 1174 g on an Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) for 6 minutes. The upper aqueous layer was discarded, and a small amount of anhydrous sodium sulphate was added to remove residual water. The chloroform phase containing the MCF derivatives was transferred to 2 ml amber CG glass vials fitted with 150 µL inserts with bottom-spring (Sigma-Aldrich, St. Louis, MO, USA) for GC-MS analyses.

4.2.5 GC-MS measurements and quality control

The derivatized samples were analysed with a gas chromatograph GC7890B coupled to a quadrupole mass spectrometer MSD5977A (Agilent Technologies, CA, USA), with a quadrupole mass selective detector (EI) operated at 70 eV. The system was equipped with a ZB-1701 GC capillary column (30 m × 250 μm id × 0.15 μm with 5 m guard column) (Phenomenex, Torrance, CA, USA). The instrumental setup parameters for MCF derivatized samples were set according to Smart et al. (2010). The GC-oven temperature was initially held at 45 °C for 2 minutes, and then raised with a series of gradient increases as following: increased 9 °C per minute to 180 °C, held for 5 minutes; increased 40 °C per minute to 220 °C, held for 5 minutes; increased 40 °C per minute to 240 °C, held for 11.5 minutes; increased 40 °C per minute to 280 °C, held for a further 2 minutes. The interface temperature was set to 250 °C, the source was set at 230 °C and the quadrupole temperature was set at 150 °C. Samples (1 μl) were injected under pulsed splitless mode with the injector temperature at 260°C. Helium was used as the carrier gas and was held at a constant flow of 1 ml per minute. The GC column was equilibrated for 6 minutes prior to each analysis. The mass spectrometer was operated in scan mode, starting after 6 minutes with mass range 38–650 AMU at 1.47 scans per second and detection threshold of 100 ion counts.

Several types of quality control (QC) samples were employed to ensure reproducibility of GC-MS measurements. The first QC samples were chloroform solvent and non-derivatized n-alkanes (C10-C40). The alkane samples were also used to check Kovats retention index and create the calibration file. Secondly, six standard amino acid mixtures (20 μL, 20 mM) were similarly derivatized and measured as the protocol for samples. Thirdly, six blank samples containing 20 μL of 10 mM d_4 alanine were extracted, derivatized and analysed using the sample protocol as above. The final two QC samples

were pooled samples from both infected and noninfected (controlled) mussels that were processed as samples. These QC samples were injected at the beginning and after every ten samples. Together, QC samples made up more than 30% of all injections performed.

4.2.6 Data processing

Raw spectra data were transformed into AIA format (.cdf) files using ChemStation (Agilent Technologies, Inc., US) and processed using automated mass spectral deconvolution and identification system (AMDIS) software (online software distributed by the National Institute of Standards and Technology, USA - http://www.amdis.net/) integrated with an automated in-house R-based package (Aggio et al., 2011). GC-MS data mining was carried out using an automated in-house R-based package (Aggio et al., 2011). Identification was performed using an in-house MS library with the minimum matching percentage of 70% based on both the MS spectrum of the metabolite and its respective retention time. Other parameters used to accomplish this analysis were: retention time (RT) window (0.2 minute), RT range (6.5-34.0 minute), component width (14), QA/QC (solvent tailing: 83 m/z, column bleed: 207 m/z) and scan sets (3). Annotated metabolites were manually checked with ChemStation software (Agilent Technologies, Inc., US) and AMDIS for the presence of contaminants. Repeats (based on ID number, match factor and retention time) and aberrant records were removed. Data were normalized to the internal standard (d_4 alanine) to compensate for potential technical variations (e.g. variable metabolite recoveries) prior to data analyses.

4.2.7 Data analyses

Metabolite profile data were analysed using MetaboAnalyst 3.0 (Xia et al., 2015). Data were generalized log (glog) transformed and mean centred to make individual features more comparable. Unsupervised principal components analysis (PCA) was used to

identify natural groupings of all tissue samples based on the underlying structure of the data. Supervised partial least squares - discriminant analysis (PLS-DA) was applied to distinguish two groups from each other and to perform a classification and regression model. The PLS-DA model performance was validated using leave one out cross validation (LOOCV), which was assessed via accuracy, multiple correlation coefficient (R^2) and cross-validated R^2 (Q^2). The important classifiers were identified via their variable importance in projection (VIP) scores. All metabolites with VIP score values greater than one were considered important for the separation among infected and control groups. For these relevant compounds, univariate analyses were performed (*t*-test and fold change) for distinguishing infected and control group in each tissue. A 95 % confidence interval ellipses was applied for all the tests.

Differences in bacterial concentrations, total haemocyte counts and viability between control and infected mussels were analysed with independent student's *t*-tests using SPSS® software (version 23.0) (IBM, Armonk, NY, USA).

4.3 RESULTS

4.3.1 Bacterial quantity, haemocyte concentrations and viability

Vibrio counts were significantly higher in haemolymph from infected mussels (1.87 × 10^6 ± 1.18 x 10^6 cfu·ml^{-1}) than those of non-infected (control) mussels (70.0 ± 35.0 cfu·ml^{-1}) (t_8 = –1.59; p = 0.033) (Fig. 4.2A). There was a significant increase in total haemocyte concentration in infected mussels (8.01 x 10^6 ± 1.18 × 10^6 cfu·ml^{-1}) compared to controlled mussels (2.68 × 10^6 ± 4.46 x 10^6 cfu·ml^{-1}) (t_{38} = –3.83; p = 0.036) (Fig. 4.2B). However, the percent viability of haemocytes was lower in infected mussels (51.94 ± 4.20 %) than in controls (71.74 ± 1.79 %) (t_{38} = 4.34; p = 0.002) (Fig. 4.2C).

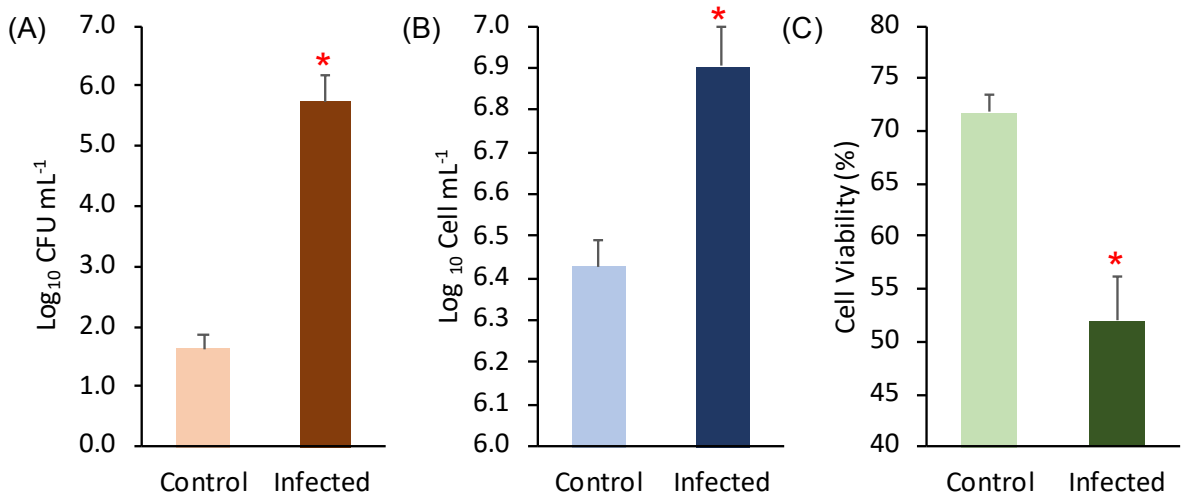

Figure 4.2 Enumeration of bacteria and haemocytes. Enumeration of *Vibrio* sp. in mussel haemolymph (Log_{10} cfu·ml^{-1}) (A). Log_{10} haemocyte concentrations (cells·ml^{-1}) in mussel haemolymph (B). Viability of haemocytes (C). Data are represented as mean ± SE (n = 6). Significant differences relative to the control are marked with an asterisk (*) (*t*-test, $p < 0.05$).

4.3.2 Metabolomics

A total of 73 metabolites that matched with the in-house library were identified from GC-MS spectra. These metabolites belong to broad chemical classes of amino acids (e.g., serine, valine, cysteine), fatty acids (e.g., DHA, EPA, oleic acid), organic acids (e.g.,

succinic acid, fumaric acid, itaconic acid), and vitamins (e.g., nicotinic acid), among others.

The PCA score plot showed a good separation between gill, hepatopancreas and haemolymph samples (Fig. 4.3). Supervised PLS-DA analyses were used to sharpen the discrimination between control and infected groups in all tissues/organs and to build the classification and prediction model. The PLS-DA model of gill tissues showed an accuracy of 94.4 %, an R^2 value of 92.0 % and a Q^2 value of 74.7 %. Similarly, the PLS-DA model for hepatopancreas had high accuracy (94.4 %), R^2 (92.0 %) and Q^2 (74.7 %). These parameters indicate optimal fitness and prediction performance of the PLS-DA models for gill and hepatopancreas tissues. For the haemolymph PLS-DA model, the accuracy (82.1 %), R^2 value (76.1 %) and Q^2 value (55.0 %) were lower than those of the gill and hepatopancreas models, but they were still considered reasonable for classification and predictive purposes.

Furthermore, PLS-DA also provided the list of metabolites that are important classifiers of these models, based on their VIP scores. With VIP scores > 1.0, there were 29, 28 and 28 metabolites in haemolymph, gills and hepatopancreas, respectively, that were identified as contributing strongly towards class discriminations. Ten of these metabolites were common important model contributors across all tissues. In addition, gills shared 11 and 2 common important classifiers with haemolymph and hepatopancreas, respectively. Similarly, haemolymph and hepatopancreas have 3 common metabolites that strongly contributed to the classification models (Fig. 4.4). T-test analyses ($p < 0.05$) of these metabolites revealed 26 metabolites that were differently expressed in haemolymph of control and infected mussels with 2 up-regulated and 24 down-regulated metabolites. Gills showed the alteration of all 28 important classifiers with 2 up-regulated and 26

down-regulated. For the hepatopancreas samples, there were increases in 19 metabolites and decreases in 6 metabolites. Details of *t*-test analyses and fold change values of these metabolites are presented in Table A.1, 2 & 3 in the appendix.

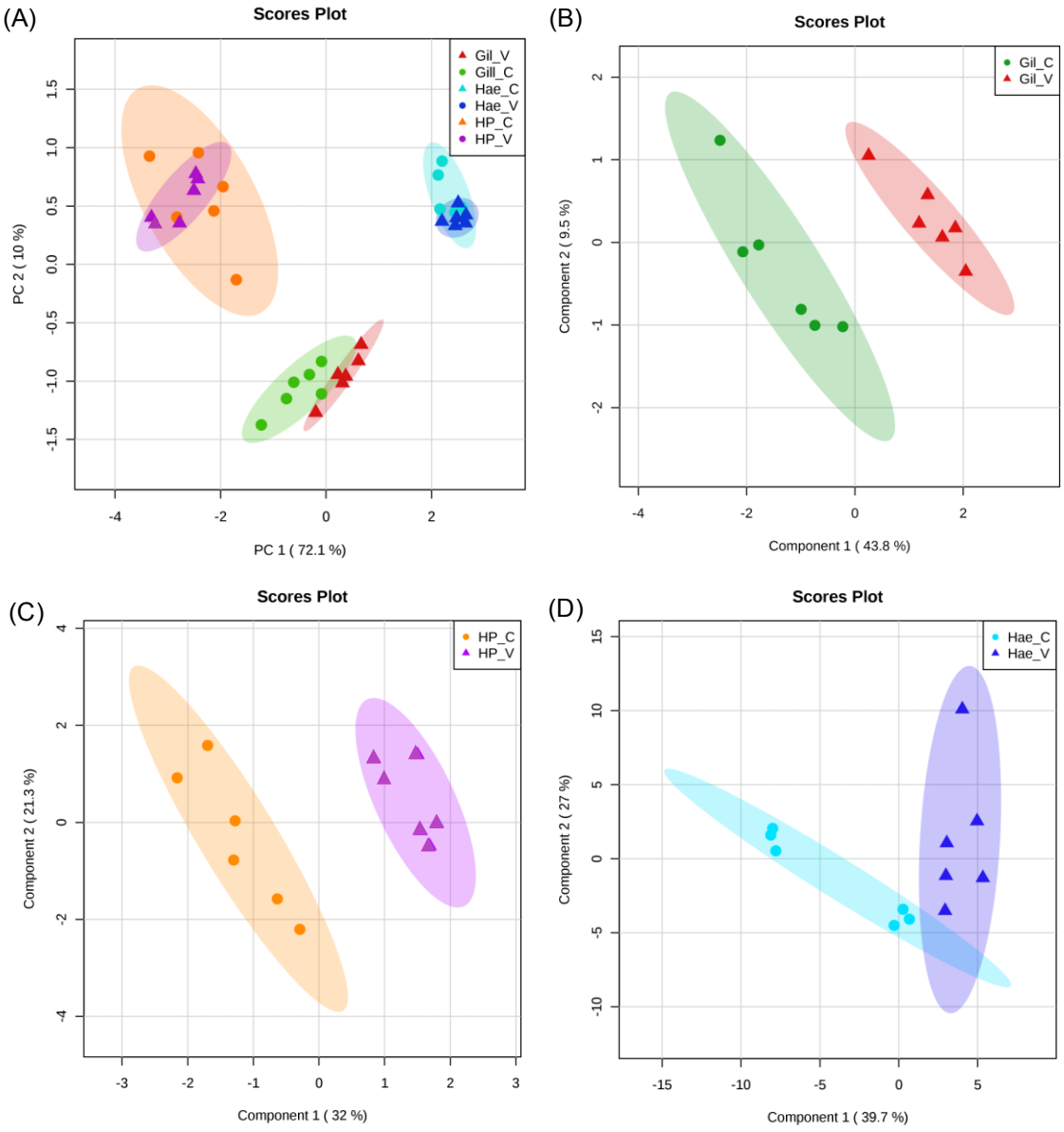

Figure 4.3 Multivariate data analysis of haemolymph, gills and hepatopancreas in infected and non-infected (control) mussels. PCA score plot of gill, hepatopancreas and haemolymph in infected and control mussels (A). PLS-DA score plot of gill tissue (B), hepatopancreas (C) and haemolymph (D). *Gil, gill tissues; Hae, haemolymph; HP, hepatopancreas; C, control (PBS injected; non-infected); V, Vibrio bacteria (Vibrio DO1 injected; infected).*

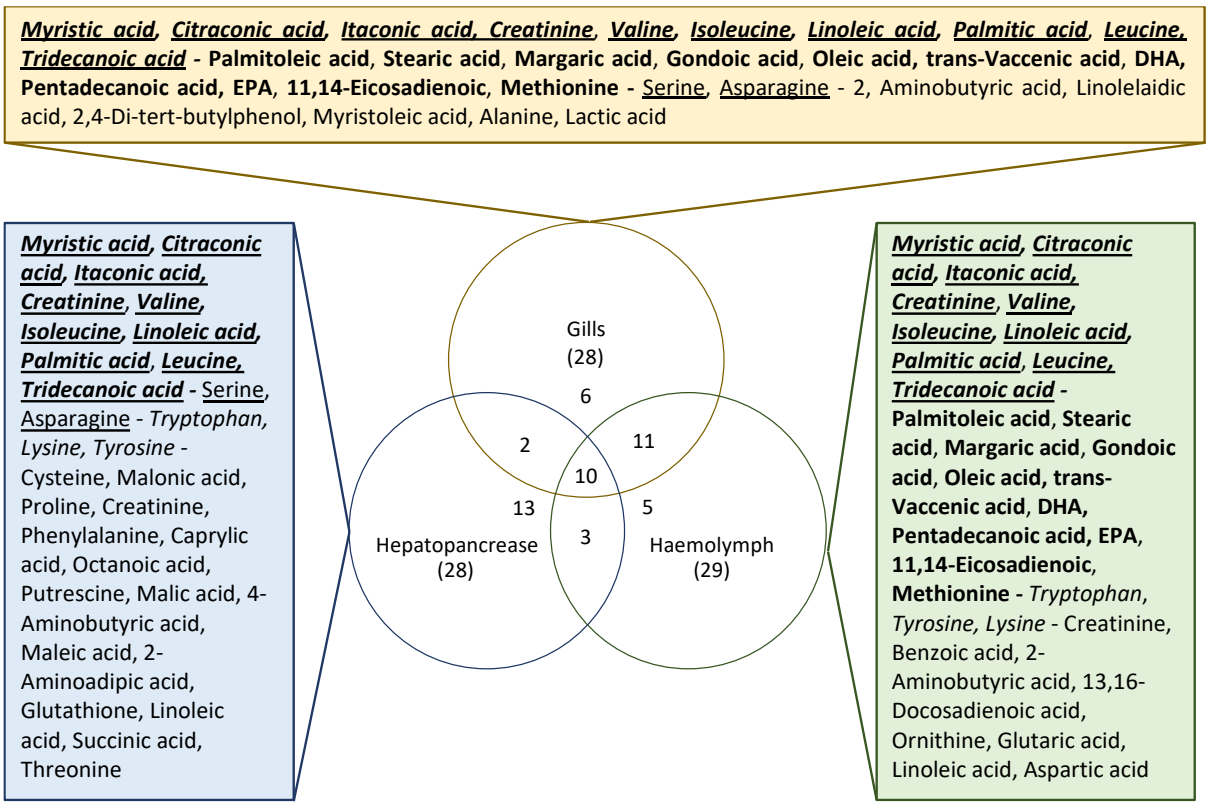

Figure 4.4 List of metabolites that were identified as contributing strongly to classification models based PLS-DA VIP Score (> 1) of haemolymph, gills and hepatopancreas. ***Metabolites*** in **bold**, *italic* and underlined are common compounds among the three tissues. **Metabolites** in **bold** are common metabolites between gills and haemolymph samples, while metabolites in *italic* are common classifiers between haemolymph and hepatopancreas. Underlined metabolites represent the common metabolites between hepatopancreas and gills.

4.4 DISCUSSION

V. coralliilyticus is a ubiquitous bivalve pathogen that has contributed to dramatic losses in shellfish aquaculture worldwide in recent years (Nguyen et al., 2018a). In this study, we challenged mussels with *Vibrio* sp. DO1 (*V. coralliilyticus*-like isolate) and observed significantly higher *Vibrio* bacterial loads and lower haemocyte viability in haemolymph of infected mussels compared to non-infected control mussels after 24 hours. Furthermore, circulating haemocyte numbers were higher in infected mussels, which is consistent with other reports of bivalves exposed to pathogens (Allam et al., 2001) or environmental stressors (Pipe et al., 1999). Since haemocytes participate in phagocytosis and other important immune responses, the increase of haemocytes in infected mussels is suggestive of a stimulation of the immune system.

Metabolically, we observed differences in metabolite profiles among haemolymph, gills and hepatopancreas, and between infected mussels and control mussels in each tissue. There were 10 common metabolites identified as contributing strongly towards classification models, but their regulations were different from tissue to tissue. While there were increases in itaconic acid and decreases in free fatty acids (e.g., myristic acid, linoleic acid, palmitic acid, tridecanoic acid) in all tissues, free amino acids (e.g., valine, isoleucine, leucine, creatinine) decreased in gills and haemolymph, but increased in hepatopancreas. These results suggest common and different metabolic responses among these tissues of mussel challenged with *Vibrio* sp.

For haemolymph, we found significant increases in itaconic acid and glutaric acid, and decreases in 24 metabolites of infected mussels compared to those in the control group. Itaconic acid is an antimicrobial metabolite that is synthesised *de novo* during pathogen infections in mammalian macrophages (Cordes et al., 2015), and more recently

demonstrated in invertebrate cells (Nguyen et al., 2018b, Nguyen et al., 2018a, Young et al., 2017). Furthermore, previous studies on mussels following *Vibrio*-challenge have shown that itaconic acid may participate in regulation of the tricarboxylic acid cycle and inflammation (Nguyen et al., 2018a). Hence, the increase in itaconic acid suggests an important role of itaconic acid in antimicrobial and other immune responses of mussel haemocytes against the *Vibrio* infection.

Glutaric acid is a metabolic product of some amino acids, including lysine and tryptophan and key metabolites in glutaryl co-enzyme (Gholson et al., 1959, Neuberger and Sanger, 1944, Sauer et al., 2005). The involvement of glutaric acid in the pathogenesis of diseases has been demonstrated in several vertebrate studies. For example, glutaric acid reportedly plays roles in the activation or facilitation of excitotoxic mechanisms and oxidative stress (de Oliveira Marques et al., 2003, Kölker et al., 2004), the imbalance in glutamatergic and GABAergic neurotransmission (Porciúncula et al., 2000), the inhibition of energy metabolism (Ullrich et al., 1999), and the uncompetitive inhibition of the tricarboxylic acid cycle (TCA) (Sauer et al., 2005). Although less is known about glutaric acid function in invertebrates, the increased level of GA and decreases in a number of amino acids, including lysine and tryptophan in the gills and haemolymph of infected mussels, may suggest an involvement of glutaric acid in the pathogenesis of *Vibrio* sp. Thus, future investigations are needed to discover functions of this metabolite in the internal defense mechanism of invertebrates.

Among the altered metabolites, all fatty acids were decreased (Table 4.A.1). Since immune responses of marine bivalves against pathogenic infections are known to be energetically costly (Flye-Sainte-Marie et al., 2007), this result may suggest the use of fatty acids as an energy source for the host due to high energy demands of mussels in

response to *Vibrio* sp. infection. Branched-chain amino acids (BCAAs), including leucine, isoleucine and valine are essential metabolites for the immune system, which provide energy and act as the precursors for biosynthesis of proteins (Calder, 2006). The decrease in BCAAs has been previously reported in mussel haemolymph challenged with *Vibrio* sp. DO1 (Nguyen et al., 2018a). In the present study, the decreased level of BCAAs in infected mussel haemolymph may indicate high demands for BCAAs as energy sources and for immune activities. In marine molluscs, amino acids can be oxidized to generate energy (Viant et al., 2003). Hence, the reduction of others amino acids in haemolymph of *Vibrio*-infected mussels may suggest the use of amino acids for the high energy demands of the immune response.

For the gills, a similar pattern of metabolite alterations during the infection was found as in haemolymph. They include the 11 common metabolites identified as important classifiers, the increases in itaconic acid, and decreases in fatty acids (e.g., gondoic acid, DHA, EPA**)** and amino acids (e.g., tryptophan, lysine, GABA) (Table 4.A.2). In addition to common features with haemolymph, the infected gills showed a decrease in other fatty acids (myristoleic acid and tridecanoic acid) and other amino acids (methionine, alanine, lactic acid). Together, these results suggest the elevated metabolic demands for the activated immune system of mussels during *Vibrio* infection. These similar patterns between gills and haemolymph may reflect the fact that the gills have rich haemolymph supplies, and thus, the metabolic responses of the gills are somewhat similar to those of haemocytes. Another possibility is that the challenge method by injection of *Vibrio* sp. directly to the adductor muscle may not activate specific responses of other cell types in gills (e.g., epithelial cells, mucocytes) in the same way that gills would respond to water-borne pathogens in the aquatic environment. However, future investigations are needed to confirm these hypotheses.

The metabolic responses of hepatopancreas to *Vibrio* sp. challenge was found to share some common features with the gills and haemolymph (Table 4.A.3). For example, the accumulations of itaconic acid in the hepatopancreas, gills and haemolymph of infected mussels suggest this is a common feature across the body of mussels. Similarly, the decreases in fatty acids (tridecanoic acid, myristic acid, palmitic acid and linoleic acid) indicate the increased energetic demand in infected mussels.

In contrast to gills and haemolymph, hepatopancreas showed increased levels in many major amino acids (e.g., BCAAs, tyrosine, lysine, cysteine and glutathione). In agreement to these findings, elevated amounts of amino acids in hepatopancreas have been reported in marine bivalves exposed to pathogens (Liu et al., 2013, Lu et al., 2017) or environmental stress (Lu et al., 2016). For example, elevated BCAAs were observed in hepatopancreas of Manila clams (*Ruditapes philippinarum*) following a *V. harveyi*-challenge (Liu et al., 2013). The increases in other amino acids (e.g., lysine, choline and glutamine) have been reported in hepatopancreas of abalone exposed to *V. parahaemolyticus* (Lu et al., 2017). Generally, the accumulation of these amino acids is known to result from a disturbance in energy metabolism during infection (Ji et al., 2013). In the present study, amino acids increased in hepatopancreas, but decreased in gills and haemolymph, which may suggest that hepatopancreas could produce amino acids to provide to other tissues. Since hepatopancreas is an integrated organ of immunity and metabolism, a metabolic shift in hepatopancreas can fuel immune responses in molluscs (Rőszer, 2014).

From the elevated amino acids in the hepatopancreas, there were increases in metabolites involved in glutathione synthesis (e.g. serine, cysteine, glutathione). Glutathione is an important and abundant low-molecular-mass thiol and antioxidant that plays diverse roles

in many cellular processes, including amino acid transport and synthesis, protein synthesis, regulation of enzyme activity and metabolism of xenobiotics, carcinogens and ROS (Grant and Dawes, 1996). Glutathione in a reduced form (GSH) can react with electrophilic oxidants (e.g. ROS) by converting two GSH molecules into its oxidized form (GSSG) (Espinosa-Diez et al., 2015). The role of glutathione and its synthesis pathway in regulation of oxidative stress has been recently demonstrated in marine bivalves following the *Vibrio* sp. challenge (Nguyen et al., 2018a). In adition, the supplementation of serine was reported to alleviate oxidative stress in mice via supporting the methionine cycle (transsulfuration pathway) and glutathione synthesis (Zhou et al., 2017). Cysteine is a glutathione residue and its free sulfhydryl groups can be easily oxidized in response to a wide range of ROS (Chen et al., 2016, Ma, 2013, Reina et al., 2016, Templeton et al., 2010). Hence, elevated levels of glutathione, serine and cysteine indicate the activated glutathione pathway and thus an oxidative stress in mussel hepatopancreas caused by *Vibrio* infection. Together, the increases in amino acids in infected mussel hepatopancreas tissues suggest the activated pathways of their immune system.

4.5 CONCLUSION

In summary, this is the first study that demonstrates the application of GC-MS-based metabolomics to characterize the tissue-specific immune responses in haemolymph, gill and hepatopancreas of *P. canaliculus* following *Vibrio* sp. infection. Overall, there were increases in itaconic acid and decreases in fatty acids in all tissues. However, amino acids were decreased in haemolymph and gills, but increased in hepatopancreas. The alterations of these metabolites indicate high energy demands and oxidative stress in infected mussels. The endogenous metabolite changes in different tissues provide insights into host-pathogen interactions at the metabolic level. Among the altered metabolites, the elevation of itaconic acid in all tissues suggests the important role of this compound in the bivalve defence mechanism. In a recent surprising discovery, itaconic acid was recognized as an immune-supportive metabolite in mammalian immune cells. However, the role this metabolite in invertebrate cells is unclear. Therefore, it would be critical for future investigations to establish whether itaconic acid has an antimicrobial role in bivalves, and how it might be involved in regulation of immune-related metabolism. That would lead to the better understanding of bivalve immune system and the use of itaconic acid as the important metabolite biomarker for early detection of pathogen infection and the health status of the host. Such kind of knowledge and approach would be contribute to the development of effective management strategies for infectious diseases in bivalves such as the summer mortality events affecting New Zealand Greenshell^{TM} mussels (*P. canaliculus*).

4.6 APPENDIXES

Table 4.A.1 List of metabolites with PLS-DA VIP Scores (> 1) and its *p* value (*t*-test) and Log_2 FC in haemolymph.

Compounds	PLS-DA VIP Scores	P value (t-test)	Log_2 FC	*Vibrio* effects
Valine	1.62	0.006	-3.36	↓
Tryptophan	1.50	0.006	-2.90	↓
Stearic acid	1.46	0.004	-7.95	↓
Leucine	1.46	0.010	-2.84	↓
Isoleucine	1.46	0.011	-2.65	↓
Myristic acid	1.45	0.007	-6.85	↓
11,14-Eicosadienoic	1.45	0.017	-8.16	↓
Margaric acid	1.43	0.005	-7.94	↓
Pentadecanoic acid	1.42	0.005	-7.91	↓
Palmitoleic acid	1.42	0.008	-8.03	↓
Creatinine	1.42	0.022	-1.98	↓
Benzoic acid	1.41	0.005	-4.13	↓
2-Aminobutyric acid	1.41	0.028	-2.45	↓
Palmitic acid	1.39	0.004	-7.90	↓
13,16-Docosadienoic acid	1.36	0.017	-5.52	↓
Tyrosine	1.35	0.058	-2.38	-
trans-Vaccenic acid	1.34	0.007	-7.86	↓
Ornithine	1.34	0.037	-2.62	↓
Oleic acid	1.34	0.007	-7.86	↓
Gondoic acid	1.32	0.024	-8.04	↓
Glutaric acid	1.28	0.007	7.31	↑
Linoleic acid	1.20	0.015	-7.76	↓
Itaconic acid	1.17	0.023	7.61	↑
Lysine	1.12	0.035	-7.85	↓
Aspartic acid	1.12	0.044	-2.67	↓
DHA	1.10	0.042	-7.86	↓
EPA	1.08	0.040	-7.80	↓
Methionine	1.07	0.073	-1.84	-
Tridecanoic acid	1.04	0.087	-1.57	-

Table 4.A.2 List of metabolites with PLS-DA VIP Scores (> 1) and its *p* value (*t*-test) and Log_2 FC in gill tissues.

Compounds	PLS-DA VIP Scores	P value (t-test)	Log_2 FC	*Vibrio* Effects
Myristic acid	1.57	0.001	-6.37	↓
Itaconic acid	1.56	<0.001	8.50	↑
Creatinine	1.53	0.001	-8.67	↓
Valine	1.50	0.001	-8.44	↓
2-Aminobutyric acid	1.47	0.001	-8.42	↓
Isoleucine	1.47	<0.001	-8.41	↓
Linolelaidic acid	1.43	0.003	-8.43	↓
Palmitoleic acid	1.37	0.003	-8.40	↓
2,4-Di-tert-butylphenol	1.37	0.007	-3.56	↓
Linoleic acid	1.33	0.004	-8.42	↓
Myristoleic acid	1.23	0.021	-6.37	↓
Methionine	1.23	0.012	-2.92	↓
Stearic acid	1.22	0.008	-4.99	↓
Margaric acid	1.20	0.014	-4.45	↓
Serine	1.19	0.044	1.43	↑
Gondoic acid	1.18	0.010	-8.01	↓
Oleic acid	1.15	0.012	-8.27	↓
trans-Vaccenic acid	1.15	0.012	-8.27	↓
Alanine	1.13	0.022	-3.16	↓
DHA	1.13	0.020	-3.17	↓
Pentadecanoic acid	1.13	0.032	-3.04	↓
EPA	1.10	0.013	-4.45	↓
Palmitic acid	1.08	0.019	-6.22	↓
Lactic acid	1.08	0.033	-2.58	↓
Leucine	1.07	0.013	-7.95	↓
Asparagine	1.07	0.026	-6.47	↓
Tridecanoic acid	1.05	0.024	-3.22	↓
11,14-Eicosadienoic	1.04	0.020	-3.56	↓

Table 4.A.3 List of metabolites with PLS-DA VIP Scores (> 1) and its *p* value (*t*-test) and Log_2 FC in hepatopancreas tissues.

Compounds	PLS-DA VIP Scores	P value (t-test)	Log_2 FC	*Vibrio* Effects
2-Aminoadipic acid	1.14	0.043	6.90	↑
4-Aminobutyric acid	1.24	0.528	4.24	-
Asparagine	1.36	0.052	-1.13	↓
Caprylic acid	1.32	0.055	-8.45	↓
Creatinine	1.35	0.013	4.13	↑
Cysteine	1.59	0.005	8.44	↑
Glutathione	1.13	0.048	2.39	↑
Isoleucine	1.25	0.042	2.46	↑
Itaconic acid	1.66	0.005	8.63	↑
Leucine	1.54	0.002	3.37	↑
Linoleic acid	1.09	0.123	-8.33	↓
Lysine	1.46	0.023	5.63	↑
Maleic acid	1.14	0.037	5.67	↑
Malic acid	1.25	0.016	8.10	↑
Malonic acid	1.46	0.012	2.27	↑
Myristic acid	1.23	0.067	-7.61	↓
Octanoic acid	1.32	0.055	-8.45	-
Palmitic acid	1.21	0.062	-8.16	↓
Phenylalanine	1.35	0.012	3.34	↑
Proline	1.44	0.023	2.80	↑
Putrescine	1.31	0.013	8.16	↑
Serine	1.56	0.002	8.31	↑
Succinic acid	1.09	0.053	8.24	-
Threonine	1.07	0.038	7.93	↑
Tridecanoic acid	1.57	0.017	-8.39	↓
Tryptophan	1.54	0.007	8.42	↑
Tyrosine	1.10	0.039	5.24	↑
Valine	1.60	0.007	3.56	↑

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Chapter 5

Metabolic and immunological responses of male and female New Zealand Greenshell™ mussels (*Perna canaliculus*) to *Vibrio* sp. infection

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ABSTRACT

Massive mortalities due to pathogens are routinely reported in bivalve cultivation that have significant economic consequences for the global aquaculture industry. However, host-pathogen interactions and infection mechanisms that mediate these interactions are poorly understood. In addition, gender-specific immunological responses have been reported for some species, but the reasons for such differences have not been elucidated. In this study, we used a gas chromatography-mass spectrometry (GC-MS)-based metabolomics platform and flow cytometry approach to characterize metabolic and immunological responses in haemolymph of male and female mussels (*Perna canaliculus*) experimentally infected with *Vibrio* sp. Sex-based differences in immunological responses were identified, with male mussels displaying higher percentage of mortality, oxidative stress and apoptosis after pathogen exposure than female mussels. However, central metabolic processes appeared to be similar between two sexes at 24 h post injection with *Vibrio* sp. DO1. Significant alterations in relative levels of 37 metabolites were detected between infected and uninfected mussels. These metabolites are involved in major perturbations on the host's innate immune system. In addition, there were alterations of seven metabolites in profiles of mussels sampled on the second day and mussels that survived six days after exposure. These metabolites include itaconic acid, isoleucine, phenylalanine, creatinine, malonic acid, glutaric acid and hydroxyproline. Among these, itaconic acid has the potential to be an important biomarker for *Vibrio* sp. DO1 infection. These findings provide new insights on the mechanistic relationship between a bivalve host and a pathogenic bacterium, and highlight the need to consider host sex as a biological variable in future immunological studies.

5.1 INTRODUCTION

Sex-specific differences in immune responses of hosts toward foreign antigens (e.g., fungi, bacteria, viruses) and self-antigens have been reported for a number of invertebrates (Kurtz et al., 2000, Matozzo and Marin, 2010) and vertebrates (Fargallo et al., 2007, Pap et al., 2010). In vertebrates, sex-based differences in immune responses have been well documented (Klein and Flanagan, 2016) with differences described between males and females for both innate (Berghöfer et al., 2006, Griesbeck et al., 2015, Marriott et al., 2006, Pisitkun et al., 2006, Rettew et al., 2008, Torcia et al., 2012) and adaptive immune responses (Abdullah et al., 2012, Lee et al., 1996, Leposavic et al., 2011, Leposavić et al., 1996). Generally, immune responses are stronger in females than males (Klein and Flanagan, 2016, Roved et al., 2017, Zuk, 1990, Zuk and McKean, 1996). These sex-based differences contribute to variation in susceptibility of the hosts to infectious diseases, occurrence of auto-immune diseases and malignancies and responses to vaccines (Klein and Flanagan, 2016).

In invertebrates, sex differences in immuno-competence and immune responses have been studied in a number of insect species. For example, studies on X (chromosome)-linked immune genes of the common fruit fly (*Drosophila melanogaster*) showed that genetic variations in many of the genes is associated with sex differences, and sex-specific induction was observed following fungal or bacterial infection (Hill-Burns and Clark, 2009, Taylor and Kimbrell, 2007). Female scorpionflies (*Panorpa vulgaris*) have been reported to have higher lysozyme-like and phagocytosis activities than males (Kurtz et al., 2000), as well as higher activity of phenoloxidases (PO) and enzymes catalyzing the formation of melanins during wound healing and parasite encapsulation, suggesting higher immunocompetence (Kurtz and Sauer, 2001). The influence of sex on immunocompetence via PO activity was also reported in crickets (*Gryllus texensis*)

exposed to the bacterium *Serratia marcescens*; immunocompetence increased in reproductively active females (Adamo, 2004). In contrast, females of the dampwood termites (*Zootermopsis angusticollis*) were found to have lower encapsulation ability than males during colony foundation, which may be due to the high demands of oocyte maturation and egg production in reproductive females (Calleri et al., 2007). In addition to insects, sex-linked differences in immunocompetence have been reported in invertebrates such as fairy shrimp (*Streptocephalus dichotomus*) (Radhika et al., 1998) and sea cucumbers (*Apostichopus japonicas*) (Jiang et al., 2017). These studies suggest that sex-based differences in disease susceptibility and immune function are a common feature of fauna.

Many studies of bivalves have reported impacts of external factors, such as temperature (Monari et al., 2007), salinity (Matozzo et al., 2007) and season (Duchemin et al., 2007) on haemocyte immune parameters, however, there have been few reported investigations of sex-related differences in immune response (Dang et al., 2012, Duchemin et al., 2007, Matozzo and Marin, 2010). The first evidence of sex-related differences in immune parameters of bivalves were reported by Duchemin et al. (2007) who observed significantly higher phagocytic activities in female triploids than in male triploid and female and male diploid Pacific oysters, *Crassostrea gigas*. Female clams (*Ruditapes philippinarum*) have been shown to have significantly higher numbers of granulocytes in the haemolymph than males during the pre-spawning period (Matozzo and Marin, 2010). Since granulocytes are considered to be the major effector cells of the innate immune system in bivalve molluscs (Foley and Cheng, 1975, López et al., 1997, Tripp, 1992), the higher proportion of active haemocytes in female clams is thought to lead to higher endocytotic and haemolymph lysozyme activities and more efficient defence pathways against oxidative stress than those of males (Matozzo and Marin, 2010). In oysters (e.g.,

Saccostrea glomerata and *Pinctada fucata*), although sex-related differences have not been observed for total and differential haemocyte counts or phagocytosis capacity, the intracellular oxidative metabolism is reportedly higher in males than females (Dang et al., 2012). These studies have provided evidence of sex-based differences in some immune parameters. However, whether there are differences in immunological responses of male and female bivalves toward a pathogen infection remain uncertain.

To fill this gap, the aim of this study is to use an integrated approach of flow cytometry (cell viability, oxidative stress and apoptosis) and gas chromatography-mass spectrometry (GC-MS)-based metabolomics to characterize the sex-specific differences in immunological and metabolic responses in haemolymph of Greenshell™ mussels (*Perna canaliculus*) after exposure to *Vibrio* sp. DO1 (*V. coralliilyticus*/*neptunius*-like isolate). This study also provides insight into bivalve immunology and host-pathogen interactions between a bivalve and a *Vibrio* species at the metabolic level.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

Adult mussels (weight: 52.29 ± 9.37 g and shell length: 8.86 ± 0.55 cm) were obtained from Westpac Mussels Distributors Ltd. (Auckland, New Zealand). Upon arrival to the Auckland University of Technology Aquaculture Laboratory, mussels were separated by sex after opening valves slightly to observe gonad colouration. The gonad colour of male mussels is milky white while that of female mussels is yellow orange (Fig. 5.1). Male and female mussels were acclimatized separately in two 50 l tanks for 7 days on a recirculation system as condition described in Chapter 4 (4.2.1). A pool of 20 male mussels (weight: 58.75 ± 14.36 g and shell length: 9.14 ± 0.74 cm) were selected and randomly divided into two equal groups (control male group [n = 10] and infected male group [n = 10]). Females (weight: 46.56 ± 3.56 g and shell length: 8.51 ± 0.40 cm) were similarly grouped. All mussels were placed individually in 10 l plastic tanks (n = 40 tanks) containing 5 l of FSW seawater that was continuously aerated with air stones and maintained at 16 °C.

Mussels within the infection treatment groups were injected in the adductor muscle with 50 µl of *Vibrio* sp. DO1 suspension (10^6 cells·ml^{-1}) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The bacterial strain (*Vibrio* sp. DO1; 99.5 % 16S sequence similarity with *V. corallyliticus* and *V. neptunius*; Genbank: EU358784) was kindly provided by Cawthron Institute (Nelson, New Zealand) and bacterial suspensions were prepared in accordance with the protocol described in Chapter 4 (4.2.1). Mussels in the control group were only injected with 50 µl of PBS. After injection, all animals were returned to their respective tanks. After 24 hour post inoculation (hpi), 1 ml of haemolymph was collected from each animal by gently inserting a needle (25 gauge and 5/8″ needle) attached to a 3 ml sterile syringe

(Terumo, Japan) into the posterior adductor muscle and transferred to 2 ml Eppendorf tubes kept on ice for flow cytometry assays and bacterial counts. Aliquots of 300 µl were additionally transferred to 2 ml cryovials (BioStor™), snap frozen in liquid nitrogen, and stored at –80 °C for metabolomics analyses. After haemolymph withdrawal, mussels were returned to their tanks to observe mortality for an additional 5 days. At the end of the experiment (n = 6 days), additional haemolymph samples were taken from all surviving mussels for metabolomic analyses (n = 4 for infected female mussels and n = 8 for uninfected mussels).

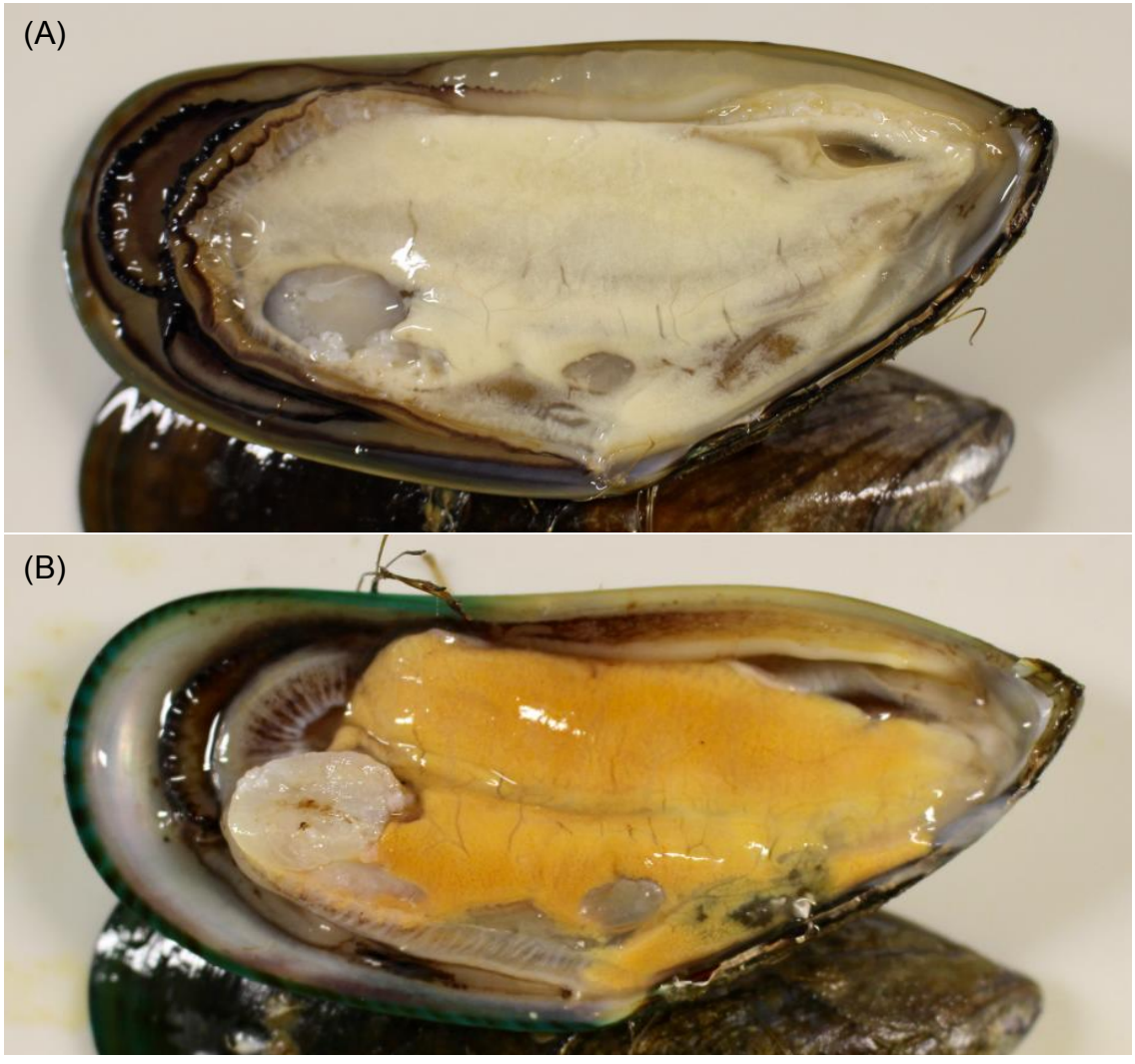

Figure 5.1 Differences in matured gonads of male (A – milky white) and female (B – yellow orange) New Zealand Greenshell™ mussels (*Perna canaliculus*).

5.2.2 Flow cytometric analyses

Flow cytometric analyses of total cell count, haemocyte viability, production of intracellular reactive oxygen species (ROS), and apoptosis were performed using the Muse® Cell Analyzer (Millipore, Hayward, CA, USA) and commercially available assay kits.

Total haemocyte count and viability parameters were measured using the Muse® cell Count and Viability kit (Catalog No. MCH100102, Merck Millipore, USA), following the manufacturer's specifications, as previously described (Chapter 4 – 4.2.3).

Intracellular ROS production (namely superoxide radicals) were measured using the Muse® Oxidative Stress kit (Catalog No. MCH100111, Merck Millipore, USA), following the manufacturer's specifications, as previously described (Grandiosa et al., 2016). This kit uses dihydroethidium (DHE), a reagent that has been extensively used to detect ROS in cells (Bindokas et al., 1996). In brief, haemolymph samples from infected and control mussel groups were diluted in 1X assay buffer at an optimal concentration of 1×10^6 haemocytes per ml, mixed with the Muse® Oxidative Stress working solution (1:20 dilution) and incubated for 30 minutes at 37 °C. The samples were mixed thoroughly and run on the Muse® Cell Analyzer (flow rate = 0.59 $\mu l \cdot sec^{-1}$, number of events = 1042-8058, FSC threshold = 10). The assay provide the quantity and percentage of cells with ROS (ROS +) and without ROS (ROS –).

The proportion of healthy non-apoptotic, early- and late-stage apoptotic and dead haemocytes were quantified using the Muse® Annexin V & Dead Cell kit (Catalog No. MCH100105, Merck Millipore, USA) following the manufacturers specifications. The assay is based on annexin V (annexin V) and 7-aminoactinomycin D (7-AAD) co-staining to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. Bivariate

analysis of annexin V/7-AAD staining distinction of four populations of cells, including non-apoptotic cells (annexin V^-, 7-AAD^-), early-stage apoptotic cells (annexin V^+, 7-AAD^-), late-stage apoptotic cells (Annexin V^+, 7-AAD^+) and dead cells (annexin V^-, 7-AAD^+). Briefly, 100 µl haemolymph was incubated with 100 µl of Muse® Annexin V & Dead Cell reagent for 20 minutes (min) at room temperature prior to flow cytometric analysis on the Muse® Cell Analyzer. The assay was performed at a flow rate = 0.59 $\mu l \cdot sec^{-1}$ and FSC threshold = 29.

5.2.3 Metabolite profiling

Metabolite extractions, derivatizations of mussel samples, GC-MS measurement and quality control were conducted as described in Chapter 4 (4.2.4-5). Raw spectra were processed using automated mass spectral deconvolution and identification system (AMDIS) software (version 2.66) integrated with the MassOmics R-based package (The University of Auckland) and ChemStation software (Agilent Technologies) (Details in Chapter 4 – 4.2.6).

5.2.4 Statistical analyses

Statistical analyses for the log-transformed count data and flow cytometric assays were performed using two-way ANOVA with IBM® SPSS® Statistics software (version 23).

Statistical analyses of metabolite data were conducted using Metaboanalyst 3.0 (Xia et al., 2015). Peak intensity data were normalized by generalized log (glog)-transforming and mean centering. A two-way ANOVA was used to identify differences in metabolite profiles between sex and treatments after 24 hpi (2nd day) and 144 hpi (6th day). To further access the difference between infected and control mussels with a larger data set, we grouped male and female mussel samples after 24 hpi together and performed univariate and multivariate analyses to compare the metabolite profile of infected and uninfected

mussels. The fold change analysis was performed to compare the absolute value of change between control and infected group. Multivariate data analyses including unsupervised principal components analysis (PCA), supervised projection to latent structures discriminant analysis (PLS-DA) and validation of PLS-DA model performance were conducted in accordance with the protocol described in Chapter 4 (4.2.7).

5.2.5 Pathway analysis

Quantitative enrichment analysis (QEA) using global test algorithm (Xia and Wishart, 2010) and network topology analysis (NTA) using relative-betweeness centrality (Nikiforova and Willmitzer, 2007) were performed to investigate functional relationships among the annotated metabolites for pathway analyses. Pathways involving two or more annotated metabolites that matched with the Kyoto encyclopedia of genes and genomes (KEGG) database (Kanehisa and Goto, 2000) with simultaneous QEA p-values < 0.05, QEA false discovery rates (FDRs) < 0.1, and with NTA pathway impact (PI) scores > 0.1 were considered as potential primary pathways of interest, as previously described (Young et al., 2017).

5.3 RESULTS

5.3.1 Mussel mortality

Strong effects of *Vibrio* treatments and host sex were identified within the mussel mortality data (Fig. 5.2A). At 24 hpi, 10 % mortality was recorded in infected groups, while no mortality was observed in control treatments and sex-related differences were not apparent at this time. However, after 36 hpi, infected male mussels experienced 60 % mortality, and 100 % mortality was observed at 144 hpi (6^{th} day). The mortality of infected female mussels increased slowly compared to that of male mussels and reached 60 % at 148 hpi and remained unchanged until the end of the experiment. No mortality was recorded in uninfected female mussels, while uninfected male mussels suffered 20 % mortality by the end of the experiment.

5.3.2 Bacterial accumulation in mussel haemolymph

There was no significant difference in bacterial counts between male and female mussels in both control and infected groups ($p > 0.05$) at 24 hpi. The concentration of *Vibrio* bacteria in the haemolymph of control and infected mussels were 2.46×10^3 and 5.85×10^6 CFU·ml^{-1}, respectively, and these values were significantly different from each other ($F_{1,20} = 101{,}573$; $p < 0.001$). Bacterial colonies on thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates from haemolymph samples of control and infected mussels at 24 hpi and $\log_{10}$ bacterial concentrations are represented in Fig. 5.2B,C. No interaction between sex and treatments was recorded ($p > 0.05$).

5.3.3 Flow cytometric analyses of mussel haemolymph

Although there was no interaction between the effects of sex and bacterial treatments on all parameters (cell viability, ROS production and apoptotic profile) ($p > 0.05$), the effects of *Vibrio* sp. DO1 on mussels were strongly impacted by host sex.

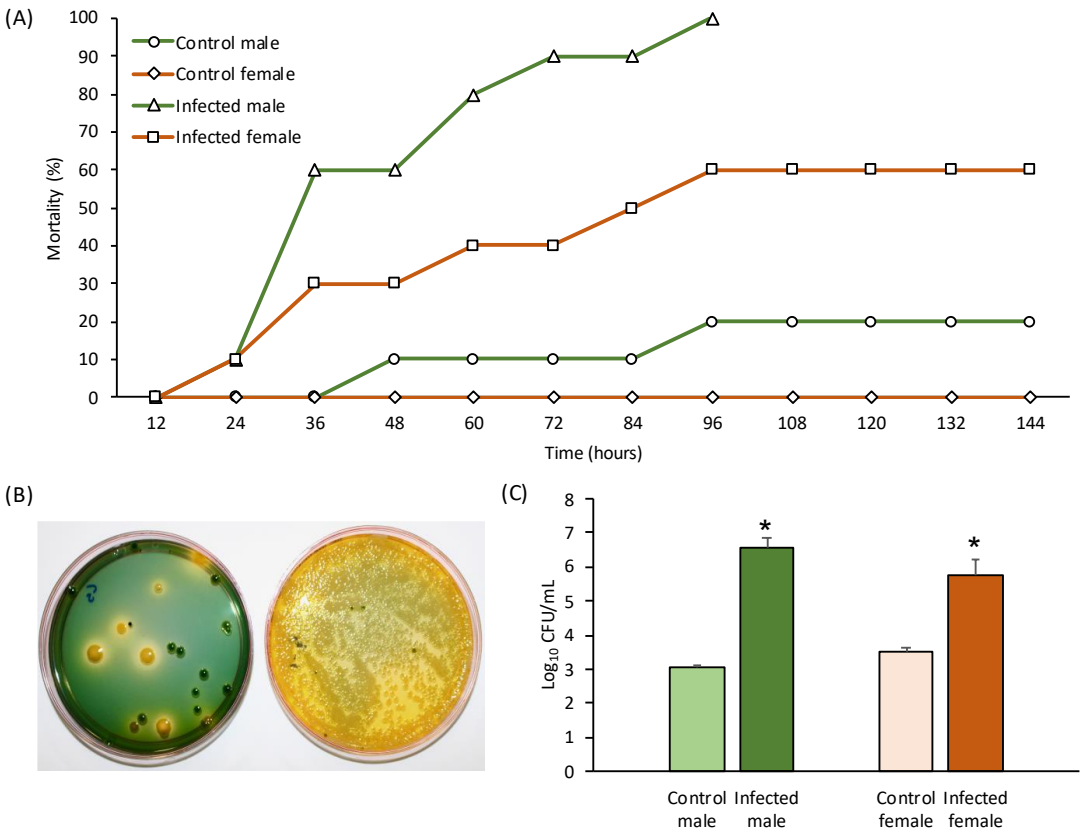

Figure 5.2 (A) Percent mortality of male and female mussels within control and infected groups during the six-day experiment. (B) *Vibrio* colonies on TCBS agar plates from haemolymph samples of control (left, dilution: 10^{0}) and infected mussels (right, dilution: 10^{-3}) at 24 hpi. (C) Log_{10} CFU·ml^{-1} in mussel haemolymph at 24 h post-injection. The error bars represent the standard errors. Significant differences relative to the control and males are marked with an asterisk (*) and two asterisks (**), respectively.

The cell viability profile showed no significant difference between male and female mussels at 24 hpi (p = 0.108), but there was a remarkable decrease in cell viability in infected compared to control mussels ($F_{1,31}$ = 23.12, p < 0.001) (Fig. 5.3A). The percent viability of haemocytes in infected and uninfected mussels were 45.0 % ± 5.4 % and 66.4 ± 3.1 %, respectively.

We observed a significant increase of intracellular ROS production in both male and female mussels exposed to *Vibrio* sp. DO1 compared to their non-exposed counterparts ($F_{1,25}$ = 19.12, p < 0.001) (Fig. 5.3B). There was also a significantly higher level of ROS production in male compared to female mussels in both infected and control groups ($F_{1,25}$

= 4.78, p = 0.038). These findings indicate that infection of *Vibrio* sp. DO1 induced an increase of intracellular ROS level in both male and female groups with higher ROS production in male mussels.

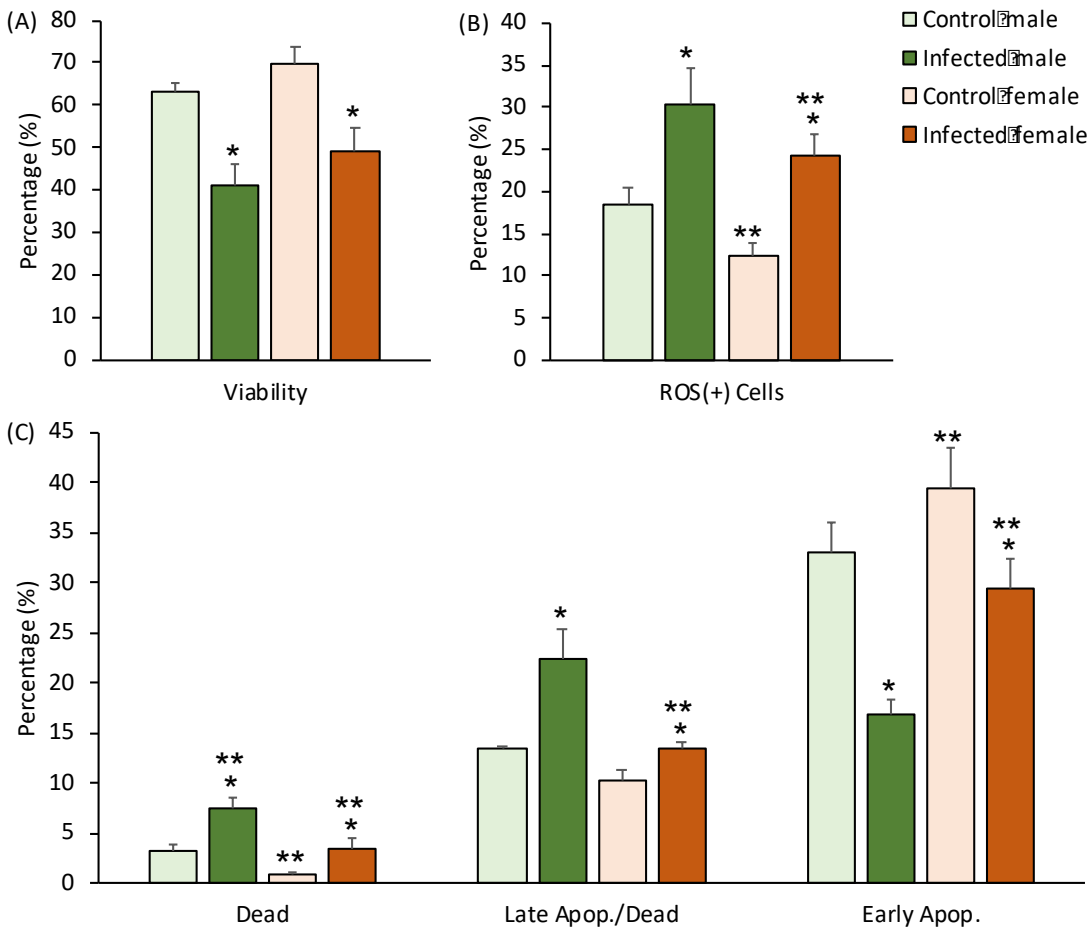

Figure 5.3 Flow cytometric parameters of mussel haemolymph at 24 h post-injection with *Vibrio* sp. A) Viability of haemocytes. B) ROS production. C) Apoptotic cell subpopulations. The error bars represent standard errors. Significant differences relative to the control and males are marked with an asterisk (*) and two asterisks (**), respectively.

There was a significantly higher percentage of dead haemocytes in infected mussels compared to control animals ($F_{1,20}$ = 10.26, p = 0.004), and males had higher numbers of dead cells compared to females ($F_{1,20}$ = 8.74, p = 0.008). Similarly, there were more late apoptotic cells in infected mussels than uninfected mussels ($F_{1,20}$ = 5.80, p = 0.026) and more in infected males than infected females ($F_{1,20}$ = 5.87, p = 0.025). On the other hand,

lower numbers of early apoptotic cells were detected in infected mussels compared to controls ($F_{1,20} = 22.22$, $p < 0.001$). In addition, female mussels in both infected and control groups had a higher percentage of early apoptotic cells than male mussels ($F_{1,20} = 10.81$, $p = 0.004$).

5.3.4 Metabolite profiles of male and female mussels

We identified and annotated 63 metabolites from spectra of mussel haemolymph samples. Two-way ANOVA analyses of metabolite profiles revealed non-significant differences between males and females in both control and *Vibrio* sp. infected groups. There was also no interaction between treatment and sex. This result indicates that there was little effect of sex on the metabolic responses in mussel haemolymph as a result of the *Vibrio* sp. challenge.

5.3.5 Metabolomics responses of mussels to *Vibrio* sp. DO1

Although metabolite profiles of male and female mussels were similar post-pathogen challenge, exposure to *Vibrio* sp. DO1 led to alterations in 37 metabolites in infected mussels compared to control mussels (two-way ANOVA; $p < 0.05$). The list of these metabolites with fold change values ($\log_2$ fold change [infected/control] of the untransformed data) is represented in Fig. 5.4A. Among them, 13 metabolites were up-regulated, while 24 metabolites were down-regulated in infected mussels, compared to uninfected controls.

Using the combined male and female mussel samples, we performed multivariate analyses to compare the metabolite profile of infected and uninfected mussels. A PCA revealed a clear separation between infected and uninfected mussels along PC1 (Fig. 5.4B). The PCA score plot corresponding to the first two principal components demonstrated that PC1 and PC2 explained 50.0 % and 24.9 %, respectively, of the dataset

total variance. Compared to the distribution of uninfected mussels in the score plot, that of infected mussels is more dispersed which may indicate different infection levels in

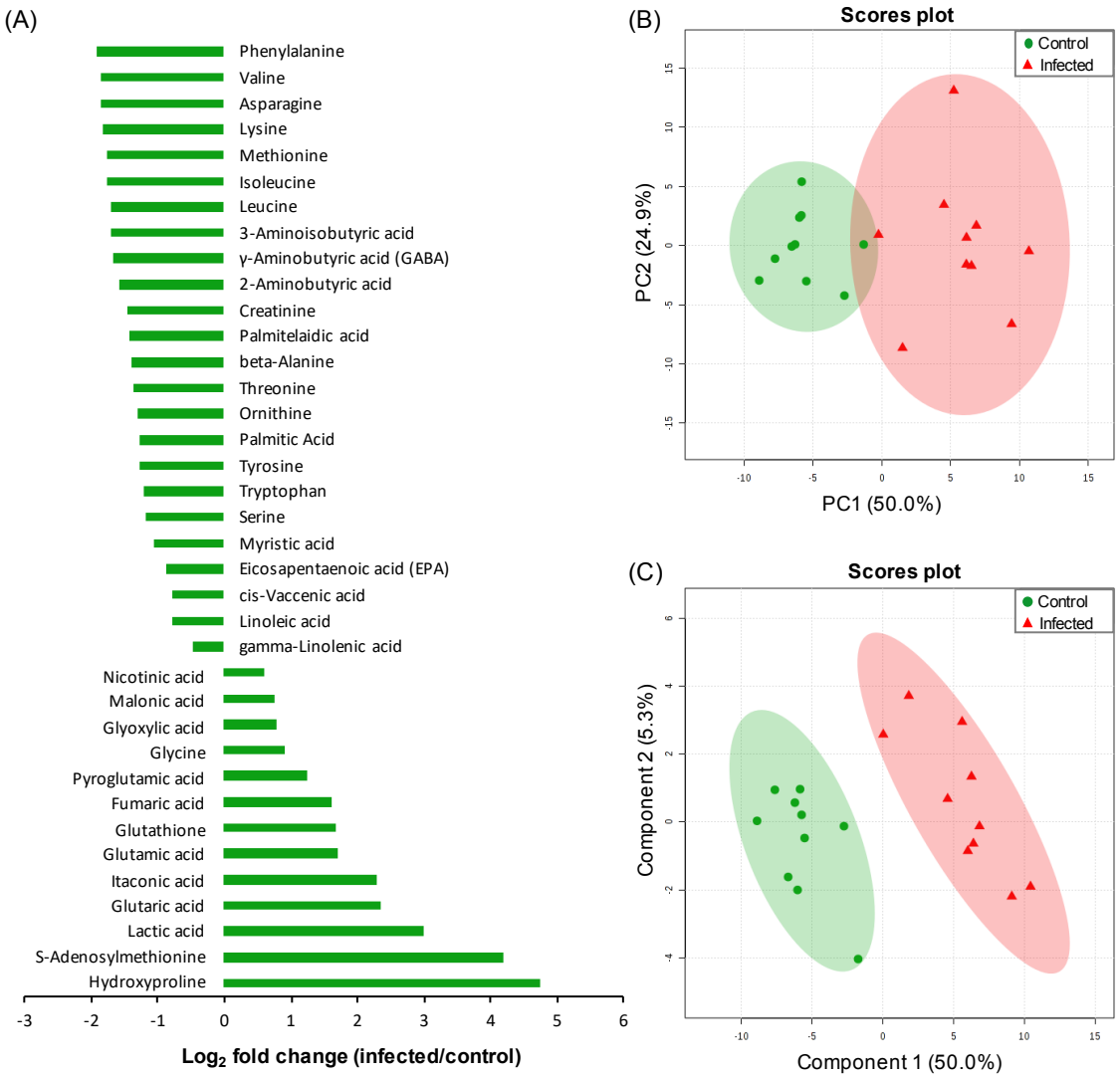

Figure 5.4 Univariate and multivariate analyses of metabolite profiles from infected and uninfected mussels at 24 h post-injection. (A) List of 43 metabolites identified as differently expressed between the two mussel groups (two-way ANOVA; $p < 0.05$); where x axis = $\log_2$ fold change (infected/control) of the untransformed data. (B) Principal component analysis (PCA) 2D score plot (95 % confidence interval ellipses). (C) Projection to latent structure discriminant analysis (PLS-DA) 2D score plot (95 % confidence interval ellipses).

mussels exposed to *Vibrio* sp. DO1. In addition, a PLS-DA model (Fig. 5.4C) showed high discriminative and predictive capability with an accuracy of 93 %, R^2 of 90 %, and Q^2 of 78 % (three component model). Twenty-seven metabolites with VIP scores > 1.0 were identified as contributing strongly to the model (Table 5.A.1 in Appendix). All of

these important classifiers were identified as differently expressed between infected and control mussels by two-way ANOVA.

5.3.6 Pathway analysis

Combined QEA and NTA analysis identified 42 biochemical pathways within the KEGG database that were relevant based on the list of metabolites we obtained. Among these, 15 pathways involving at least two annotated metabolites with simultaneous QEA p-values < 0.05, QEA FDR values < 0.1, and NTA pathway impact (PI) values > 0.1 were screened as potential primary target pathways of interest relating to the treatment effect (Table 5.1).

Table 5.1 List of altered metabolic pathways that were identified as primary target pathways in metabolite profiles of mussels exposed to *Vibrio* sp.

Pathways	Hits/ Total compounds	Raw p	FDR	Impact
Glycine, serine and threonine metabolism	5/31	<0.001	<0.001	0.570
Cysteine and methionine metabolism (transsulfuraiton pathway)	4/29	<0.001	<0.001	0.338
Valine, leucine and isoleucine biosynthesis	5/13	<0.001	<0.001	1.000
Aminoacyl-tRNA biosynthesis	17/67	<0.001	<0.001	0.103
Arginine and proline metabolism	6/43	<0.001	<0.001	0.322
Methane metabolism	2/9	<0.001	<0.001	0.400
Alanine, aspartate and glutamate metabolism	7/24	<0.001	<0.001	0.570
Phenylalanine, tyrosine and tryptophan biosynthesis	2/4	<0.001	0.001	1.000
Phenylalanine metabolism	2/11	<0.001	0.001	0.407
Tyrosine metabolism	4/44	<0.001	0.001	0.164
Glutathione metabolism	6/26	0.005	0.008	0.418
beta-Alanine metabolism	2/16	0.013	0.017	0.395
Citrate cycle (TCA cycle)	4/20	0.014	0.018	0.136
Glyoxylate and dicarboxylate metabolism	3/18	0.039	0.048	0.148
Tryptophan metabolism	2/39	0.041	0.049	0.155

5.3.7 Metabolomics profiling of female mussels that recovered from *Vibrio* infection

Forty percent of female mussels were alive 6 days after injection with *Vibrio* sp. DO1. These mussels had shown signs of infection (e.g., slow closing valves when disturbed, low haemocyte viability) after 24 hpi, but there were signs of recovery (e.g. faster closing valves when disturbed) on the sixth day. Haemolymph metabolite profiles from these mussels revealed significant changes in seven metabolites compared to those of uninfected controls, and from infected mussels at 48 hpi. These metabolites included itaconic acid, isoleucine, phenylalanine, creatinine, malonic acid, glutaric acid and hydroxyproline. Among them, itaconic acid was identified as highly different by both treatment and sampling time ($p < 0.05$). Indeed, itaconic acid was significantly higher in infected mussels compared to infected mussels after 24 hpi, but it was decreased on the sixth day. Isoleucine, phenylalanine and creatinine were only determined as different between two sampling times, whereas malonic acid, glutaric acid and hydroxyproline were only different between treatments (Fig. 5.5). Two-way ANOVA showed no interaction effect between treatments and sampling times ($p > 0.05$).

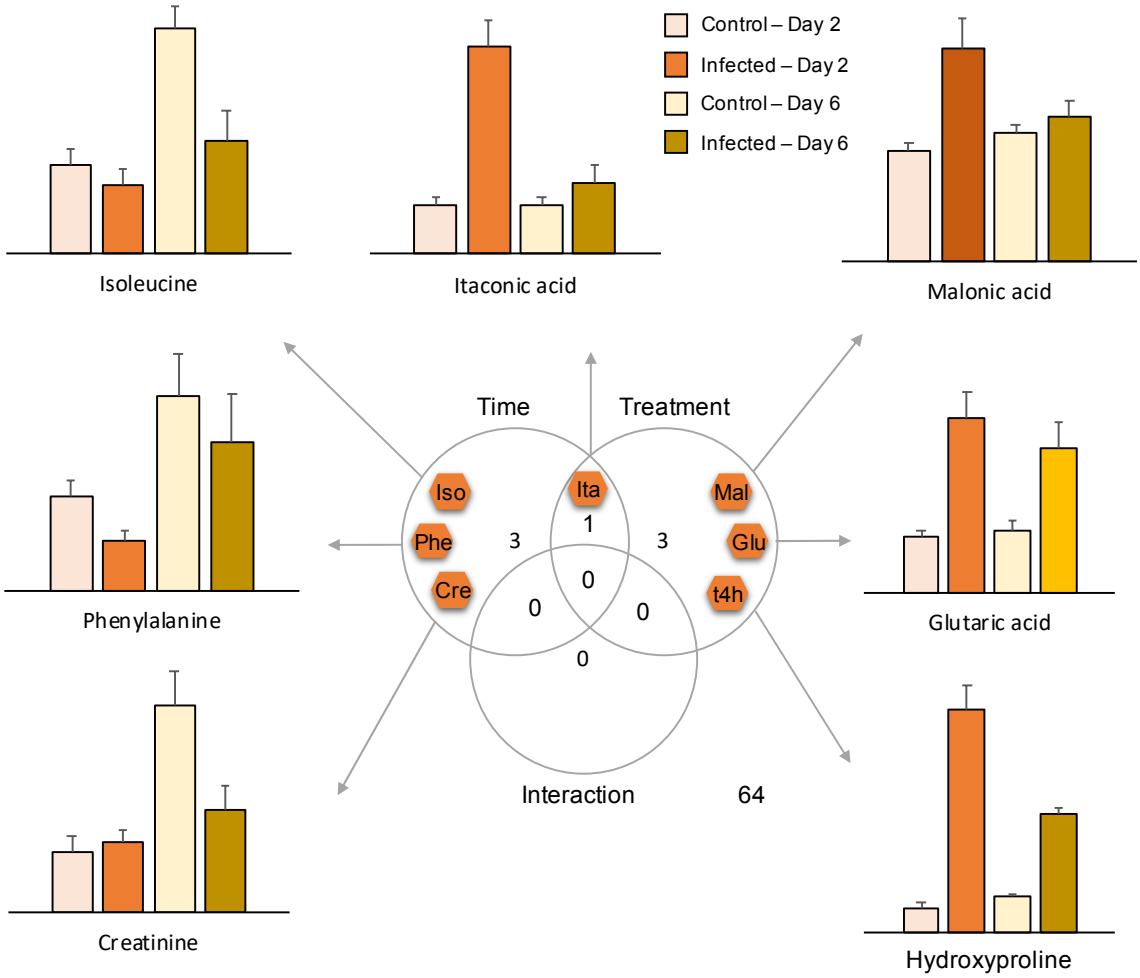

Figure 5.5 Alterations of metabolites in haemolymph of female mussels at 2nd and 6th day post-infection identified by two-way ANOVA ($p < 0.05$). The error bars represent standard errors.

5.4 DISCUSSION

Bacteria belonging to the genus *Vibrio* (e.g. *V. aestuarianus*, *V. coralliilyticus, V. tubiashii*, *V. tapetis, V. spendidus* and *V. harveyi*) have been associated with a number of infectious diseases in marine bivalves (reviewed by Travers et al. 2015). Among them, infections with *V. coralliilyticus* have been reported in several marine organisms, including coral (*Pocillopora damicornis)* (Ben-Haim et al., 2003, Rozenblat and Rosenberg, 2004), fish (Austin et al., 2005) and shellfish (Kesarcodi-Watson et al., 2012, Richards et al., 2015). A *V. corallyliticus*-like isolate (*Vibrio* sp. DO1, 99.5 % 16S rDNA sequence similarity with *V. corallyliticus*, GenBank: EU358784) was identified as a highly pathogenic species for New Zealand Greenshell™ mussel larvae (*P. canaliculus*) (Kesarcodi-Watson et al., 2009a, Kesarcodi-Watson et al., 2009b). In the present study, we challenged *P. canaliculus* with injections of 50 µl of *Vibrio* sp. at a concentration of 10^6 cells·ml^{-1} ($5x10^4$ cells/mussel) and observed mortality of 100 % in males and 60 % in females after six days. The successful challenge protocol allowed us to investigate whether there were sex-based responses elicited by *Vibrio* sp. in *P. canaliculus* and provided insights into the immunological and metabolic responses of mussels toward *Vibrio* infection.

5.4.1 Immunological responses of mussels to *Vibrio* infection

Generation of ROS at a basal level is a general protective mechanism of most organisms against stressors (e.g., pathogen, contaminants) (Torreilles et al., 1996). However, an imbalance between the production of ROS and antioxidants in favour of ROS can lead to oxidative stress, loss of cell function and cell death (Winston, 1991). The increase of ROS in haemolymph in response to pathogenic bacteria or stimulation with pathogen-associated molecular patterns has been previously reported in several marine bivalves (Buggé et al., 2007, Costa et al., 2009, Goedken and De Guise, 2004, Lambert et al.,

2003). It has also been demonstrated that accumulation of ROS could induce apoptosis under both pathologic and physiological stress (Guo et al., 2017, Pierce et al., 1991, Simon et al., 2000). For example, a study on molecular responses to copper stress in *L. vannamei* confirmed that inhibitor of apoptosis protein (IAP) interacts with caspase-3 to regulate apoptosis caused by superfluous ROS generation (Guo et al., 2017). In the present study, we observed significantly higher ROS production in *Vibrio*-infected mussels and an increased percentage of dead and late-stage apoptotic cells. This suggests that generation of ROS during a *Vibrio* infection could result in oxidative stress and eventual cell death by apoptosis.

Interestingly, there were significant differences between male and female mortality, ROS production and apoptotic profiles of haemocytes. Some sex-related differences in immune parameters of bivalve haemocytes have previously been demonstrated (Dang et al., 2012, Duchemin et al., 2007, Matozzo and Marin, 2010). Bivalve haemocytes are generally divided into three types of cells, including granulocytes, hyalinocytes and small agranulocytes (Dang et al., 2012). Among these, granulocytes are considered to be the major effector cells of the innate immune system of bivalves that participate in a number of antimicrobial activities, especially in phagocytosis (Foley and Cheng, 1975, López et al., 1997, Tripp, 1992). The higher proportion of granulocytes in females than males in the clam *R. philippinarum* was suggested to lead to higher phagocytosis capacity and more efficient defence pathways against oxidative stress in females than males (Matozzo and Marin, 2010). In the present study, we observed higher mussel mortality and ROS production of haemocytes in male mussels compared to females, suggesting that males are more susceptible to oxidative stress. Similarly, higher percentages of dead and late-stage apoptotic haemocytes were recorded in males. These results suggest that female mussels may have a more efficient apoptotic pathway against pathogen infection than

male mussels, which leads to a lower mortality in females. Hence, it is important to take into account the impact of intrinsic parameters, such as host sex when studying the immune parameters of marine bivalves.

5.4.2 Metabolic responses of mussels to *Vibrio* infection

No sex-based differences in metabolite profiles of male and female mussel haemolymph were observed after 24 h post-challenge with *Vibrio* sp. DO1. However, when all mussels were grouped by infection status, infected mussels displayed clear changes in levels of metabolites. Among these, 13 metabolites were up-regulated in infected mussels compared to control (uninfected) mussels. Accumulation of these metabolites are thought to be associated with several innate immune responses, such as oxidative stress and apoptosis. For example, the increase of itaconic acid (ITA) in infected mussels may be a result of antimicrobial activity as part of the host's defence system. ITA is an unsaturated dicarboxylic acid, and is synthesized in mammalian immune cells during macrophage activation (Sugimoto et al., 2012), and more recently was reported in marine bivalves following bacterial and viral infections (Nguyen et al., 2018, Young et al., 2017). The role of ITA has also recently been identified as an immune-supportive metabolite involved in regulation of succinate levels, mitochondrial respiration and inflammatory cytokine production during macrophage activation (Lampropoulou et al., 2016). The gene responsible for its synthesis, immune-responsive gene 1 (Irg1), was over-expressed in activated macrophages during pathogen infection (e.g., *Mycobacteria*, *S. enterica*) or LPS-stimulation (Basler et al., 2006, Li et al., 2013, Michelucci et al., 2013, Shi et al., 2005). *Irg1* encodes for immune-responsive gene 1 protein/cis-aconitic acid decarboxylase (IRG1/CAD) which catalyses production of ITA from cis-aconitate in vitro (Michelucci et al., 2013, Vuoristo et al., 2015). In the present study, ITA was identified as an important metabolite for PLS-DA classification and prediction model,

and its level was elevated in mussels exposed to *Vibrio* sp. DO1, suggesting that invertebrate immune cells are able to produce ITA similar to mammalian cells infected by bacteria. Interestingly, in infected female mussels which survived the challenge duration, ITA showed a remarkable decrease 6 days post-inoculation compared to levels at 2 days post-inoculation. These mussels were unable to close their valves when disturbed on the third day post-inoculation but showed a significant recovery (faster closing valves) in the following days. These results suggest the potential use of ITA as a biomarker for pathogen infection and/or health status in marine bivalves.

We observed accumulations of glutamic acid, glycine and hydroxyproline in haemolymph of infected mussels. Glutamic acid is a key compound in cellular metabolism, and the accumulation of extracellular glutamic acid appeared to be involved in tissue damage (Amaya et al., 2013, Hassel et al., 2014). Similarly, glycine is another amino acid in collagen, and increase of glycine were found to be relative to degraded collagen (Turban-Just and Schramm, 1998). Hydroxyproline is a major component of protein collagen (Etherington and Sims, 1981), and accumulation of hydroxyproline in serum and urine has been known to be associated with degradation of connective tissue (Buccino et al., 1969, Kelleher, 1979, Ofulue and Thurlbeck, 1988), muscle damage (Nogueira et al., 2011), depression, and stress (Lee et al., 2011). The elevated levels of these metabolites in infected mussels may suggest collagen degradation and tissue damage caused by infection of *Vibrio* sp. Interestingly, the decrease of hydroxyproline in infected mussels on the sixth day, like ITA, may be relevant to the recovery of mussels from infection.

In agreement with the finding of increased ROS production in infected mussels, accumulations of metabolites in the transsulfuration pathway (i.e., S-

Adenosylmethionine) and glutathione (GSH) metabolism (i.e., GSH, glutamic acid, pyroglutamic acid, glycine) further substantiate oxidative stress in haemocytes as a response to bacterial infections. GSH metabolism plays a critical role in regulating ROS and defending cells against oxidative stress (Hayes and McLellan, 1999). Oxidation of GSH directly scavenges ROS by metabolising hydrogen peroxide into water (Espinosa-Diez et al., 2015) or indirectly is involved in repairing ROS-induced damage (Apel and Hirt, 2004, Knight, 2000). Although the alteration of GSH as a metabolic response to pathogen infection has not yet been reported in marine bivalves, the expression of antioxidant enzymes, which regulates GSH turnover and/or the genes which encode them have been described (Corporeau et al., 2014, He et al., 2015, Schmitt et al., 2013). Changes in levels of glycine, glutamic acid and pyroglutamic acid also indicated an effect on GSH regulation, which culminated in the GSH metabolic pathway being identified as a perturbed system via secondary pathway analyses (Table 5.1). Furthermore, pronounced accumulations of S-Adenosylmethionine (SAMe) and subtle but coordinated changes in levels of methionine and serine also led to the transsulfuration pathway being detected as likely being affected. The transsulfuration pathway was identified as an altered metabolic pathway due to the *Vibrio* effect (Table 5.1). In transsulfuration, SAMe is transformed through a series of enzymatic steps to cysteine, a precursor of GSH (McBean, 2012). In combination, these results support the importance of the ROS-regulatory system in bivalve immunity to against pathogen infections.

The accumulation of malonic acid, fumaric acid, lactic acid and ITA in infected mussels suggests that the TCA cycle functioning of the host was affected by *Vibrio* sp. infection. Accumulations of TCA intermediates in activated mice macrophages are a consequence of TCA cycle interruption (Jha et al., 2015). Similarly, elevated levels of TCA intermediates have been reported in marine bivalves challenged with pathogens (Nguyen

et al., 2018, Young et al., 2017). In a previous pilot study, we challenged *P. canaliculus* with a higher dose (10^7 cells·ml^{-1}) of *Vibrio* sp. DO1 and observed significant increases in three metabolites within the TCA cycle (succinic acid, fumaric acid and malic acid). At the lower dose (10^6 cells·ml^{-1}) used in this study, we observed only accumulation of fumaric acid. However, increased levels of malonic acid, ITA and lactic acid also indicate disruption to basal cellular respiration. Malonic acid is a competitive inhibitor of succinate dehydrogenase in the TCA cycle (Pardee and Potter, 1949), which may limit oxidation of succinic acid to fumaric acid in infected mussels. The accumulation of citrate due to the break in the TCA cycle has been shown to generate ITA via the enzyme immune-responsive gene 1 (IRG1) (Michelucci et al., 2013). Production of ITA from its cis-aconitate precursor, also indicates disruption of the TCA cycle as previously reported in mammals and marine bivalves (Michelucci et al., 2013, Nguyen et al., 2018, Young et al., 2017). The increase in ITA, together with other intermediates of TCA cycle have been previously reported for Greenshell™ mussels exposed to *Vibrio* sp. (Nguyen et al., 2018). Lactic acid is the end product of anaerobic glycolysis (Bakker et al., 2013, Rogatzki et al., 2015). In addition, the TCA cycle itself was identified as being affected by *Vibrio* infection via secondary pathway analysis (Table 5.1). The accumulation of lactic acid in our study suggests that the TCA cycle was also being disrupted by a switch towards anaerobic respiration, and potentially limiting conversion of pyruvate to citrate.

Vibrio sp. DO1 led to the decrease of 29 metabolites in mussel haemolymph. Many of them are important amino acids that are involved in energy metabolism and protein synthesis. The decrease of amino acids, such as branched chain amino acids (BCAAs), creatinine and linoleic acid in infected mussels suggests the disruption of amino acid metabolism caused by *Vibrio* sp. DO1. BCAAs, including valine, leucine and isoleucine have diverse metabolic and physiological roles, such as protein synthesis, signalling

pathways, and glucose and energy metabolism, among others (Monirujjaman and Ferdouse, 2014). Pathogen infections are known to lead to remarkable increases in demand of BCAAs for energy and substrates for biosynthesis of new protective molecules by the immune system (Calder, 2006). In our study, the significant depletion of BCAAs in the haemolymph of infected mussels suggests that there was a high demand of BCAAs for host energy production in response to *Vibrio* sp. DO1 infection. Creatinine is a breakdown product of high-energy compound phosphocreatine in muscle tissues (Taylor, 1989), and is a valuable source of carbon and nitrogen (Yoshimoto et al., 2004). The decrease of creatinine in this study may also be a result of the high energy demand of infected mussels. The lower levels of free amino acids in infected mussels suggest disruptions in protein and amino acid metabolism. In fact, many amino acid metabolisms were altered in infected mussels compared to control mussels (Table 5.1). Among these amino acids, decreases in tyrosine, phenylalanine and lysine have also been reported in metabolic responses of abalone (*H. diversicolor*) to *V. parahaemolyticus* infection (Lu et al., 2017). Interestingly, levels of creatinine, isoleucine and phenylalanine increased in infected but recovering female mussels on the sixth day compared to the second day post-injection. These changes, along with the decreases in ITA and hydroxyproline towards baseline levels as discussed above, support that surviving female mussels were recovering after being exposed to *Vibrio* sp. DO1. These results also suggest that the alterations of metabolites such as ITA, hydroxyproline and amino acids could be used to indicate the health status of mussels.

5.5 CONCLUSION

This study is the first to report on sex-based differences in immune responses of mussels exposed to a pathogenic *Vibrio*. Female mussels appeared to have a more effective defence response against pathogen infection than male mussels. However, there was no significant difference in the metabolic profiles measured in haemolymph of males and females following *Vibrio* sp. DO1 infection. The infection of *Vibrio* sp. DO1 caused an alteration of 43 metabolites in the mussel haemolymph that are involved in many important immune activities, including oxidative stress, apoptosis, energy metabolism, amino acid metabolism and protein biosynthesis. We discovered that mussel immune cells are able to produce ITA similar to mammalian macrophages during bacterial infection. The change of ITA during *Vibrio* infection may be associated with mussel immune responses and infection status. Although recent studies have revealed the role of ITA as a mammalian antimicrobial metabolite, there are no previous reports on the involvement of ITA in bivalve immune system. Further studies should be directed towards ITA and its antimicrobial function in invertebrate immune cells to provide data for identification of new intervention points and develop remediation strategies for infectious diseases in aquaculture. The application of flow cytometry and metabolomics provide complementary information regarding sex differences and metabolic responses that can be used to explore new research questions in this field.

5.6 APPENDIXES

Table 5.A.1 List of metabolites identified as differently expressed between infected and control mussels.

Compounds	Two-way ANOVA			Log(FC)	PLS-DA VIP Score	*Vibrio* effect
	Treatment	Gender	Interaction			
Hydroxyproline	0.001	0.830	0.365	4.748	3.173	↑
S-Adenosylmethionine	0.001	0.779	0.730	4.184	2.539	↑
Lactic acid	0.002	0.987	0.777	3.007	1.639	↑
Asparagine	<0.001	0.334	0.530	-1.853	1.608	↓
Valine	<0.001	0.322	0.730	-1.869	1.523	↓
Phenylalanine	<0.001	0.107	0.730	-1.925	1.496	↓
Itaconic acid	0.001	0.344	0.974	2.290	1.442	↑
Isoleucine	<0.001	0.331	0.730	-1.757	1.382	↓
Glutaric acid	0.006	0.628	0.730	2.344	1.377	↑
Leucine	<0.001	0.538	0.736	-1.708	1.369	↓
3-Aminoisobutyric acid	<0.001	0.614	0.730	-1.688	1.367	↓
γ-Aminobutyric acid (GABA)	<0.001	0.628	0.740	-1.668	1.340	↓
Methionine	<0.001	0.628	0.730	-1.760	1.305	↓
Glutamic acid	0.010	0.852	0.199	1.706	1.302	↑
Tyrosine	0.002	0.107	0.530	-1.258	1.249	↓
Glutathione	0.009	0.948	0.232	1.678	1.244	↑
2-Aminobutyric acid	<0.001	0.770	0.730	-1.582	1.226	↓
Lysine	0.006	0.364	0.691	-1.812	1.213	↓
Fumaric acid	0.007	0.770	0.691	1.622	1.196	↑
Ornithine	0.010	0.779	0.530	-1.290	1.171	↓
Tryptophan	0.045	0.261	0.740	-1.203	1.160	↓
Palmitelaidic acid	0.001	0.096	0.934	-1.430	1.156	↓
Creatinine	<0.001	0.334	0.740	-1.470	1.151	↓
Threonine	<0.001	0.533	0.530	-1.372	1.142	↓
beta-Alanine	<0.001	0.322	0.416	-1.399	1.109	↓
Serine	0.005	0.852	0.416	-1.195	1.019	↓
Palmitic acid	<0.001	0.106	0.928	-1.259	1.013	↓
Pyroglutamic acid	0.032	0.948	0.265	1.240	0.985	↑
Glycine	0.003	0.183	0.099	0.895	0.974	↑
Myristic acid	0.001	0.106	0.767	-1.061	0.886	↓
EPA	0.034	0.106	0.740	-0.883	0.759	↓
cis-vaccenic acid	0.005	0.118	0.767	-0.794	0.597	↓
Linoleic acid	0.006	0.106	0.767	-0.783	0.588	↓
Glyoxylic acid	0.004	0.948	0.767	0.770	0.522	↑
Malonic acid	0.015	0.948	0.691	0.762	0.515	↑
Nicotinic acid	0.009	0.948	0.538	0.615	0.478	↑
gamma-linolenic acid	0.047	0.106	0.666	-0.472	0.324	↓

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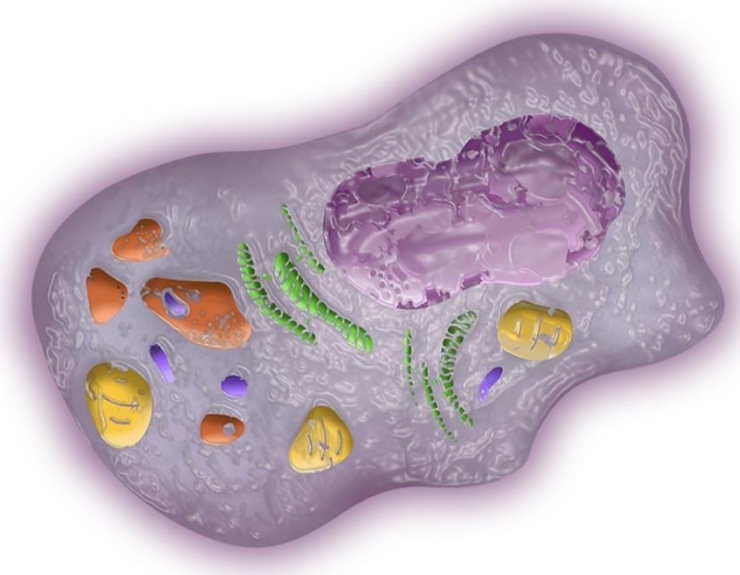
Section III

Metabolomics profile of New Zealand Greenshell™ mussel (*P. canaliculus*) haemocytes



Mussel haemocyte

In this section:

Chapter 6

Metabolic and immunological responses of mussel (*Perna canaliculus*) haemocytes to *Vibrio* sp.

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ABSTRACT

Vibrio coralliilyticus is a bacterial pathogen which can affect a range of marine organisms, such as corals, fish and shellfish, with sometimes devastating consequences. However, little is known about the mechanisms involved in the host-pathogen interaction, especially within molluscan models. We applied gas chromatography-mass spectrometry (GC-MS)-based metabolomics to characterize the physiological responses in haemolymph of New Zealand Greenshell™ mussels (*Perna canaliculus*) injected with *Vibrio* sp. DO1 *(V. coralliilyticus/neptunius*-like isolate). Univariate data analyses of metabolite profiles in *Vibrio*-exposed mussels revealed significant changes in 22 metabolites at 6 hours post-infection, compared to non-exposed mussels. Among them, 10 metabolites were up-regulated, while 12 metabolites were down-regulated in infected mussels. Multivariate analyses showed a clear distinction between infected and non-infected mussels. In addition, secondary pathway analyses indicated perturbations of the host innate immune system following infection, including oxidative stress, inflammation, and disruption of the tricarboxylic acid (TCA) cycle, change in amino acid metabolism and protein synthesis. These findings provide new insights into the pathogenic mechanisms of *Vibrio* infection of mussels, and demonstrate our ability to detect detailed and rapid host responses from haemolymph samples using a metabolomics approach.

6.1 INTRODUCTION

Bacteria in the genus *Vibrio* (e.g. *V. aestuarianus*, *V. tubiashii*, *V. coralliilyticus*, *V. tapetis, V. spendidus* and *V. harveyi*) have been associated with a number of infectious diseases in marine bivalves (reviewed by Travers et al. 2015). Among them, infections with *V. coralliilyticus* have been reported in several marine organisms. This bacterium is a well-known coral pathogen that causes tissue lysis of *Pocillopora damicornis* in the Indian Ocean and Red Sea (Ben-Haim et al., 2003, Rozenblat and Rosenberg, 2004). Recently, it has been shown to cause mortality in a number of fish and shellfish species (Austin et al., 2005, Genard et al., 2013). *V. coralliilyticus* is a gram-negative, motile and facultative anaerobic bacterium (Plumb and Hanson, 2011), which is phylogenetically related to *V. tubiashii* (Ben-Haim et al., 2003). Therefore, many marine isolates of *V. coralliilyticus* from shellfish were misidentified as *V. tubiashii* until recently when the genomes of several *V. coralliilyticus* strains have been sequenced (Kehlet-Delgado et al., 2017, Richards et al., 2014). Therefore, previous emergences of *V. tubiashii* in bivalve shellfish aquaculture on the west coast of North America (Brown, 1981, Dumbauld et al., 2011, Elston et al., 1981, Elston et al., 2008) were possibly caused by *V. coralliilyticus* (Richards et al., 2015). In previous work, pathogen challenges with *V. coralliilyticus* induced significant larval mortality in New Zealand green-lipped mussels (*P. canaliculus*) (Kesarcodi-Watson et al., 2009a, Kesarcodi-Watson et al., 2009b), great scallops (*Pecten maximus*), European flat oysters (*Ostrea edulis*) (Kesarcodi-Watson et al., 2012), Eastern oysters (*Crassostrea virginica*) and Pacific oysters (*Crassostrea gigas*) (Richards et al., 2015). These suggest that *V. coralliilyticus* is an important pathogen for bivalves that has contributed to dramatic losses in shellfish aquaculture worldwide in recent years. Despite the fact that *V. coralliilyticus* appear to be a global bivalve pathogen, there is limited information about its pathogenicity, infection mechanism and/or disease

mitigation.

As filter feeders, bivalves are always exposed to pathogen-rich environments, and mostly rely on an innate immune system with non-specific defence mechanisms composed of cellular and humoral components (Song et al., 2010). Cellular components include haemocytes and epithelial cells, whereas humoral components include various active molecules secreted by haemocytes and released into the haemolymph (Allam and Raftos, 2015, Song et al., 2010). Haemocytes are considered to be the backbone of the bivalve immune system and they play major roles in phagocytosis, encapsulation and nacrezation of invading pathogens and parasites (Allam and Raftos, 2015, Anderson and Good, 1976, Song et al., 2010). These specialized cells are also involved in other biological functions, such as wound healing, food digestion, transport of nutrients, gonad resorption, shell formation and secretion of humoral factors (Allam and Raftos, 2015, Bachère et al., 2015). Despite a recent proliferation of cellular and molecular studies focusing on bivalve immunology (Allam and Raftos, 2015, Bachère et al., 2015), immune response to pathogenic infections of bivalves is not fully understood (Bassim et al., 2015, Estes et al., 2004, Yue et al., 2013). Part of the reason for this lack of understanding may be that current immunological and molecular biology techniques are still not sufficiently advanced to clearly elucidate the complex mechanisms of infections within a host-pathogen model.

'Omics' is an encompassing term for relatively new approaches to the study of large sets of biological molecules, and comprises genomics, transcriptomics, proteomics and metabolomics (Smith et al., 2005). The advancement of high-throughput technologies and rapid development of bioinformatics have led to an expansion of omics applications in a variety of life science fields (Alfaro and Young, 2018, Gómez-Chiarri et al., 2015,

Guo and Ford, 2016). Amongst these omics, transcriptomics (the study of the transcriptome, or group of mRNA molecules present in a biological system) has been widely used in many areas of aquaculture research, such as selective breeding, stress physiology and toxicology, developmental biology, disease resistance and immunology (reviewed by Li (2014). In bivalve immunity studies, high-throughput transcriptomics has been used for finding the immune-related genes/ transcripts or pathways in oysters (Fleury et al., 2009, Rosa et al., 2012, Zhang et al., 2014), mussels (Moreira et al., 2014, Philipp et al., 2012, Venier et al., 2009), clams (Hasanuzzaman et al., 2017, Milan et al., 2011, Moreira et al., 2012) and scallops (Pauletto et al., 2014, Sun et al., 2014, Zhang et al., 2017). Compared to transcriptomics, metabolomics (study of the complete set of small-molecules or metabolites present in cells, tissue body fluids and entire organisms) is relatively new and its application in aquaculture research is limited (Alfaro and Young, 2018, Young and Alfaro, 2016). However, since metabolites are the end-products of cellular regulatory processes, the metabolome (total set of metabolites within a biological sample) is highly sensitive to environmental change. Thus, metabolomics can represent a better picture of what is actually happening in the organism at a given time (Alfaro and Young, 2018). Due to its strong predictive power of phenotypes, metabolomics may provide a novel tool for understanding endogenous metabolic changes caused by many diseases across a range of host-pathogen interactions. Furthermore, metabolomics approaches can be used in early diagnosis of diseases, leading to the development of therapies and health monitoring systems.

In the present study, we report on the application of GC-MS-based metabolomics approach integrated with flow cytometry to characterize the metabolic and immunological responses in haemolymph of New Zealand Greenshell™ mussels (*Perna canaliculus*) following infection with *V. corallyliticus*-like isolate. This is an innovative

metabolomics-based study of non-lethally collected haemolymph as the target sample to investigate the interaction between a bivalve host and a marine pathogen. The knowledge acquired from this study leads to a better understanding of *V. coralliilyticus* pathogenicity and future development of disease management strategies in aquaculture production.

6.2 MATERIALS AND METHODS

6.2.1 Animal preparation and challenge experiment

Adult mussels were obtained from Westpac Mussels Distributors Ltd (Auckland, New Zealand) and acclimatized for 7 days in a re-circulation system as the condition described in Chapter 4 (4.2.1). Only male mussels were used in this experiment to avoid potential variations due to gender-specific responses, as previously reported for other species (Liu et al., 2014b, Lu et al., 2017). A pool of 20 mussels were selected (mean weight = 75.96 ± 10.29 g; mean shell length = 10.24 ± 0.62 cm), and randomly divided into two equal groups, and placed into 50 l rectangular plastic tanks filled with continuously aerated FFSW.

The challenge experiment was performed with the bacterial suspension of *Vibrio* sp. DO1 (*V. coralliilyticus*/*neptunius*-like isolate) which was prepared as the protocol described in Chapter 4 (4.2.1). Ten mussels were injected with 50 µl of *Vibrio* sp. DO1 suspension (10^7 cells·ml^{-1}) into the adductor muscle. The ten remaining mussels (control group) were injected with 50 µl of PBS only. After injection, all animals were put back into their respective tanks. After 6 hours post-injection, approximately 1 ml of haemolymph was collected from each animal (Chapter 4 – 4.2.1). Immediately after withdrawal, samples were transferred into 2 ml Eppendorf tubes and kept on ice. 300 µl of haemolymph were subsequently pipetted into 2 ml Cryovials (BioStor™), immediately flash-frozen in liquid nitrogen, and stored at –80°C until metabolite analyses were carried out. The remaining haemolymph was stored briefly (ca. 30 min) on ice for bacterial counts and flow cytometry assays.

6.2.2 Bacterial quantification

Bacteria in each mussel group were enumerated using the spread plate method, as previously described in Chapter 4 (4.2.2).

6.2.3 Flow cytometric analyses

Sub-samples of the remaining haemolymph were used to assess total cell count, haemocyte viability, and production of intracellular reactive oxygen species (ROS) via flow cytometry using a Muse® Cell Analyzer (EMD Millipore, Hayward, CA, USA). Haemocyte concentration and viability parameters were measured for each sample using the Muse® Cell Count and Viability kit (Chapter 4 – 4.2.3), while relative quantification of reactive oxygen species (ROS) in haemocytes was estimated using the Muse® Oxidative Stress kit (Chapter 5 – 5.2.2).

6.2.4 GC-MS-based metabolomics analyses and data processing

Metabolites from haemolymph samples of both mussel groups were co-extracted with an internal standard (L-alanine-2,3,3,3-d_4) in cold methanol-water solution (MeOH:H_2O), then derivatized via methyl chloroformate (MCF) alkylation, as the method described in Chapter 4 (4.2.4-5). After derivatisation, the MCF derivatives were analysed via a GC-MS system and spectral processing was conducted in accordance with the protocol described in Chapter 4 (4.2.6).

6.2.5 Statistical and pathway analysis

Bacterial count and flow cytometric data were analysed with independent student's *t*-tests using SPSS® software (version 23.0) (IBM, Armonk, NY, USA).

Metabolite data were normalized by generalized log (glog) transforming, and mean centering prior to statistical analysis using MetaboAnalyst 3.0 software (Xia et al., 2015).

Univariate analyses were performed to identify metabolite differences between infected and control groups, including students *t*-test, significance analysis of microarrays/metabolites (SAM) and empirical bayesian analysis of microarrays/metabolites (EBAM). Multivariate data analyses of unsupervised principal components analysis (PCA) and hierarchical cluster analysis (HCA) (Euclidian distance; Ward's criterion) were used to identify natural groupings of samples based on the underlying structure of the data. The supervised projection to latent structures discriminant analysis (PLS-DA) and validation of PLS-DA model *via* leave one out cross validation (LOOCV) were performed as described in Chapter 4 (4.2.7).

Quantitative enrichment analysis (QEA) (Xia and Wishart, 2010) and network topology analysis (NTA) (Nikiforova and Willmitzer, 2007) were performed to investigate functional relationships among the annotated metabolites for pathway analyses (Chapter 5 – 5.2.5).

6.3 RESULTS

6.3.1 Bacterial cell counts

Vibrio counts were significantly higher (*t*-test; $p < 0.05$) in haemolymph from infected mussels (ca. 3.22×10^5 cfu·ml^{-1}), compared to those of non-infected (control) mussels (ca. 278.33 cfu·ml^{-1}) (Fig. 6.1A).

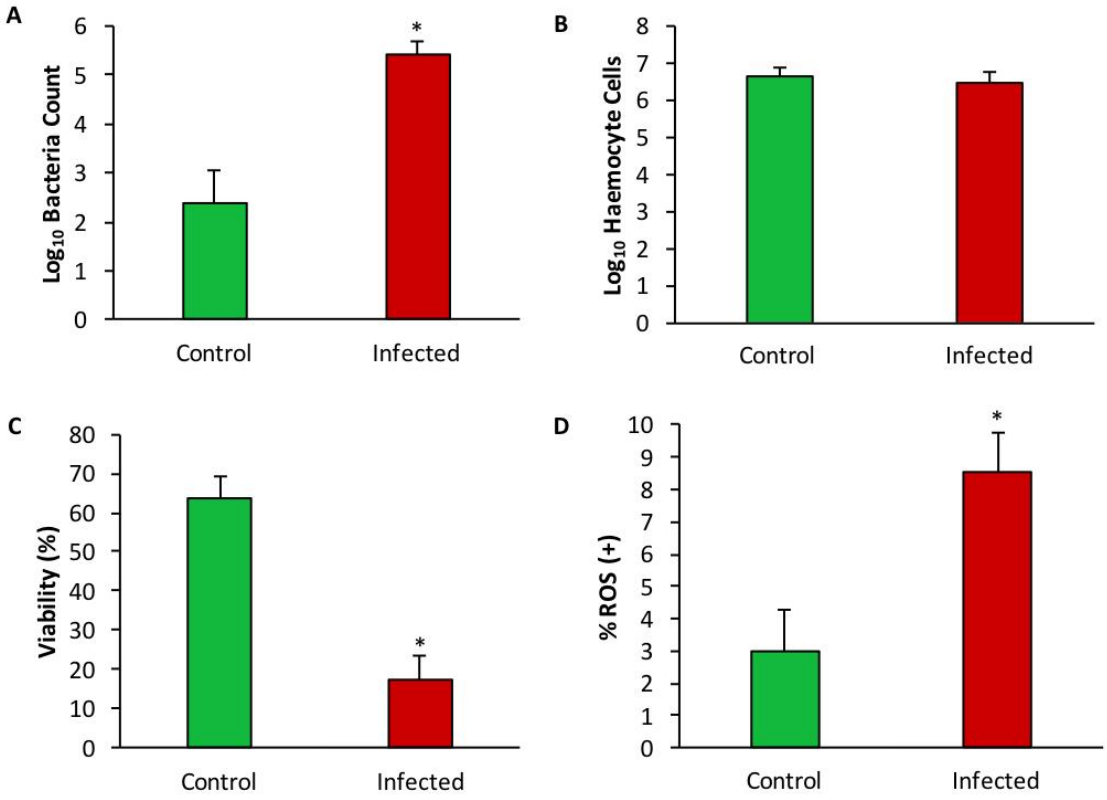

Figure 6. 1 Flow cytometric analyses of bacterial and mussel haemolymph. (A) Log_{10} bacterial cell count on TCBS agar plates. (B) Log_{10} haemocyte cell counts. (C) Viability of mussel haemocytes (%). (D) Percentage of ROS (+) cells (cells exhibiting ROS) in the haemolymph. Significant differences relative to the control are marked with an asterisk (*) (Student's *t*-test, $p < 0.05$). Error bars represent standard deviations.

6.3.2 Haemolymph cell counts and viability

Haemocyte cell concentrations in haemolymph of infected and non-infected mussels were similar (ca. 4.14×10^6 cells·ml^{-1}) (Fig. 6.1B). However, the proportion of haemocytes which was viable in haemolymph of infected mussels (ca. 18 %) was substantially lower than in non-infected mussels (ca. 64 %) (*t*-test; $p < 0.05$) (Fig. 6.1C).

6.3.3 ROS analyses

At 6 hours post-injection, the number of haemocytes generating ROS were significantly ($p < 0.05$) higher in mussels injected with *Vibrio* sp. DO1 (8.53 ± 2.36 %) compared to control mussels injected with PBS only (3.01 ± 1.25 %) (Fig. 6.1D).

6.3.4 Metabolite profiles

We reliably annotated 71 metabolites across broad chemical classes from the haemolymph spectra (e.g., amino acids, organic acids, fatty acids, sterols, tripeptides, vitamins).

Univariate data analysis

Univariate statistical analyses showed a number of differences in the metabolite profiles between infected and non-infected mussels. Firstly, *t*-test analysis identified 24 metabolites as being significantly different between infected and non-infected mussels ($p < 0.05$). Similarly, SAM and EBAM revealed an alternation of 23 metabolites that were differentially expressed between infected and non-infected mussels. With a fold-change cut-off threshold of 2, we identified 22 common metabolites that were significantly different between two treatment groups. Among them, 10 metabolites were up-regulated, while 12 metabolites were down-regulated in infected mussels (Fig. 6.2).

Multivariate data analyses

Multivariate analyses of the haemolymph metabolite profiles showed good separations between infected and non-infected mussels (Fig. 6.3). PCA correctly grouped the samples by their class labels, with the distribution of non-infected mussels (green dots) being clearly separated from that of infected mussels (red triangles) along the PC1 axis (Fig. 6.3A).

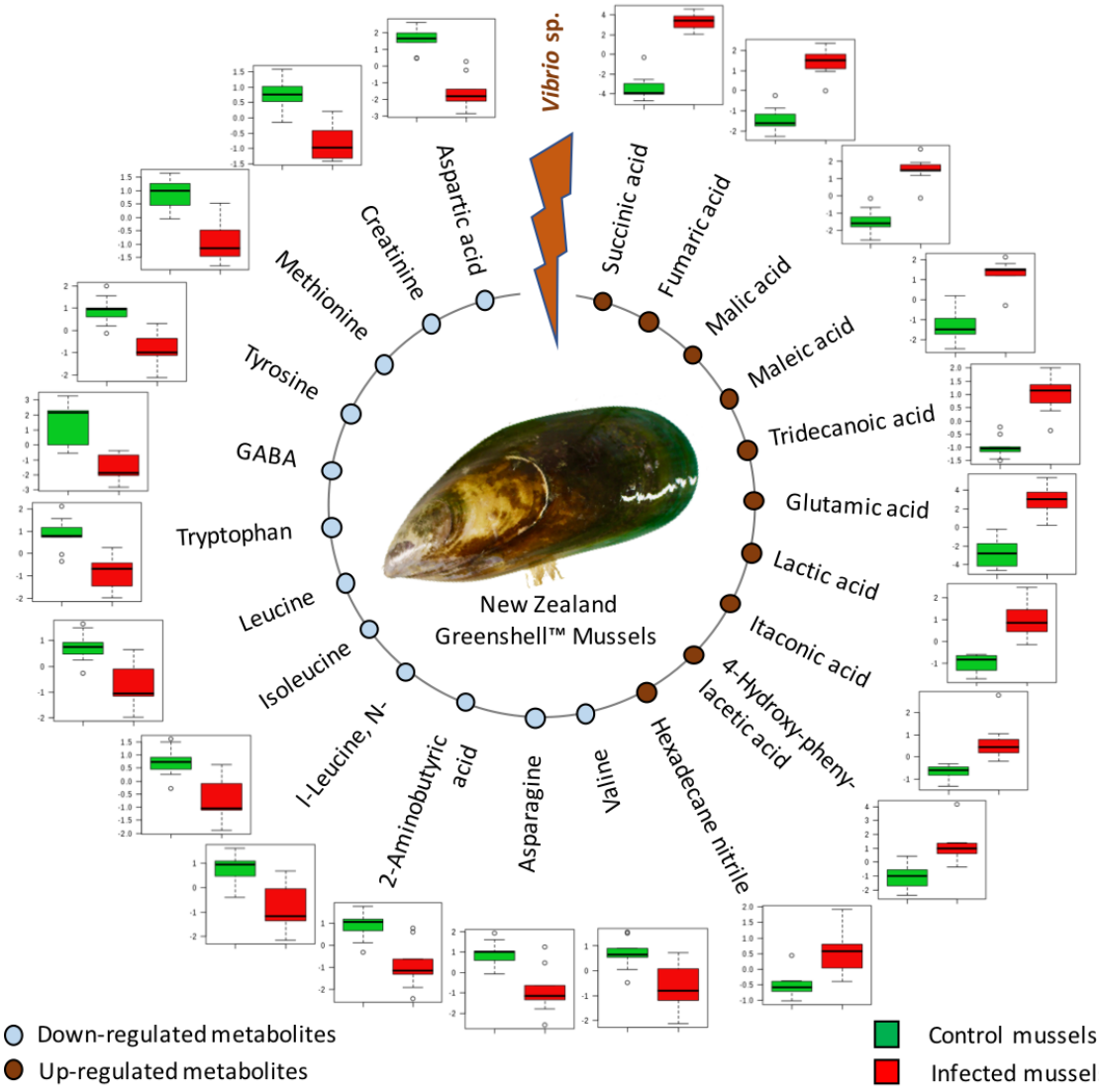

Figure 6.2 Alternations of metabolites in *Vibrio* sp. infected and non-infected mussels at 6 hrs post-infection. Box plots of metabolites are based on *t*-test analysis, showing relative abundances of metabolites in control (green) and infected mussels (red). *l-Leucine, N-: l-Leucine, N-methoxycarbonyl, methyl ester*).

These sample groupings were independently supported via unsupervised HCA (Fig. 6.3B), with the dendrogram revealing two clear discriminative clusters based on the treatment that they received. Supervised PLS-DA analysis sharpened the discrimination between control and infected groups (Fig. 6.3C). The PLS-DA model showed an accuracy of 100 %, a multiple correlation coefficient (R^2) of 95.6 %, and a cross-validated predictive ability (Q^2) of 92.3 %. These parameters indicate optimal fitness and

prediction performance of the PLS-DA model. Fifteen metabolites with VIP scores > 1.0 were identified as contributing strongly to the model (Fig. 6.3D).

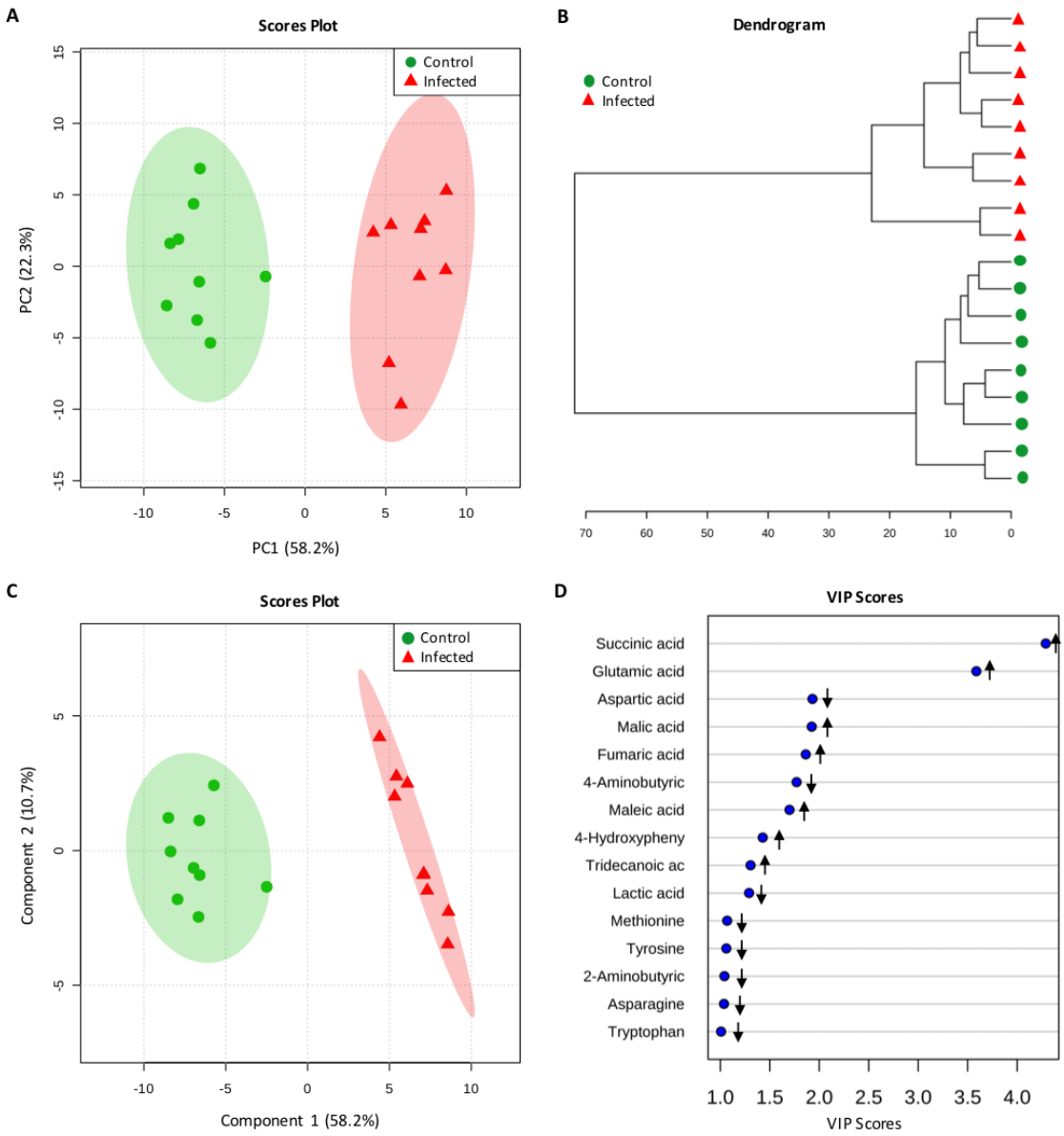

Figure 6. 3 Multivariate cluster analyses of metabolite profiles of *Vibrio* sp. infected and non-infected (control) mussels. (A) 2D PCA score plot. (B) Dendrogram (Euclidian distance; Ward's method). (C) 2D PLS-DA score plot. (D) Top 15 metabolites with VIP scores for the PLS-DA model. Up and down arrows, respectively, demonstrate increased and decreased metabolites in infected mussels compared to non-infected mussels.

Pathway analysis

Pathway topology analysis showed 44 biochemical pathways that matched the KEGG database. Among these, 15 pathways involving at least two annotated metabolites with QEA p-values < 0.05, QEA FDR values < 0.1, and NTA PI values > 0.1 were screened as potential primary target pathways of interest relating to the treatment effect (Table 6.1).

Table 6.1 List of altered metabolic pathways that identified as primary target pathways in mussel haemolymph during *Vibrio* sp. DO1 infection.

Pathways: metabolites	Hits/ Total compounds	Raw p	-LOG(p)	FDR	Impact
Alanine, aspartate and glutamate metabolism: aspartic acid, alanine, glutamic acid, gamma-aminobutyric acid, glutamine, asparagine, fumaric acid, succinic acid.	8/24	0.000	23.456	0.000	0.719
Arginine and proline metabolism: glutamine, ornithine, aspartic acid, glutamic acid, proline, gamma-aminobutyric acid, fumaric acid.	7/43	0.000	22.919	0.000	0.322
Citrate cycle (TCA cycle): succinic acid, malic acid, citric acid, fumaric acid.	4/20	0.000	19.214	0.000	0.151
Tyrosine metabolism: Tyrosine, fumatic acid, p-Hydroxyphenylacetic acid.	3/44	0.000	18.906	0.000	0.137
Histidine metabolism: Glutamic acid, histidine, aspartic acid.	3/14	0.000	18.176	0.000	0.238
Aminoacyl-tRNA biosynthesis: asparagine, histidine, phenylalanine, glutamine, cysteine, glycine, aspartic acid, serine, methionine, valine, alanine, lysine, isoleucine, leucine, threonine, tryptophan, tyrosine, proline, glutamic acid.	19/67	0.000	17.265	0.000	0.103
Glyoxylate and dicarboxylate metabolism: citric acid, malic acid.	2/18	0.000	15.184	0.000	0.296
D-Glutamine and D-glutamate metabolism: glutamic acid, glutamine	2/5	0.000	13.846	0.000	1.000
Glutathione metabolism: glutathione, glycine, cysteine, glutamic acid, ornithine, pyroglutamic acid.	6/26	0.000	13.414	0.000	0.418
beta-Alanine metabolism: beta-alanine, aspartic acid.	2/16	0.000	11.437	0.000	0.395
Phenylalanine, tyrosine and tryptophan biosynthesis: phenylalanine, tyrosine.	2/4	0.001	7.551	0.001	1.000
Phenylalanine metabolism: phenylalanine, tyrosine.	2/11	0.001	7.551	0.001	0.407
Valine, leucine and isoleucine biosynthesis: valine, leucine and isoleucine.	4/13	0.003	5.874	0.005	1.000
Cysteine and methionine metabolism: cystathionine, serine, methionine, cysteine.	4/29	0.008	4.883	0.014	0.443

6.4 DISCUSSION

Recent NMR-based metabolomics studies have shown changes in metabolites underlying several metabolic pathways in different bivalve organs, such as hepatopancreas and gills of individuals infected with pathogenic *Vibrio* species (Liu et al., 2014a, Liu et al., 2013a, Liu et al., 2014b, Liu et al., 2013b). However, metabolic alterations in the host's haemolymph during *Vibrio* infections have until now been elusive. Using a GC-MS platform, we observed many metabolic responses reflected in the haemolymph of mussels 6 hours post-infection with *Vibrio* sp. DO1. In infected mussels, these metabolic changes included up-regulation of 10 metabolites and down-regulation of 12 metabolites. These endogenous metabolites are important signatures of metabolic pathways and mechanisms (e.g. oxidative stress, inflammation, disruptions of TCA cycle, amino acid metabolism and protein synthesis) that are relevant to the innate immune response of mussels when infected with *Vibrio* sp. DO1.

6.4.1 Oxidative stress

ROS are a natural by-product of different biological processes and have important role in protective mechanism of most organisms (Jabs, 1999, Torreilles et al., 1996). However, excess of ROS production due to changes of intra- and extra-cellular environmental conditions that leads to an imbalance between ROS and antioxidants in favour of ROS can cause oxidative stress and long-term damage to cells and tissues via degradation of DNA, proteins and lipids (Jabs, 1999, Torreilles et al., 1996). In this study, we observed a significantly higher percentage of ROS-producing cells in infected mussels compared to non-infected mussels, demonstrating that *P. canaliculus* haemocytes up-regulated ROS production in response to *Vibrio* sp. DO1 infection. In agreement with this finding, previous studies have also reported increases in ROS within haemolymph samples of several other marine bivalves in response to pathogenic bacteria or stimulation of

pathogen-associated molecular patterns (PAMPs) (Buggé et al., 2007, Costa et al., 2009, Goedken and De Guise, 2004, Lambert et al., 2003).

In addition to the flow cytometry assay, metabolomic analyses also revealed some important metabolite signatures of potential oxidative stress in infected mussels, including altered glutathione metabolism and transsulfuration pathway (cysteine and methionine metabolism), decreased free methionine content, and increased levels of glutamic and succinic acids. Glutathione in reduced form (GSH) is an important and abundant antioxidant molecule, which can react with electrophilic oxidants (e.g., H_2O_2) by converting two GSH molecules into oxidized form (GSSG) (Fig. 6.4) (Espinosa-Diez et al., 2015). The GSH:GSSG ratio is thus often considered to be a biomarker for oxidative stress in various physiological and pathophysiological situations (Asensi et al., 1999, Gurer-Orhan et al., 2004, Jones, 2002, Zitka et al., 2012). In the present study, we identified total glutathione (GSH and GSSG) which was not different between infected mussels and control mussels, but we could not discriminate between GSH and GSSG due to the technical limitations of the GC-MS approach, so the GSH:GSSG ratio was not obtained. However, we identified six metabolites (glutathione, glycine, cysteine, glutamic acid, ornithine and pyroglutamic acid) within the glutathione metabolic pathway, and secondary pathway analysis screened glutathione metabolism as an affected target pathway of interest relating to the treatment effect (p = 0.000, FDR = 0.000, PI = 0.418) (Table 6.1). The transsulfuration pathway is a source of cysteine for GSH synthesis under low-mid stress conditions (McBean, 2012), and was also identified as a differentially regulated pathway (p = 0.008, FDR = 0.014, PI = 0.443). During transsulfuration, methionine acts as a precursor for homocysteine, which combines with serine to form cysteine through a cystathionine intermediate. Cysteine in turn combines with glutamic acid and then glycine to form GSH (Fig. 6.4) (McBean, 2012). Hence, the decrease in

methionine in infected mussels may suggest a high demand of methionine for the transsulfuration and glutathione pathways. The elevated levels of glutamic acid (precursor of GSH) in infected mussel haemolymph could affect GSH synthesis. Increases of intracellular glutamic acid has been reported to decrease GSH levels and eventually trigger ROS production in Riluzole-treated cells (Seol et al., 2016). These combined results may indirectly suggest the involvement of GSH in regulating elevated ROS production in haemolymph of *Vibrio*-infected mussels.

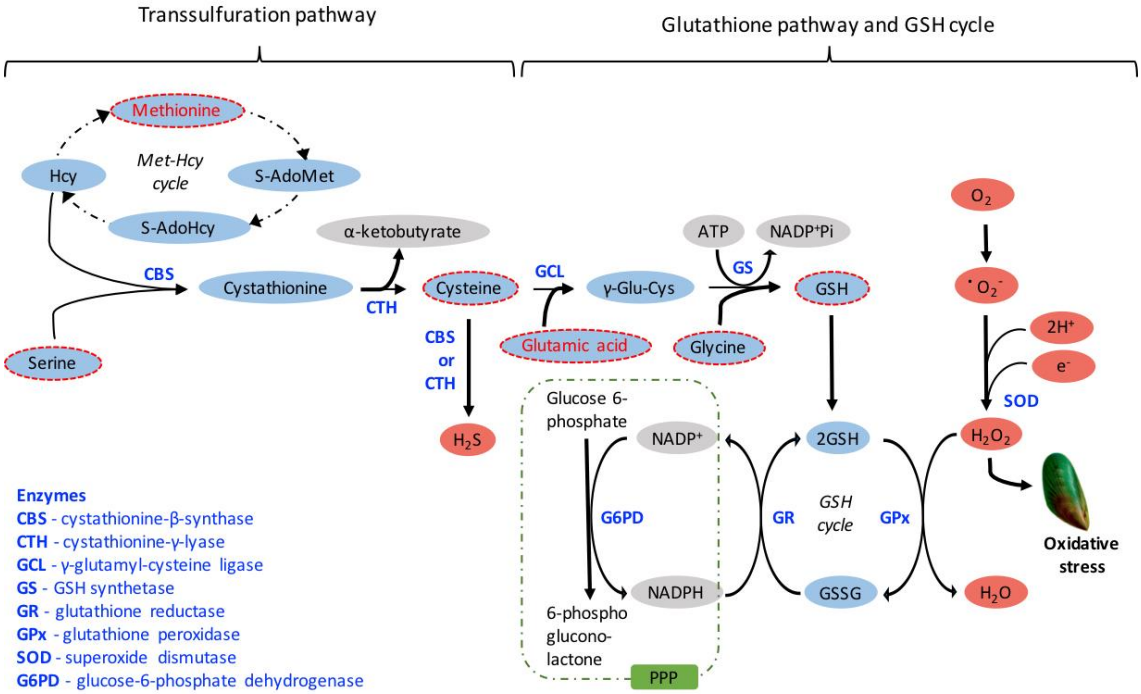

Figure 6. 4 Schematic representation of the relationship between transsulfuration pathway, glutathione pathway and oxidative stress. Compounds with red dash outlines are metabolites that were identified in this data set whereas compounds in red characters and red dash outlines were significant different between control and infected mussels. Enzymes are shown in bold and blue characters. *Hcy, homocysteine; S-AdoMet, S-adenosylmethionine; S-AdoHcy, S-adenosylhomocysteine; Met-Hcy cycle, Methionine- homocysteine cycle*.

Succinic acid is an intermediate of TCA cycle, but also plays an important role in several other metabolic pathways, including the formation and elimination of ROS (reviewed by Tretter et al. 2016). Succinic acid has been shown to drive reverse electron transport

(RET) and generate ROS production by RET during ischemia-reperfusion injury (Chouchani et al., 2014). Recently, Mills et al. (2016) found that increased succinate-driven oxidation by succinate dehydrogenase (SDH) and an elevated mitochondrial membrane potential combine to drive mitochondrial ROS production. In this study, we observed an increase of both ROS and succinic acid, which may suggest the significant contribution of succinic acid towards a greater production of mitochondrial ROS in infected mussels. The flow cytometry and metabolomics results herein suggest that *P. canaliculus* haemocytes are capable of producing ROS in response to *Vibrio* infection, and that may lead to oxidative stress in infected mussels.

6.4.2 Citric cycle

The citric acid cycle (TCA cycle) is a key metabolic pathway in all aerobic organisms that generates energy through oxidation of fuel molecules (e.g. glucose, fatty acids and certain amino acids) into carbon dioxide. Interruption of the TCA cycle in mammalian macrophages is known to lead to massive accumulations of TCA intermediates (citric acid, succinic acid, fumaric acid and malic acid) (Jha et al., 2015). These accumulations are due to two characteristic breaking points in the TCA cycle (Jha et al., 2015, Tannahill et al., 2013). The first break occurs at isocitrate dehydrogenase (IDH), which catalyzes the oxidative decarboxylation of isocitrate into alpha-ketoglutarate. Thus, the IDH break leads to accumulation of citric acid that can act as a precursor to generate fatty acids for membrane biogenesis, prostaglandin production (Infantino et al., 2011, O'Neill et al., 2016) and itaconic acid (ITA) (Michelucci et al., 2013). In this study, we did not observe a significant increase of citric acid, but the accumulation of ITA suggests that any excess citric acid was used as its precursor during infection (Fig. 6.5).

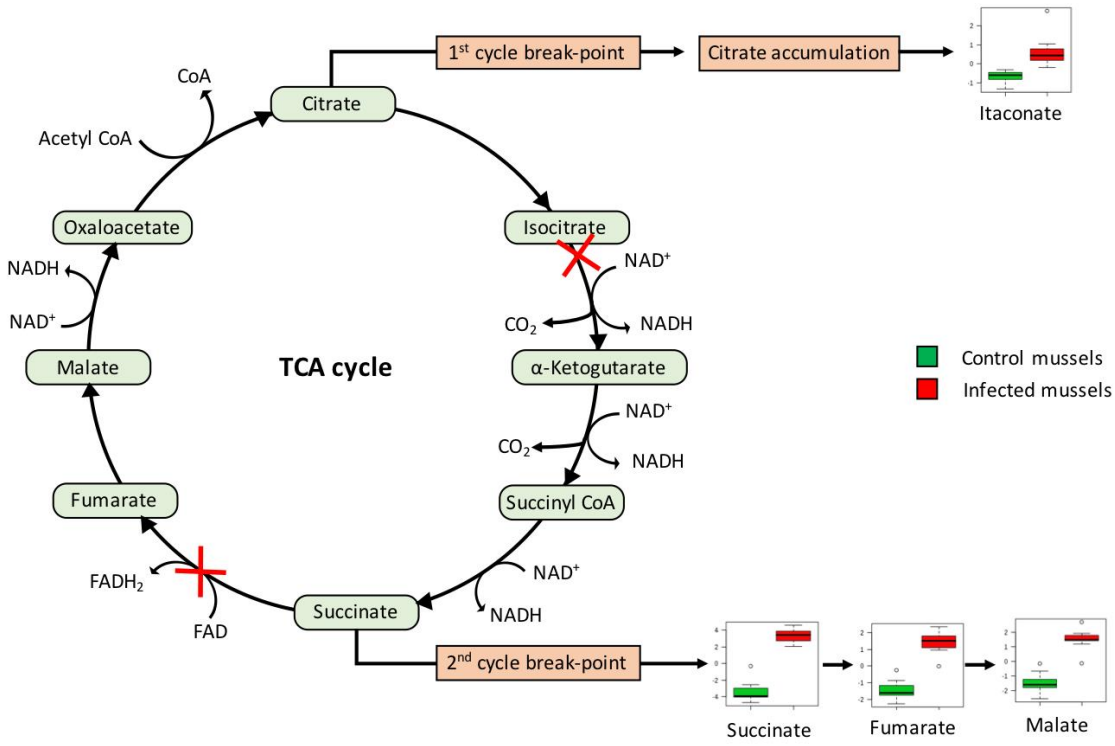

Figure 6. 5 Two TCA cycle break-points in *V. coralliilyticus* infected mussels. The first break was after citrate, resulting in accumulation of citrate, which was used to generate itaconate in mussel haemolymph. The second characteristic break-point of TCA cycle occurred at succinate dehydrogenase/respiratory Complex II (SDH/CII) that leads to the accumulation of succinate. Elevated levels of succinate replenishes production of fumarate and malate. Box plots of metabolites are based on *t*-test analysis, showing relative abundances of metabolites in control (green) and infected (red) mussels.

The second characteristic break-point of the TCA cycle occurs at the succinate dehydrogenase/respiratory Complex II (SDH/CII), which can lead to an accumulation of succinic acid (reviewed by Gaber et al. 2017). As a consequence, it has been suggested that the presence of an active variant of the aspartate-arginosuccinate shunt replenishes production of fumaric acid and malic acid, which inhibits the activity of succinate dehydrogenase (Gaber et al., 2017, Jha et al., 2015). In mussels challenged with *Vibrio* sp. DO1, we observed significantly elevated levels of succinic acid, fumaric acid and malic acid, which could be due to a similar second break-point feature of the TCA cycle in vertebrate macrophages/haemocytes. Previous studies have also reported the

accumulation of ITA and succinic acid in virus-exposed oysters (Young et al., 2017). These results suggest that a similar mechanism of TCA cycle interruption may be a consequence of pathogenic infections across diverse taxa.

6.4.3 Inflammation

Accumulation of ROS, succinic acid and ITA in mussels exposed to *Vibrio* sp. DO1 may suggest inflammatory responses in infected mussels. Inflammation is a complex biological response of tissues to harmful stimuli (i.e. pathogens, irritants) and is considered to be a critical first line of defence of both vertebrates and invertebrates (Rowley, 1996). Inflammation also is a protective mechanism involving humoral and cell mediators of the immune system. In mammalians, well described molecular mediators are cytokines, prostaglandins, NO and ROS produced by both innate and adaptive immune cells (Mills and O'Neill, 2014). Numerous studies on the biology and pathology of molluscs over the last decades has predominantly revealed that the inflammatory response in molluscs may be driven by a pool of functionally conserved molecules, such as those observed in vertebrates (De Vico and Carella, 2012, Humphries and Yoshino, 2003, Ottaviani et al., 2010, Rowley, 1996).

ROS are signalling molecules and mediators that play a central role in the progression of inflammatory responses (Mittal et al., 2014). An increase in ROS, which leads to acute inflammation has been well described in many studies (Fubini and Hubbard, 2003, Mittal et al., 2014, Zhou et al., 2010). Hence, the increase of ROS production observed in infected mussels in this study may also trigger inflammation.

Succinic acid is known as a metabolic signal in inflammation (Mills and O'Neill, 2014, Tannahill et al., 2013). In mammalians, succinic acid directly stabilizes the transcription factor hypoxia-inducible factor-1α (HIF-1α) (Mills and O'Neill, 2014, Tannahill et al.,

2013) or indirectly stabilizes HIF-1α by accumulation of mitochondrial ROS levels via oxidation of succinic acid to form fumaric acid by succinate dehydrogenase (SDH) (Mills et al., 2016). Once stabilized, HIF-1α activates target genes that support the inflammation (Mills et al., 2016, Tannahill, 2013). In marine bivalves, the critical role of HIF-α in regulation of respiratory burst activity of the Pacific oyster (*Crassostrea gigas*) hemocytes has been demonstrated (Choi et al., 2013). Thus, accumulation of succinic acid in *Vibrio*-exposed mussels may play an important role in inflammatory responses of these mussels in this study.

ITA is a highly induced metabolite during macrophage activation, and it presently has been recognised as a major physiological regulator of inflammation (Lampropoulou et al., 2016). Lampropoulou et al. (2016) investigated the role of ITA in LPS-activated macrophages, and found that ITA regulates succinic acid levels, mitochondrial respiration and ROS production. Similarly, we observed a significant increase in ITA, succinic acid and ROS production in mussels exposed to *Vibrio* sp. DO1. This suggests that a similar critical role of ITA in inflammatory responses may exist in marine bivalves. However, further investigation is needed to confirm the involvement of ITA in regulation of succinic acid and inflammation in invertebrates.

The amino acid gamma-aminobutyric acid (GABA), is well known as an important inhibitory neurotransmitter in the central nervous system for inflammation suppression of both vertebrates and invertebrates (Eriksson and Panula, 1994). Molecular cloning of GABAA receptor-associated protein (GABARAP) from abalone (*Haliotis diversicolor*) and its expression in different abalone tissues after bacterial challenge suggest its vital role in the innate immune system of molluscs (Bai et al., 2012). Indeed, a decrease in GABA was found in the haemolymph of Pacific oysters (*Crassostrea gigas)* 6 h after

LPS stimulation (Li et al., 2016). Interestingly, GABA could indirectly inhibit cellular immune and humoral immune responses by reducing the production of pro-inflammatory cytokines (CgIL-17 and CgTNF) and immune effectors (CgSOD and CgBPI) (Li et al., 2016). In our case, we also observed the decrease of GABA in infected mussels, suggesting that this metabolite may have a function in suppression of the immune-mediated pro-inflammatory reactions.

6.4.4 Amino acid metabolism and protein synthesis

Decreases in a number of amino acids in infected mussels suggest diverse disruptions of amino acid metabolism and protein biosynthesis. For example, the branched-chain amino acids (BCAA), including leucine, isoleucine, and valine are essential metabolites for the immune system, which provide energy and act as the precursors for biosynthesis of new molecules and cells, especially lymphocytes (Calder, 2006). When infection occurs, there is a high demand of BCAA for lymphocytes to synthesize protein, RNA, and DNA and other immune cell functions (Calder, 2006). Similarly, we observed a decrease of BCAAs in haemolymph of mussels challenged with *Vibrio* sp. DO1, which suggest the high demand in BCAAs for immune activities. In contrast, Liu et al. (2013a) found an increase of BCAAs in the hepatopancreas of clams exposed to *V. anguillarum*. These differences in results from tissue and haemolymph analyses may be due to tissue-specific responses of hosts to infections, which have been reported in several studies (Liu et al., 2014a, Lu et al., 2017). In addition to BCAAs, we found significant decreases in other amino acids, such as tryptophan and aspartic acid. Tryptophan is an essential amino acid that is required by all life forms for the regulation of protein synthesis. It is also a substrate for other major biosynthetic routes, such as the synthesis of serotonin, essential cellular factors and the kynurenine pathway (Schröcksnadel et al., 2006). Aspartic acid, is an α-amino acid that is used in protein biosynthesis (Voet et al., 2016). Hence, the decrease in

these important amino acids in haemolymph of infected mussels suggests an immune stimulation by *Vibrio* sp. DO1 infection and the disturbance of amino acid metabolism and protein biosynthesis by the pathogen.

6.5 CONCLUSION

We report the first evidence of metabolic profiling of the New Zealand Greenshell™ mussel (*P. canaliculus*) haemolymph exposed to *Vibrio* sp. DO1. We found several major perturbations on the host innate immune system of mussels resulting from *Vibrio* sp. DO1 infection, including oxidative stress, inflammation, disruption of TCA cycle, changes in amino acid metabolism and protein synthesis. We identified significant alteration in a number of important metabolites. Among them, succinic acid, itaconic acid and BCAAs are involved in diverse metabolic and physiologic roles and could potentially be considered as biomarkers of *Vibrio* infection in bivalves. Hence, further characterization of these metabolites would be essential for development of health biomarkers.

6.6 REFERENCES

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Chapter 7

In vitro study of apoptosis in mussel (*Perna canaliculus*) haemocytes induced by lipopolysaccharide

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ABSTRACT

Apoptosis or programmed cell death is a fundamental process essential for an organism's development and homeostasis in immune system of both vertebrates and invertebrates. However, little is known about apoptotic process in marine bivalves which require investigations to elucidate mechanisms and identify molecular effectors of cell death pathways. In this study, we characterized cellular and molecular mechanisms of apoptosis induced by lipopolysaccharide (LPS) in New Zealand Greenshell™ mussel (*Perna canaliculus*) haemocytes. Mussel haemocyte samples were exposed to different LPS concentrations (0, 50 and 100 $\mu g \cdot ml^{-1}$) and incubated at 19 °C for 3 h prior to assessment of various cell health parameters *via* flow cytometry assays and gas chromatography-mass spectrometry (GC–MS)-based metabolomic analyses. Flow cytometry results showed slightly higher, but non-significant differences in production of reactive oxygen species between LPS-exposed and control (no LPS) samples. However, percentages of apoptotic cells determined *via* depolarization of the mitochondrial membrane potential and caspase-3/7 activity in LPS-exposed samples were significantly higher than in control samples, providing mechanistic information regarding initiation and progression of the apoptotic cascade. The metabolite profile of LPS-exposed haemocytes showed elevated levels of 11 metabolites compared to that of the control. These metabolites may be involved in protein and lipid degradation as a consequence of apoptosis and other immune or physiological responses. This study demonstrates that LPS could trigger apoptosis in mussel haemocytes and provides insights into apoptotic processes in mussel haemocytes. Such knowledge could be useful for understanding the immune responses of farmed bivalves to waterborne pathogens and identification of molecular biomarkers for disease management in aquaculture.

7.1 INTRODUCTION

Bivalves are important cultured species in New Zealand aquaculture industry (FAO, 2019b). The country produced 95,983 metric tonnes of bivalves in 2016, contributing to 88 % of the national aquaculture harvest. Two bivalves, including Pacific oysters (*Crassostrea gigas*) and Greenshell™ mussels (*P. canaliculus*), together with King salmon (*Oncorhynchus tshawytscha*) are the most important cultivated seafood species in the country (FAO, 2019b). Among these species, *P. canaliculus* is endemic to New Zealand which naturally distribute throughout the country but more common in the North Island. Farming of *P. canaliculus* is carried out on longlines, which Coromandel, Marlborough Sounds, and Stewart Island are major farming areas. In 2016, production of *P. canaliculus* was 94,037 metric tonnes, which equated to more than 86 % of the aquaculture production of New Zealand in that year (FAO, 2019a). However, high mortality events effecting mussels during summer months are major challenges for this industry. The causes of these mortality events are currently not clear, but are thought to be associated with pathogens and environment factors (e.g., elevated temperature, ocean acidification). Pathogens and diseases associated with farmed *P. canaliculus* mussels that have been recorded in New Zealand include apicomplexan parasite X (APX), *Bonamia exitiosa*, digestive epithelial virosis (DEV), invasive ciliates, shell-boring worms (mudworms) and *Vibrio* sp. bacteria (Castinel et al., 2014).

Like other invertebrates, bivalves mostly rely on the innate immune system which includes cellular and humoral components (Song et al., 2010). As part of the internal innate immune system, haemocytes act as mediators of cellular defences whereas various active molecules in haemolymph secreted by haemocytes are major components of humoral defences (Allam and Raftos, 2015, Song et al., 2010). Thus, haemocytes represent the foundation of the bivalve innate immune system and haemocyte-mediated

immunity is considered to be the main internal defence mechanism of marine bivalves (Zannella et al., 2017). Haemocytes play a key role in phagocytosis, but also in encapsulation, nacrezation of biotic and abiotic foreign particles (Allam and Raftos, 2015, Anderson and Good, 1976, Bachere et al., 1995, Song et al., 2010) and apoptosis (Romero et al., 2011, Sunila and LaBanca, 2003).

Apoptosis or programmed cell death plays a fundamental role in homeostasis and the development of many organisms and tissue systems in both vertebrates and invertebrates (Kiss, 2010, Sokolova, 2009). In addition, apoptosis is known as a host defence mechanism against pathogen infections (DeLeo, 2004, Koyama et al., 2000). The apoptosis process in mollusc is conversed to that of vertebrates, and is regulated *via* two major pathways: the intrinsic pathway (or mitochondrial-mediated apoptosis) which is activated in response to various types of intracellular stressors, and the extrinsic pathway (or death receptor-mediated apoptosis) which is initiated by external cell stimuli (Estevez-Calvar et al., 2013, Kiss, 2010, Sokolova, 2009, Terahara and Takahashi, 2008, Zhang et al., 2011). A number of studies have demonstrated that apoptosis in bivalve cells can be triggered by exposure to different stress factors (e.g., heavy metals, UV light, pathogens) (Gervais et al., 2016, Gervais et al., 2015, Nguyen et al., 2018a, Sokolova et al., 2004). However, the knowledge of apoptosis in bivalve molluscs remains limited, especially at the metabolic level.

Metabolomics is the broad-scale analysis of metabolites in biological samples, and it is a rapidly growing field with significant applicability in aquaculture research, such as larval production, nutrition and diet, disease and immunology and post-harvest quality control (Alfaro and Young, 2018). Metabolomics provides a novel tool for understanding endogenous metabolic changes of an organism throughout the course of a biological

process promoted by disease or environmental perturbation (Klassen et al., 2017). We recently demonstrated that metabolomics analyses of bivalve haemocytes can be used to provide insights into the molecular mechanisms of bivalve biological responses against pathogens (Nguyen et al., 2018b, Nguyen et al., 2018c), water contaminants (Nguyen et al., 2018a) and aerial exposure (Alfaro et al., 2019). In addition, the flow cytometry analyses using a Muse® Cell Analyzer for rapid and accurate characterization of health parameters of bivalve haemocytes (e.g., viability, oxidative stress, apoptosis) have been successfully reported in our recent studies (Grandiosa et al., 2018, Nguyen et al., 2018b, Nguyen et al., 2018c, Nguyen et al., 2018d). Hence, the combination of metabolomics approach and flow cytometry analysis would provide inside into host-pathogen interactions at both cellular and molecular levels.

In the present study, we applied GC-MS-based metabolomics combined with flow cytometry to characterize apoptosis in LPS-exposed haemocytes from New Zealand Greenshell™ mussel (*P. canaliculus*). The flow cytometry analyses were used to measure the alteration of two apoptosis biomarkers including depolarization of the mitochondrial membrane potential and caspase-3/7 activity which were successfully demonstrated in *P. canaliculus* haemocytes exposed to copper (Nguyen et al., 2018a). The untargeted metabolomics analysis was performed to compare metabolite profiles of mussel haemolymph in the LPS exposure and the control. Generation of a metabolic signature specific to apoptosis induced by LPS exposure could provide valuable information to elucidate the mechanisms of apoptosis in marine bivalves and could be used to generate signatures of other immune responses, leading to better management of aquaculture stock.

7.2 MATERIALS AND METHODS

7.2.1 Experimental design

Adult mussels (shell length: 7.86 ± 0.35 cm) were obtained from Westpac Mussel Distributors Ltd. (Auckland, New Zealand) and acclimatized for 7 days using the same protocol previously described in Chapter 4 (4.2.1). Approximately 1–2 ml of haemolymph were collected from each animal by gently inserting a needle (23 gauge x 1.5") attached to a 3 ml sterile syringe (Terumo, Japan) into the posterior adductor muscle. Immediately after withdrawal, haemolymph samples were transferred into 10 ml Eppendorf tubes and kept on ice. For each treatment, haemolymph samples from five mussels were pooled and homogeneously mixed to reduce inter-individual variations, and to provide enough haemolymph for subsequent laboratory tests (Gagnaire et al., 2006). A total of 15 mussels were used for three treatments (0, 50 and 100 $\mu g \cdot ml^{-1}$) with 7 replicates for each treatment.

Cold artificial seawater (ASW) was mixed with mussel haemolymph at the ratio 1:1 as the anti-coagulant (Gagnaire et al., 2006, Nguyen et al., 2018b, Zhou et al., 2017) and *Salmonella*-derived LPS (Sigma-Aldrich, New Zealand) was used to stimulate immune responses of mussel haemocytes (Hernroth, 2003, Xian et al., 2009). Hence, LPS stock solutions, including 100 and 200 $\mu g \cdot ml^{-1}$ were prepared by adding 1 mg and 2 mg LPS to 10 ml of 0.22 µm-filtered ASW, respectively, and then kept at 19 °C. The exposure experiment was conducted by adding 1 ml of haemolymph with 1 ml of different 19 °C LPS solutions. The final working concentrations of LPS after adding the haemolymph were 50 and 100 $\mu g \cdot ml^{-1}$. The LPS concentrations were determined based on previous publications (Costa et al., 2009, Ordás et al., 2000) and our pilot study. The control treatment was prepared by adding 1 ml of haemolymph with 1 ml of cold ASW. There

were seven replicates per treatment. The haemolymph samples were incubated at 19 °C for 3 h prior for flow cytometric and metabolomics analyses.

Since Cu^{2+} has been reported to induce reactive oxygen species (ROS) production and apoptosis in bivalve haemocytes (Nguyen et al., 2018b), a copper (II) sulfate pentahydrate solution (3.20 $\mu g \cdot ml^{-1}$ Cu^{2+}) was added to three additional haemolymph samples and incubated at 19 °C for 3 h as positive controls for ROS production and apoptosis. Hence, the Cu^{2+}exposed samples were used to establish the correct instrument settings based on the manufacturer's specifications.

7.2.2 Flow cytometry

The effect of LPS on haemocyte health parameters were assessed on a Muse® Cell Analyzer (EMD Millipore, Hayward, CA, USA) using four different assays: Muse® Cell Count & Viability, Muse® Oxidative Stress, Muse® MitoPotential, and the Muse® Caspase-3/7. All the assay kits were purchased from Merck Millipore (Abacus dx, New Zealand). These assays were conducted according to the manufacturer's specifications with modifications and performed in 1.5 ml LightSafe micro centrifuge tubes (Sigma-Aldrich, Germany) to protect the reagents from light degradation.

The Muse® Cell Count and Viability kit was used to quantitatively analyse the count and viability of haemocytes (Chapter 4 – 4.2.3) while the Muse® Oxidative Stress kit was used to gain quantitative measurements of reactive oxygen species (ROS) (Chapter 5 – 5.2.2).

The Muse® MitoPotential assay utilizes the MitoPotential dye to detect changes in the mitochondrial membrane potential (MMP) and 7-AAD as a dead cell marker during the early stages of apoptosis. Briefly, 100 µl of haemolymph (10^5 haemocytes per ml) was

incubated with 95 µl Muse® MitoPotential working solution at 37 °C for 20 min. Then, 5 µl 7-AAD dye was added and incubated for 5 min at room temperature (20 °C) prior to measuring with the Muse® Cell Analyzer (flow rate = 0.59 µl·sec^{-1} and FSC threshold = 135). The assay provided four distinguishable populations of cells including: (LL) Live cells with depolarized mitochondrial membranes [MitoPotential (-) and 7-AAD (-)], (LR) Live cells with intact mitochondrial membranes [MitoPotential (+) and 7-AAD (-)], (UR) dead cells with depolarized mitochondrial membranes [MitoPotential (+) and 7-AAD (+)] and (UL) dead cells with intact mitochondrial membranes [MitoPotential (-) and 7-AAD (+)].

The Muse® Caspase-3/7 assay utilizes a novel Muse® Caspase-3/7 reagent NucView™ for the detection of caspase-3/7 activity and a dead cell marker (7-AAD) as an indicator of cell membrane structural integrity (cell death). Briefly, 5 µl of Muse™ Caspase-3/7 working solution was mixed with 50 µl of haemolymph and incubated at 37 °C for 30 min. It was followed by addition of 150 µl of 7-AAD working solution and incubation for 5 min at room temperature (20 °C) prior to measuring with the Muse® Cell Analyzer (flow rate = 0.59 µl·sec^{-1} and FSC threshold = 61). The assay provides relative percentages of live cells [Caspase-3/7(–) and 7-AAD(–)], early apoptotic cells [Caspase-3/7 (+) and 7-AAD(–)], late apoptotic cells [Caspase-3/7(+) and 7-AAD(+)] and dead cells [Caspase-3/7(–) and 7-AAD(+)].

7.2.3 GC-MS-based metabolomics analysis and data processing

Only the treatment with 100 µg·ml^{-1} LPS and the control were sampled for metabolomics purposes at 3 h post incubation (n = 7). A total of 500 µl of each haemolymph sample were pipetted into 2 ml BioStor™ cryovials (National Scientific Supply Company, Carlifornia, USA), immediately flash-frozen in liquid nitrogen and stored at –80 °C until

they could be analyzed. The method for metabolite extraction, derivatization, GC-MS measurement, quality control and spectral processing were performed as described in Chapter 4 (4.2.4-6).

7.2.4 Statistical analyses

Statistical analyses for flow cytometric assays were performed using one-way ANOVAs (Tukey post hoc test) with IBM® SPSS® Statistics software (version 23).

Statistical analyses of metabolite data were conducted using Metaboanalyst 4.0 (Chong et al., 2018, Xia et al., 2015). Resulting data were normalized by generalized log (glog) transforming and mean centring prior to statistical analyses. Univariate students *t*-tests were used to identify differences between metabolite profiles of the LPS-exposed heamolymph and control samples. Multivariate date analysis of partial least squares-discriminant analysis (PLS-DA), validation of PLS-DA model, identification of important classifiers were conducted as described in Chapter 4 (4.2.7).

7.3 RESULTS

7.3.1 Effect of LPS on haemocyte mortality and ROS production

The total haemocyte count in haemolymph samples was 1.42×10^6 which was not significantly different among three treatments ($p > 0.05$). However, LPS exposure increased haemocyte mortality in a dose-dependent manner; from 11.4 ± 1.3 % in the control to 16.6 ± 0.8 % in the 50 $\mu g \cdot ml^{-1}$ LPS treatment and 26.9 ± 2.8 % in the 100 $\mu g \cdot ml^{-1}$ LPS treatment after 3 h incubation at 19 °C (Fig. 7.1A). However, a significant difference was only found between the control and the 100 $\mu g \cdot ml^{-1}$ LPS treatment (p = 0.010).

Although ROS production was slightly higher in LPS treatments compared to that of the control, there was no significant difference between treatments (p = 0.533). ROS production in the control, 50 and 100 $\mu g \cdot ml^{-1}$ LPS treatments was 20.8 ± 2.52 %, 25.0 ± 3.8 % and 24.8 ± 1.64 %, respectively (Fig. 7.1A).

7.3.2 Effect of LPS on haemocyte apoptosis

LPS exposure increased the percent of cells with depolarized mitochondrial membranes in a dose-dependent manner. The percent of live depolarized cells increased from 18.6 ± 2.1 % in the control to 23.8 ± 2.0 % in the 50 $\mu g \cdot ml^{-1}$ LPS treatment and 30.0 ± 1.0 % in the 100 $\mu g \cdot ml^{-1}$ LPS treatment, with statistically significant difference between control and 100 $\mu g \cdot ml^{-1}$ LPS treatment (p = 0.008). Percentages of dead depolarized cells were low (< 3 %) and no different between the control and treatments (p = 0.105). Together, the total number of depolarized cells were significantly different only between the control (20.8 ± 2.1 %) and 100 $\mu g \cdot ml^{-1}$ LPS treatment (32.6 ± 0.3 %) (p = 0.007) (Fig. 7.1B).

Similarly, the effect of LPS on the apoptosis profile of mussel haemocytes *via* caspase 3/7 activity increased the percent of dead apoptotic cells from 4.0 ± 0.4 % in the control

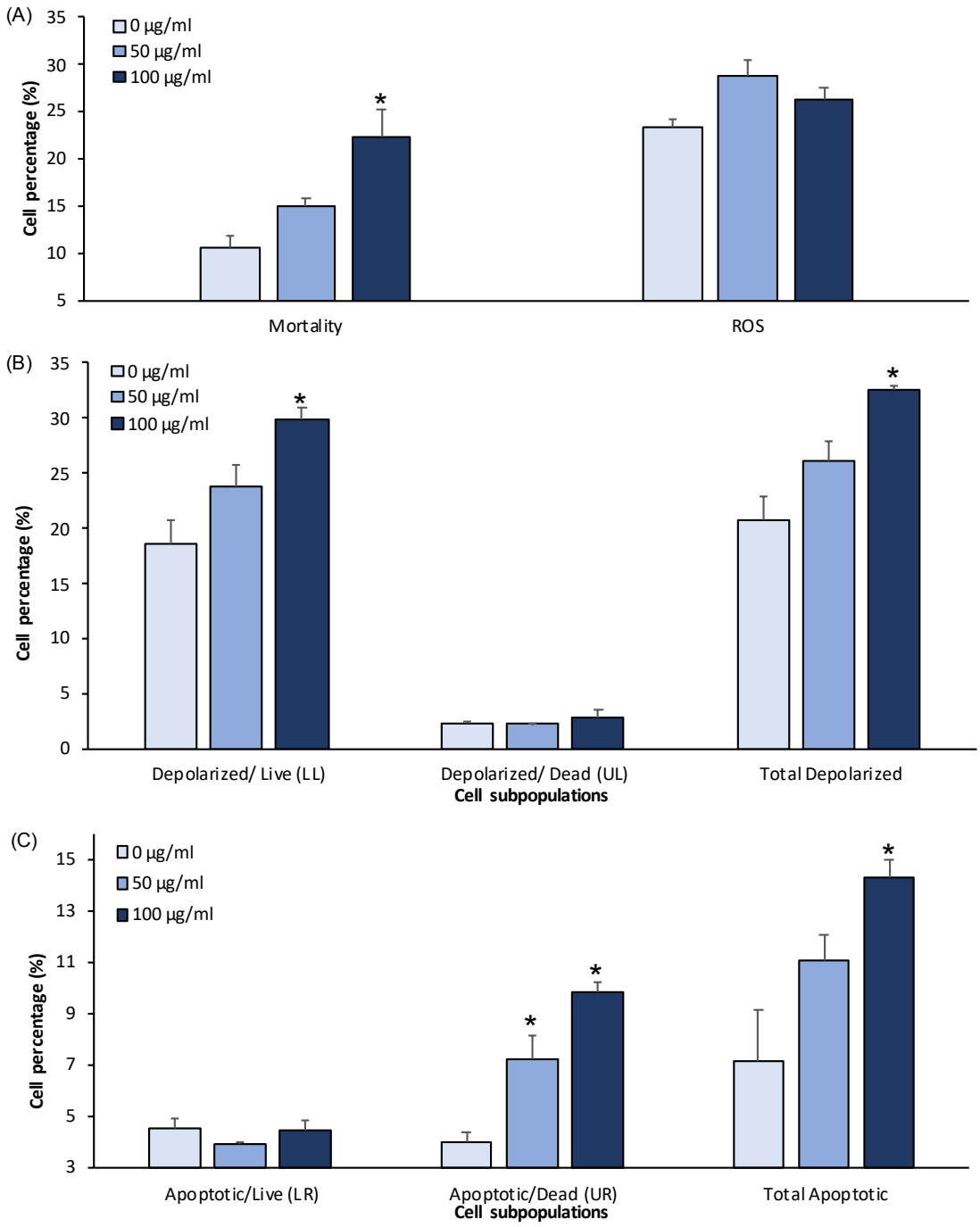

Figure 7.1 Effect of LPS on health parameters of mussel haemocytes determined *via* flow cytometry analyses. (A) Haemocyte mortality and ROS production in haemocytes. (B) Percentage of subpopulations of depolarized haemocytes. (C) Percentage of subpopulations of apoptotic haemocytes. Data are shown as the mean ± S.E. (n=3). Significant differences relative to the control are marked with an asterisk (*) ($p < 0.05$).

to 7.2 ± 0.9 % in the 50 $\mu g \cdot ml^{-1}$ LPS treatment and 9.8 % in 100 $\mu g \cdot ml^{-1}$ in the LPS treatment (Fig. 7.1C). However, a significant difference was only found between the control and the 100 $\mu g \cdot ml^{-1}$ LPS treatment (p = 0.001). There was no significant difference in the percent of live apoptotic cells in the control and treatments, which were less than 4.0 % (p = 0.639). The percentage of total apoptotic cells was significantly different only between the control (7.2 ± 2.0 %) and the 100 $\mu g \cdot ml^{-1}$ LPS treatment (14.3 ± 0.7 %) (p = 0.011).

7.3.3 Effect of LPS on metabolite profiles

A total of 387 features were detected in the chromatograms of mussel haemolymph samples of both the 100 $\mu g \cdot ml^{-1}$ LPS-exposed treatment and the control. Among them, 55 metabolites were reliably annotated as identified metabolites. There were 11 metabolites that showed significant differences between paired groups (100 $\mu g \cdot ml^{-1}$ and the control), which were all higher in the LPS-exposed haemolymph compared to the control (t-test, $p < 0.05$). These metabolites include three branched-chain amino acids (leucine, isoleucine and valine), lysine, phenylalanine, histidine, tryptophan, tyrosine, proline, methionine and creatinine (Table 7.1).

PLS-DA of metabolite profiles showed clear discrimination between the LPS-exposed haemolymph and the control (Fig. 7.2A). The distribution of non-treated haemolymph (green-filled circles) was clearly separated from that of LPS-exposed haemolymph (red-filled triangles). The PLS-DA model showed an accuracy of 92.86 %, a multiple correlation coefficient (R^2) of 80.99 %, and a cross-validated predictive ability (Q^2) of 62.41 %, which indicate good cross-validated model performance. PLS-DA similarly recognised the 11 metabolites that were significantly different between the LPS-exposed treatment and the control with a t-test, and these were the most important metabolites for

the classification model *via* their VIP scores (> 1.0). In addition to these metabolites, PLS-DA also recognised arachidic acid, cis-vaccenic acid, myristic acid, succinic acid, threonine as being important classifiers with their VIP scores greater than 1 (Fig. 7.2B). A combined heatmap and cluster analysis of these 16 metabolites revealed that they were generally increased in LPS-exposed haemocytes/haemolymph (Fig. 7.2C).

Table 7.1 List of altered metabolites identified by *t*-test ($p < 0.05$) and fold change (LPS/control) and PLS-DA VIP score of each metabolite. FDR, false discovery rate; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection.

Metabolites	*t*-test			Fold change (LPS/control)	PLS-DA – VIP score
	t.stat	p.value	FDR		
Phenylalanine	-8.88	<0.001	<0.001	117.85	1.94
Valine	-8.13	<0.001	<0.001	116.37	1.92
Isoleucine	-7.80	<0.001	<0.001	115.62	1.90
Leucine	-7.41	<0.001	<0.001	114.6	1.89
Tryptophan	-5.62	<0.001	0.001	49.56	1.77
Creatinine	-5.33	<0.001	0.002	73.38	1.75
Tyrosine	-5.25	<0.001	0.002	21.41	1.74
Lysine	-5.22	<0.001	0.002	113.62	1.73
Proline	-4.85	<0.001	0.003	102.96	1.69
Methionine	-4.58	0.001	0.004	104.45	1.66
Histidine	-3.66	0.003	0.016	9.52	1.51

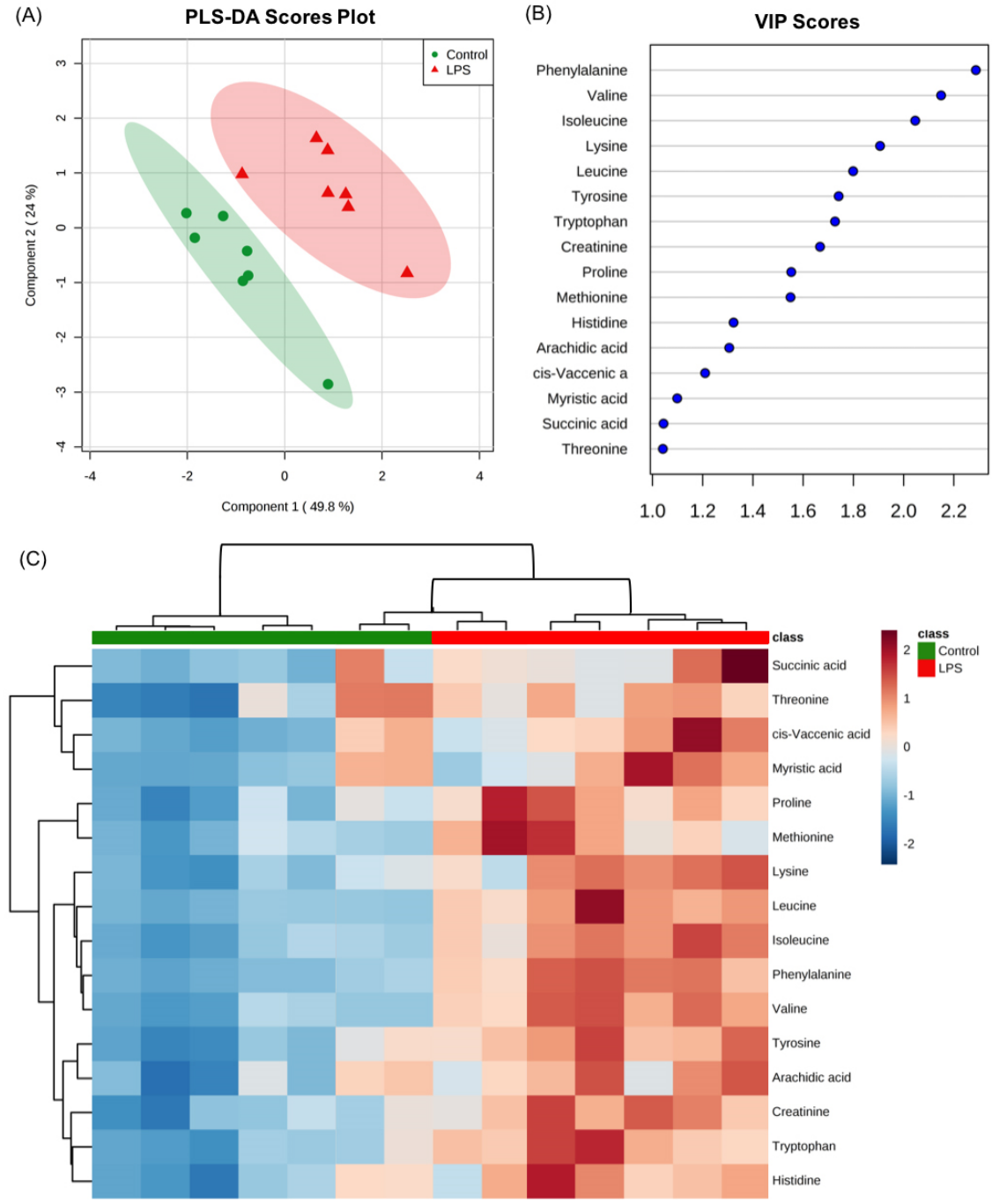

Figure 7.2 Multivariate data analyses of non-targeted metabolomics of LPS-exposed mussel haemolymph (100 $\mu g \cdot ml^{-1}$) and the controls. (A) PLS-DA score plot. (B) PLS-DA VIP Scores of top 16 metabolites with VIP Scores greater than 1.0. (C). Heatmap of 16 important classifiers identified by PLS-DA VIP scores.

7.4 DISCUSSION

Pathogen-associated molecular patterns (PAMPs) are conserved microbial molecules that are recognized by receptors of the innate immune system, such as toll-like receptors and other pattern recognition receptors in macrophages and antigen-presenting cells (Merien, 2016, Silva-Gomes et al., 2016). One of the most widely studied PAMPs is LPS, which is a highly abundant glycolipid of the outer walls of Gram-negative bacteria (Raetz and Whitfield, 2002). LPS is a powerful stimulator of innate immunity in eukaryotic species (Ulevitch and Tobias, 1995) and has been used extensively to elicit immune responses in vertebrates (Bahador and Cross, 2007, Laiakis et al., 2012, Opal, 2007, Rittirsch et al., 2007). In marine bivalves, LPS has been used to stimulate immune responses in mussel (*Mytilus* spp.) haemocytes, such as antibacterial peptide responses (Hernroth, 2003), expression of a membrane molecule related to the α chain of the IL-2 receptor (Cao et al., 2004) and expression of immune genes (e.g., myticin C, mytilin B and lysozyme) (Costa et al., 2009). This suggests that LPS could be an effective proxy for pathogen effects in bivalve studies. However, the effect of LPS on apoptosis in bivalve cells has not been extensively investigated. In our study, we confirmed that LPS induces activation of caspase-3/7 proteases in mussel haemocytes and causes depolarisation of the mitochondrial membrane potential; both key mechanisms of programmed cell death.

Mitochondria produce adenosine triphosphate and are responsible for controlling cellular energetics, and the initiation and execution of apoptosis (Estaquier et al., 2012, Green and Reed, 1998). Most of the important events in the apoptosis process appear to be related to changes of mitochondrial membrane potential (MMP) (Green and Reed, 1998, Ly et al., 2003). Perturbation of MMP associated with apoptosis has been well documented in vertebrate models (Ferreira et al., 2013, Petit et al., 1995, Vayssiere et al., 1994, Zamzami et al., 1995), and it has been recently reported in several invertebrate models, such as

Drosophila melanogaster (Zimmermann et al., 2002), *Lymnea stagnalis* (Russo and Madec, 2007), and *Penaeus monodon* (Xian et al., 2010). In marine molluscs, genes of the mitochondrial apoptotic pathway have been extensively analysed in *Crassostrea gigas* (Zhang et al., 2011) and *Mytilus galloprovincialis* (Estevez-Calvar et al., 2013). Recently, the mitochondrial-mediated pathway of apoptosis was demonstrated in oyster (*Ostrea edulis* and *C. gigas*) haemocytes exposed to UV radiation (Gervais et al., 2015, Li et al., 2017). Hence, the increased proportion of cells expressing depolarised MMPs in LPS-treated haemocytes suggests that LPS can stimulate apoptosis *via* a mitochondrial-mediated apoptotic pathway.

Caspases (cysteinyl-directed aspartate-specific proteases) are a family of protease enzymes that play a vital role in apoptosis (Cohen, 1997, Fan et al., 2005). Among them, caspase-3 is a frequently activated death protease in mammalian apoptotic cells (Porter and Jänicke, 1999), and also has been reported in invertebrates (Guo et al., 2017, Lacoste et al., 2002, Sokolova et al., 2004). Hence, activation of caspase-3 is considered to be an indicator of apoptosis (Porter and Jänicke, 1999). In bivalves, caspase-3 activity has been reported in individuals exposed to various heavy metals (Kefaloyianni et al., 2005, Sokolova et al., 2004) and organic chemicals (Lacoste et al., 2002). Hence, a significant increase of apoptotic cells *via* the presence of active caspase-3 in LPS-treated haemocytes compared to the controls suggests LPS exposure induced apoptosis in a caspase-dependent pathway.

LPS-induced ROS generation has been reported in various vertebrate cell types (Cheong et al., 2017, Lee et al., 2013, Wang et al., 2004, Yamada et al., 2006). However, LPS was not effective at stimulating ROS and nitrite production in other cell types, such as peritoneal macrophages from diabetic rats (de Souza et al., 2007). Although the

generation of ROS production during phagocytosis has been widely observed in molluscs (Bramble and Anderson, 1997, Buggé et al., 2007, Labreuche et al., 2006, Lambert et al., 2003), there is no report of LPS-induced ROS generation in marine molluscs. In the present study, we did not observe a significant difference in ROS production between the LPS-exposed haemocytes and the controls. In agreement with this finding, previous studies found that LPS did not provoke nitric oxide (NO) and ROS production in haemocytes of other bivalves (Costa et al., 2009, Novas et al., 2004). Taking together, this may indicate that LPS is ineffective to stimulate ROS in bivalve haemocytes. Furthermore, accumulation of ROS is known to trigger apoptosis in both vertebrates (Brodská and Holoubek, 2011, Higuchi et al., 1998, Luzio et al., 2013, Orrenius, 2007, Zhang et al., 2015) and invertebrates (Guo et al., 2017). In this study, apoptotic cells were higher in the LPS-exposed treatment (100 $\mu g \cdot ml^{-1}$) compared to the controls, whereas LPS-induced ROS production were not differently different among the LPS treatments. These results suggest that LPS may stimulate apoptosis in a ROS-independent pathway in mussel haemocytes.

Apoptosis is often accompanied by degradation of cellular and nuclear contents (He et al., 2009, Nagata et al., 2003). Hence, increased levels of free amino acids (cis-vaccenic acid, myristic acid and arachidic acid) in our study may signal the occurrence of lipid damage. In addition, caspase-mediated proteolysis is known to occur during the apoptotic process (He et al., 2009, Sanghavi et al., 1998), and the accumulation of numerous free amino acids that we observed in LPS-exposed haemolyph/haemocytes is consistent with the occurence of protein degradation. Some of these amino acids are also known to have specific functional roles which may have relevance in this case. For example, BCAAs (leucine, isoleucine and valine) are involved in mechanisms of protein synthesis and turnover (Monirujjaman and Ferdouse, 2014), inflammation, ROS production, and

mitochondrial dysfunction (García-Martínez et al., 1995, Moreira et al., 2017, Zhenyukh et al., 2017). Methionine is a key metabolite in the transsulfuration pathway which plays an important role in regulating overproduction of ROS and mitigation of oxidative stress (McBean, 2012, Nguyen et al., 2018e). The change of methionine due to *Vibrio* spp. infections has been previously reported in mussel (*P. canaliculus*) haemocytes (Nguyen et al., 2018e). Increased levels of tryptophan and proline in mice serum after LPS injection has been associated with having antimicrobial roles (Bera et al., 2015, Larsson et al., 2016), and accumulations of creatinine in serum, an important marker of renal dysfunction (Wyss and Kaddurah-Daouk, 2000), can inhibit bacterial replication (McDonald et al., 2012). The increase of these metabolites in LPS-exposed haemocytes may indicate occurrence of other immune or physiological responses of haemocytes to LPS, such as successful regulation of oxidative status, provision of antimicrobial activity, and changes in other currently unknown processes/functions.

7.5 CONCLUSION

This study successfully applied an integrated approach that combines GC-MS-bases metabolomics and flow cytometry to investigate apoptosis in mussel haemocytes induced by LPS-exposure. We observed significant increases in caspase-3/7 activation and MMP in LPS-exposed haemocytes, which suggests LPS induced apoptosis *via* both caspase- and mitochondria-dependent mechanisms. Furthermore, accumulations of free fatty acids and free amino acids in LPS-exposed haemolymph indicate degradation of lipids and proteins, and the potential influence on other immune-related processes. This study demonstrates the capacity of fast and accurate characterization of apoptotic processes using a flow cytometry approach and provides supporting evidence at the metabolic level *via* GC-MS-based metabolomics. This opens up new perspectives on applying this integrated approach to other aspects of aquaculture research to provide insights into complex biological processes, such as pathogen infections and environmental disturbances. However, the results presented in this study were from short term *in vitro* exposure of mussel haemocytes to LPS. *In vivo* expression of apoptosis signals induced by LPS in mussel haemocytes in comparation to real pathogen effects needs to be expressed in the future studies to provide a comprehensive understanding of apoptosis in marine bivalves. Such knowledge would be important for development of disease management strategies and biomarkers for early detection of pathogens in aquaculture.

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Chapter 8

Copper-induced immunomodulation in mussel (*Perna canaliculus*) haemocytes

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ABSTRACT

Copper is a common contaminant in aquatic environments, which may cause physiological dysfunction in marine organisms. However, the toxicity mechanisms of copper in marine bivalves is not fully understood. In this study, we applied an integrated approach that combines flow cytometry and gas chromatography-mass spectrometry (GC-MS)-based metabolomics to characterize cellular and molecular mechanisms of copper immunotoxicity in New Zealand Greenshell™ mussel (*Perna canaliculus*) haemolymph. Flow cytometric results showed significant increases in haemocyte mortality, production of reactive oxygen species and apoptosis (*via* alteration of caspase 3/7 and mitochondrial membrane potential) of haemocytes exposed to increasing total concentrations of Cu^{2+} (62.5, 125.0 and 187.5 μM) compared to a low Cu^{2+} concentration (25 μM) and control (0.0 μM). In addition to flow cytometric data, our metabolomics results showed alterations of 25 metabolites within the metabolite profile of Cu^{2+}-exposed haemolymph (125.0 μM) compared to those of control samples. Changes in levels of these metabolites may be considered important signatures of oxidative stress (e.g., glutathione) and apoptosis processes (e.g., alanine, glutamic acid). This study provides insights into the cellular and molecular mechanisms of oxidative stress and apoptosis in marine bivalves and highlights the applicability and reliability of metabolomic techniques for immunotoxicological studies in marine organisms.

8.1 INTRODUCTION

Increased pollution in natural and farmed environments has resulted in a growing focus on the adverse effects of toxic substances and the mechanisms that underpin biological responses in a wide range of organisms. Heavy metals (e.g., copper, cadmium) are not degradable and persist for long periods of time when introduced into aquatic ecosystems and may pose serious physiological problems by affecting the defence system of aquatic organisms (e.g., immunosuppression). Among heavy metals, copper is widely used in industry and agriculture and is a common chemical contaminant in aquatic environments. Copper, at low concentrations, is an essential trace element that is vitally important for numerous physiological processes of all living organisms (Loftleidir, 2005), including facilitation of a well-functioning immune system (Djoko et al., 2015). Although precise roles of Cu^{2+} in innate immune function are not yet well defined, it has been hypothesized that phagocytes may concentrate Cu^{2+} intracellularly to reduce survival of phagocytised pathogens due to its bactericidal activity (Djoko et al., 2015). However, accumulation of copper at high concentrations is known to have toxic effects (Flemming and Trevors, 1989, Nielsen and Wium-Andersen, 1970). Aquatic organisms, especially filter feeding marine bivalves, are likely to accumulate high levels of copper over their life time. Also, they have relatively long life spans and high numerical abundances (Bayne, 1989, Eisler, 2009). Hence, bivalves have been extensively used as model organisms for immunotoxicological studies. Indeed, the toxic effects of copper on bivalves have been intensively investigated during the last decades (Foster et al., 2011, Giacomin et al., 2014, Gómez-Mendikute and Cajaraville, 2003, Manley and Davenport, 1979, Suresh and Mohandas, 1990). However, applications of metabolomics approaches to characterize molecular mechanisms involved in these toxic effects of copper on bivalves still remain limited.

Metabolomics is one of the newest omics, which has been rapidly growing in the last decade. Metabolomics generates information about metabolites, which are end products of the metabolism, thus provides a strong reflection of phenotypes (Alfaro and Young, 2018). Such a phenotyping tool can be used to understand endogenous metabolic changes of an organism throughout the course of a biological process triggered by disease or environmental perturbation (Klassen et al., 2017, Villas-Boas et al., 2007). Therefore, metabolomics is an emerging approach in environmental science to characterize the interactions of organisms with their environment (Bundy et al., 2008). Different analytical platforms, such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) have been developed for environment studies. While NMR, with its specific advantages, has been the primary analytical tool for environmental metabolomics, mass spectrometry with the higher sensitivity begins to revolutionise our ability in this growing field (Viant and Sommer, 2013). Within MS-based metabolomics, several approaches have been used in environmental metabolomics, including non-targeted gas chromatography-MS (GC-MS), non-targeted directed infusion MS, and both non-targeted and targeted liquid chromatography–MS. Among them, non-targeted GC-MS-based metabolomics, which allows a wide range of metabolites to be detected, has become the most commonly used technique in metabolomics, including environmental metabolomics (Viant and Sommer, 2013, Young and Alfaro, 2016). This approach has been applied in diverse aspects of environmental science, such as organismal ecophysiology (Allen et al., 2011), ecotoxicology (Booth et al., 2011), environmental stress in trees (Wallis et al., 2011), plant–animal interactions (Robert et al., 2010) and host-pathogen interactions in marine shellfish (Nguyen et al., 2018). Although the use of NMR to study the metal toxicity is gaining importance in aquatic organisms, especially

marine bivalves (García-Sevillano et al., 2015), there is currently no report on the application of GC-MS approaches on copper toxicity on mussel haemocytes.

In addition, flow cytometry is a laser-based technology, which is a popular approach in marine science to obtain information about cellular processes. Recent studies have successfully demonstrated applications of a novel flow cytometric approach based on a robust and compact Muse® Cell Analyzer (Merck Millipore, Germany) for fast and accurate characterization of the mollusc haemocytes (Grandiosa et al., 2016, Nguyen et al., 2018). The combination of this flow cytometry approach with metabolomics technique could provide valuable insights into the toxic mechanism of heavy metals in marine bivalves at both the cellular and molecular levels. In this study, we provide the first report on the combined approach of GC-MS-based metabolomics and novel flow cytometry to characterize the toxic effects and elucidate the toxicity mechanism of copper on New Zealand Greenshell™ mussel (*Perna canaliculus*) haemocytes.

8.2 MATERIALS AND METHODS

8.2.1 Mussel samples

Adult mussels (shell length: 8.06 ± 0.45 cm) were obtained from Westpac Mussels Distributors Ltd. (Auckland, New Zealand). Mussels were acclimatized for 7 days in a 50 l tank on a seawater recirculation system as condition described in Chapter 4 (4.2.1).

Haemolymph was collected from each animal by gently inserting a needle (23 gauge × 1.5") attached to a 3 ml sterile syringe (Terumo, Japan) into the posterior adductor muscle. For each mussel, 1–2 ml of haemolymph was collected. Immediately after withdrawal, haemolymph samples were transferred into 10 ml Eppendorf tubes and kept on ice. Haemolymph samples from five mussels were pooled and homogeneously mixed to reduce inter-individual and sex variations, and to provide enough haemolymph for subsequent laboratory tests.

8.2.2 Copper exposure

A 0.05 M Cu^{2+} stock solution was prepared by dissolving 5 g copper (II) sulfate pentahydrate $CuSO_4.5H_2O$ (ECP, Auckland, New Zealand) in 100 ml 0.22 µm filtered artificial seawater (ASW). Four Cu^{2+} working solutions (50, 125, 250 and 375 µM) were subsequently prepared by diluting the stock solution with 10 ml filtered ASW.

Copper treatments were administered *in vitro* by mixing 0.5 ml of haemolymph with 0.5 ml of working Cu^{2+} solution at 4 °C. Following addition of haemolymph, the final exposure concentrations of Cu^{2+} were 25.0, 62.5, 125.0 and 187.5 µM. The negative control was prepared by adding 0.5 ml of haemolymph to 0.5 ml of cold ASW. Each treatment was conducted with three replicates. Treated haemolymph samples were incubated at 19 °C for 3 h, then assessed for haemocyte mortality, ROS production, and apoptosis. For metabolomics, 500 µl of each sample from the 125.0 µM Cu^{2+} treatment

and control were pipetted into 2 ml BioStor™ Cryovials (National Scientific Supply Company, California, USA), immediately flash-frozen in liquid nitrogen and stored at –80 °C until metabolite analyses could be performed.

8.2.3 Effect of copper on haemocyte viability and ROS production

Following copper exposures (see above), cell viability was measured for each sample with a Muse® Cell Count and Viability kit (Merck Millipore, USA), as previously described (Chapter 4 – 4.2.3). The Muse® Oxidative Stress kit (Merck Millipore, USA) was used to gain quantitative measurements of ROS, namely superoxide radicals in cells undergoing oxidative stress (Chapter 5 – 5.2.2).

To assess ROS production kinetics, haemocytes were exposed to different levels of Cu^{2+} (0.0, 25.0 and 125.0 µM), incubated at 19 °C, and assessed for ROS production at 0.25, 0.5, 1.0, 3.0, 12.0, and 24.0 hours post-incubation. ROS production was measured by the Muse® Oxidative Stress kit using the DHE, as described above.

8.2.4 Effect of copper on apoptosis

The effect of copper on apoptosis was assessed by two flow cytometry assays (Muse® MitoPotential kit and Muse® Caspase-3/7 kit), and read by Muse® Cell Analyzer (Merck Millipore, Abacus dx, New Zealand) (Chapter 7 – 7.2.2).

8.2.5 GC-MS-based metabolomics analyses and data processing

Metabolite extractions, derivatizations and GC-MS analyses, quality control and spectral processing were performed as previously described (Chapter 4 – 4.2.4-6).

8.2.6 Statistical analyses

Statistical analyses for flow cytometric assays were performed using one-way ANOVAs and two-way ANOVAs with IBM® SPSS® Statistics software (version 23).

Statistical analyses of metabolite data were conducted using Metaboanalyst 3.0 (Xia et al., 2015). Normalized peak intensity data were generalized logarithm (glog) transformed and mean centered. Then, univariate analyses were performed using students *t*-tests to identify metabolite differences between Cu^{2+}-exposed and control groups. Multivariate date analysis of partial least squares-discriminant analysis (PLS-DA), validation of PLS-DA model, identification of important classifiers were conducted as described in Chapter 4 (4.2.7).

8.2.7 Pathway analyses

Combined quantitative enrichment analysis (QEA) (Xia and Wishart, 2010) and network topology analysis (NTA) (Nikiforova and Willmitzer, 2007) were performed to investigate treatment effects on functionally related metabolites, as described in Chapter 5 (5.2.5).

8.3 RESULTS

8.3.1 Effect of copper on haemocyte viability and ROS production

The increased Cu^{2+} concentrations caused a significant effect on haemocyte viability after 3 h exposure. Except for the 25.0 µM Cu^{2+} treatment, which had no significant difference in mortality compared to the control (one-way ANOVA, $p = 0.848$), significant increases in mortality were observed in all other treatments (62.5, 125.0 and 187.5 µM) (one-way ANOVA, $p < 0.001$) (Fig. 8.1). The haemocyte mortalities were 19.0, 16.6, 32.1, 86.5 and 94.5 % in 0.0, 25.0, 62.5, 125.0 and 187.5 µM Cu^{2+} groups, respectively.

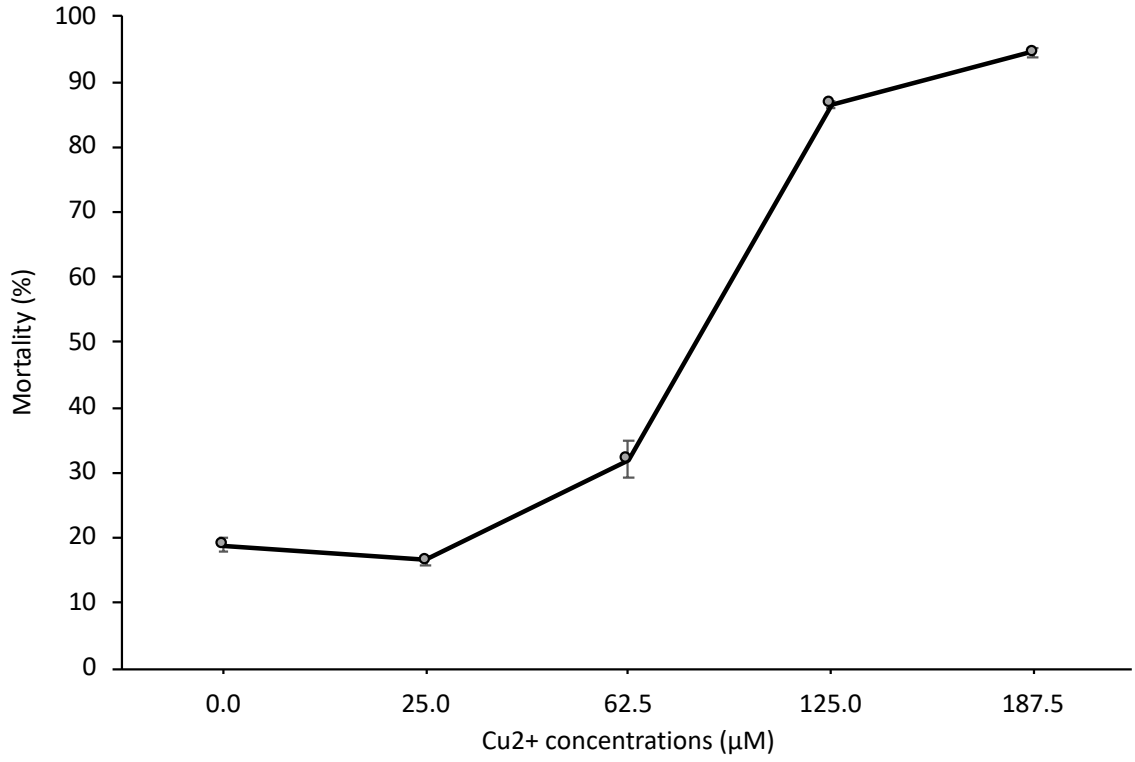


Figure 8.1 Mortality of mussel haemocytes incubated with 5 different concentrations of Cu^{2+} (0.0, 25.0, 62.5, 125.0 and 187.5 µM, respectively) for 3 h. Data are presented as mean percent (± SE, n = 3).

Generally, ROS production showed a significant increase in a dose-dependent manner (one-way ANOVA, $F_{4,36} = 214.4$, $p < 0.001$). The proportion of cells that produced ROS significantly increased with increasing Cu^{2+} concentration from 14.60 % in the 25.0 µM Cu^{2+} treatment to 26.15, 60.84 and 82.03 % in the treatments with 62.5, 125.0 and 187.5 µM Cu^{2+}, respectively, after 3 h incubation at 19 °C (Fig. 8.2A). There was no difference in ROS production between the control and the treatment with 25.0 µM Cu^{2+} (one-way ANOVA, $p = 0.433$). Based on this experiment, we selected one low (25.0 µM) and one high Cu^{2+} concentration (125.0 µM) to perform over-induction of ROS in mussel haemolymph following Cu^{2+} exposure.

ROS production kinetics showed a higher ROS level in the 125.0 µM Cu^{2+} treatment and lower in the 25.0 µM Cu^{2+} treatment compared to the control (two-way ANOVA, $F_{2,384} = 631$, $p < 0.001$) (Fig. 8.2B). In all treatments, ROS reached a peak after 30 minutes, then it declined and peaked for a second time after 24 h. In fact, haemocytes exposed to 125.0 µM of Cu^{2+} resulted in the highest ROS level (53.69 %) after 30 minutes exposure, followed by a 13 % decrease after 1 h and an increase to the second peak of 63.55 % at 24 h. In the control, ROS reached 44.04 % at 30 minutes and then declined to 14.24 % after 3h.

It was then increased to 43.23 % after 24 h. In the treatment with 25.0 µM Cu^{2+}, ROS went up to 27.13 % after 30 minutes post-incubation, then decreased to a basal level of 5.58 % after 12 h, followed by a slight increase to 20.18 % after 24 h of incubation.

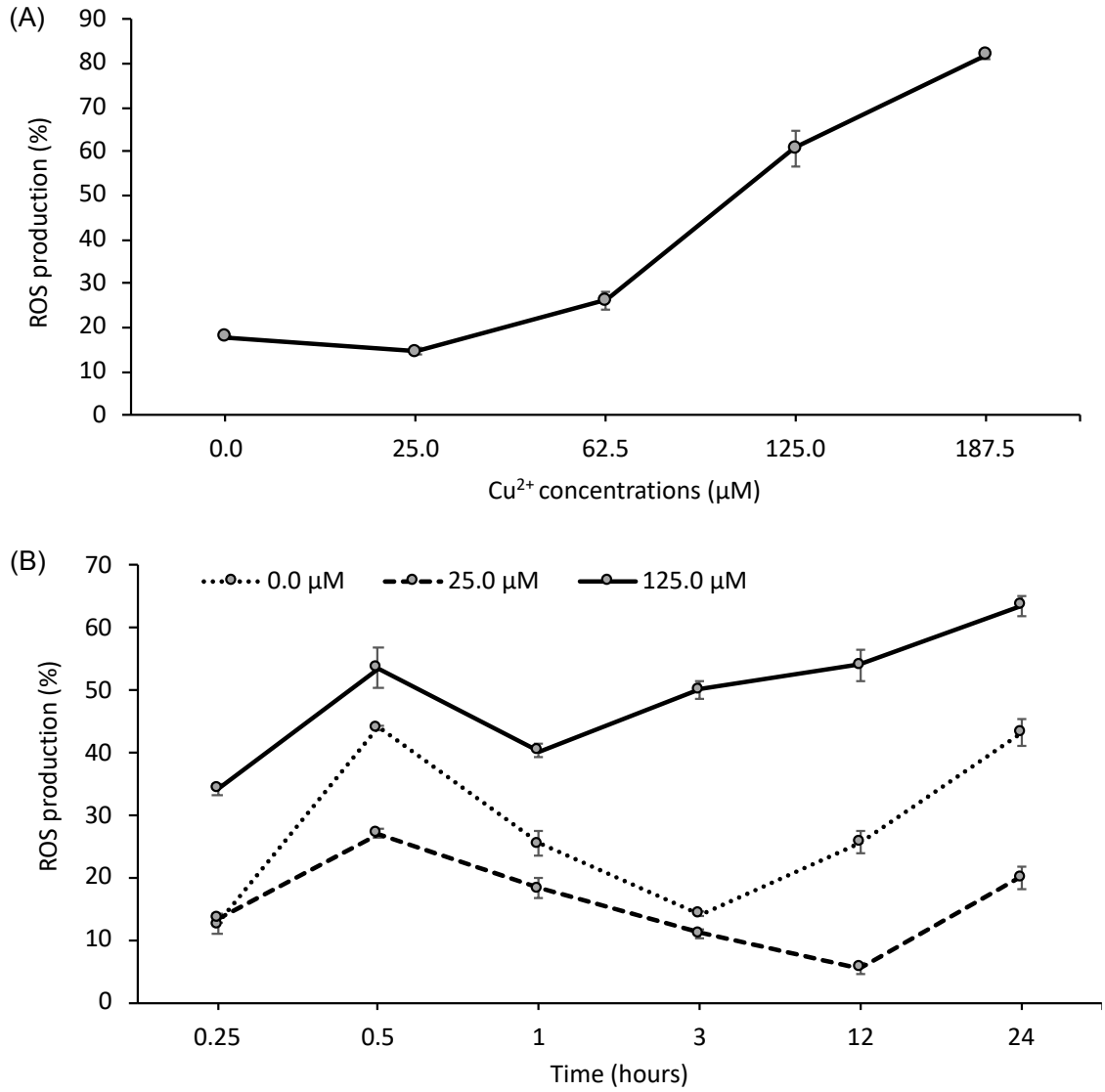


Figure 8.2 Effect of Cu^{2+} on ROS production in mussel haemocytes. (A) ROS profiles of haemocytes after 3 h incubation at 19 °C with increasing concentrations of Cu^{2+} (0.0, 25.0, 62.5, 125.0 and 187.5 μM). (B) Kinetics of ROS production (mean ± SE, n = 3) in mussel haemocytes exposed to different Cu^{2+} concentrations (0, 25.0 and 125.0 μM) at 19 °C.

8.3.2 Effect of copper on apoptosis

Caspase-3/7 activation

Generally, increased Cu^{2+} concentrations caused a simultaneous increase of apoptotic cells in a dose-dependent manner (Fig. 8.3). The percentage of apoptotic cell death (late apoptosis) increased from 1.63 % in the control to 2.40 %, 13.87 %, 26.40 % and 36.08 % in treatments with 25.0, 62.5, 125.0 and 187.5 µM Cu^{2+}, respectively. However, the proportion of live apoptotic cells (early apoptosis) was very low in all treatments (less than 1.38 %). Hence, the late apoptotic sub-population approximately represents the total apoptotic haemocytes.

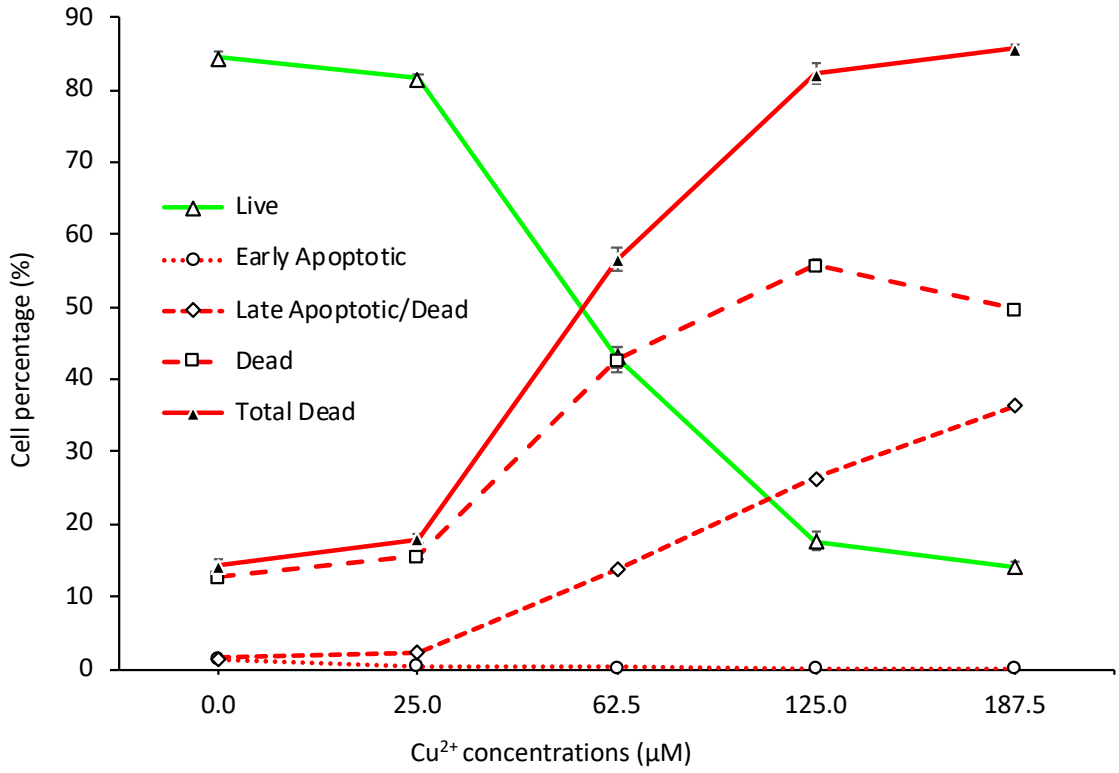


Figure 8.3 Effect of Cu^{2+} on caspase 3/7 activation in mussel haemocytes. Percentages of different cell sub-populations (mean ± SE, n = 3) following treatment with 5 different Cu^{2+} concentrations (0, 25.0, 62.5, 125.0 and 187.5 µM).

Mitochondrial membrane potential

The exposure of Cu^{2+} showed effects on mitochondrial potential and cellular plasma membrane permeabilization with a simultaneous increasing number of depolarized cells in a dose-dependent manner (Fig. 8.4). The low Cu^{2+} concentrations (25.0 and 62.5 µM) caused a significant increase in depolarised live cells compared to those in the control, while the high concentrations of Cu^{2+} (125.0 and 187.5 µM) led to a remarkable increase of depolarized dead cells. Together, the total depolarized cells showed a dose-dependent pattern, which rose from 17.75 % in the control to 28.85, 43.55 and 99.85 % in the treatments with 25.0, 62.5, 125.0 and 187.5 µM Cu^{2+}, respectively.

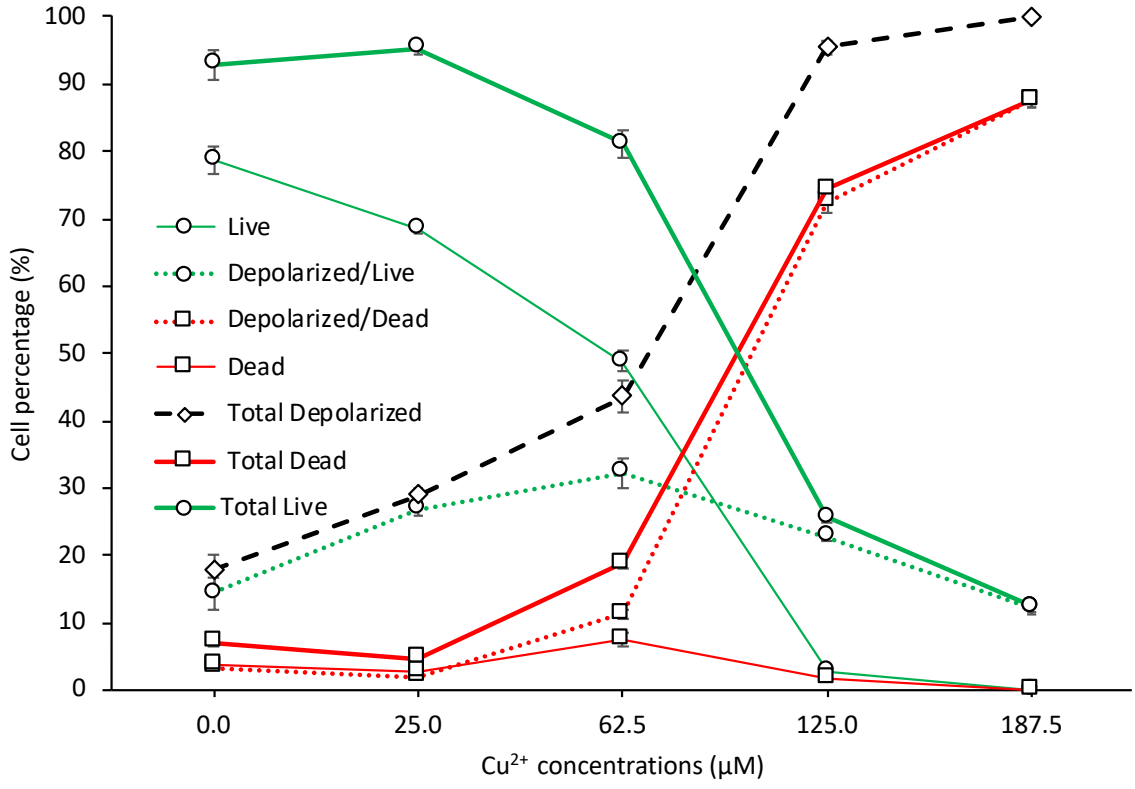
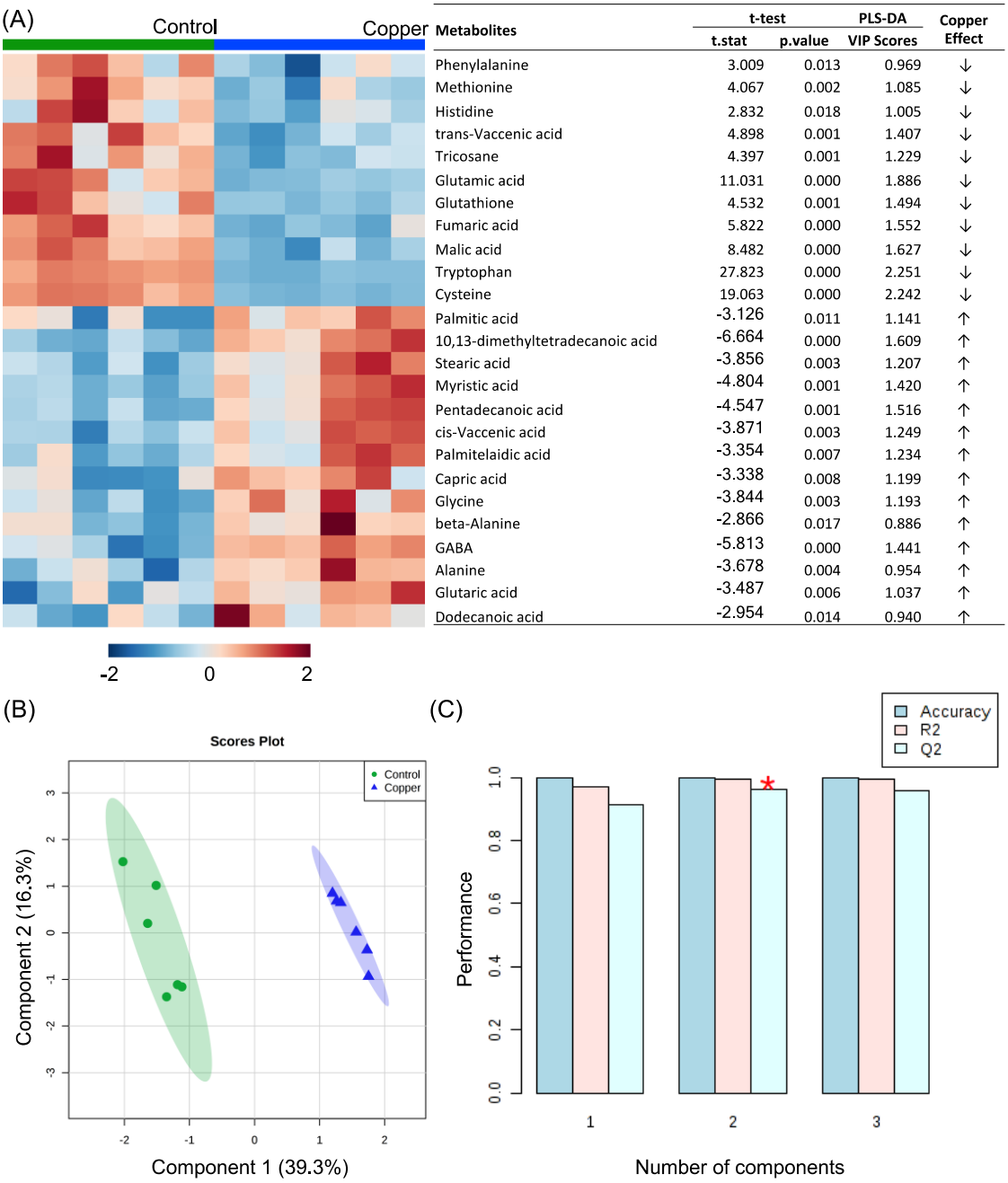


Figure 8.4 Effect of Cu^{2+} on mitochondrial membrane potential in mussel haemocytes. Percentages of different cell sub-populations (mean ± SE, n = 3) following treatment with 5 different Cu^{2+} concentrations (0, 25.0, 62.5, 125.0 and 187.5 µM).

8.3.3 Effect of copper on metabolite profiles

A total of 64 metabolites were reliably annotated across the haemolymph samples. A *t*-test analysis revealed a significant difference of 25 compounds between the Cu^{2+}-exposed and control groups (*t*-test, $p < 0.05$) (Fig. 8.5A). A heatmap cluster analysis of these 25 metabolites showed a clear separation between the Cu^{2+}-exposed group and the control group. In addition, the heatmap also separated these compounds into two groups: a group of 11 down-regulated metabolites in the Cu^{2+}-exposed haemolymph (e.g., phenylalanine, methionine, histidine) and another group of 14 metabolites which were highly expressed in Cu^{2+}-exposed haemolymph (e.g., palmitic acid, glycine, GABA).

A PLS-DA of the entire metabolite profiles showed clear discrimination between the Cu^{2+}-exposed and control groups (Fig. 8.5B). The distribution of control mussels (green dots) was clearly separated from that of Cu^{2+}-exposed mussels (blue triangles) along the LV1 axis. The PLS-DA model showed an accuracy of 100 %, a multiple correlation coefficient (R^2) of 98.6 %, and a cross-validated predictive ability (Q^2) of 94.4 % (Fig. 8.6C). These parameters indicate optimal fitness and prediction performance of the PLS-DA model.



Metabolites	t-test		PLS-DA	Copper Effect
	t.stat	p.value	VIP Scores	
Phenylalanine	3.009	0.013	0.969	↓
Methionine	4.067	0.002	1.085	↓
Histidine	2.832	0.018	1.005	↓
trans-Vaccenic acid	4.898	0.001	1.407	↓
Tricosane	4.397	0.001	1.229	↓
Glutamic acid	11.031	0.000	1.886	↓
Glutathione	4.532	0.001	1.494	↓
Fumaric acid	5.822	0.000	1.552	↓
Malic acid	8.482	0.000	1.627	↓
Tryptophan	27.823	0.000	2.251	↓
Cysteine	19.063	0.000	2.242	↓
Palmitic acid	-3.126	0.011	1.141	↑
10,13-dimethyltetradecanoic acid	-6.664	0.000	1.609	↑
Stearic acid	-3.856	0.003	1.207	↑
Myristic acid	-4.804	0.001	1.420	↑
Pentadecanoic acid	-4.547	0.001	1.516	↑
cis-Vaccenic acid	-3.871	0.003	1.249	↑
Palmitelaidic acid	-3.354	0.007	1.234	↑
Capric acid	-3.338	0.008	1.199	↑
Glycine	-3.844	0.003	1.193	↑
beta-Alanine	-2.866	0.017	0.886	↑
GABA	-5.813	0.000	1.441	↑
Alanine	-3.678	0.004	0.954	↑
Glutaric acid	-3.487	0.006	1.037	↑
Dodecanoic acid	-2.954	0.014	0.940	↑

Figure 8.5 Analysis of metabolite profiles in mussel haemolymph exposed to 125.0 µM Cu^{2+} for 3 h. (A) Heatmap of the top 25 metabolites identified as being significantly different between groups (*t*-test; $p < 0.05$), with their relative expressions and VIP scores in the PLS-DA model. (B) PLS-DA score plot (ellipses represent 95 % confidence intervals). (C) Cross validation (LLOCV) of the PLS-DA model.

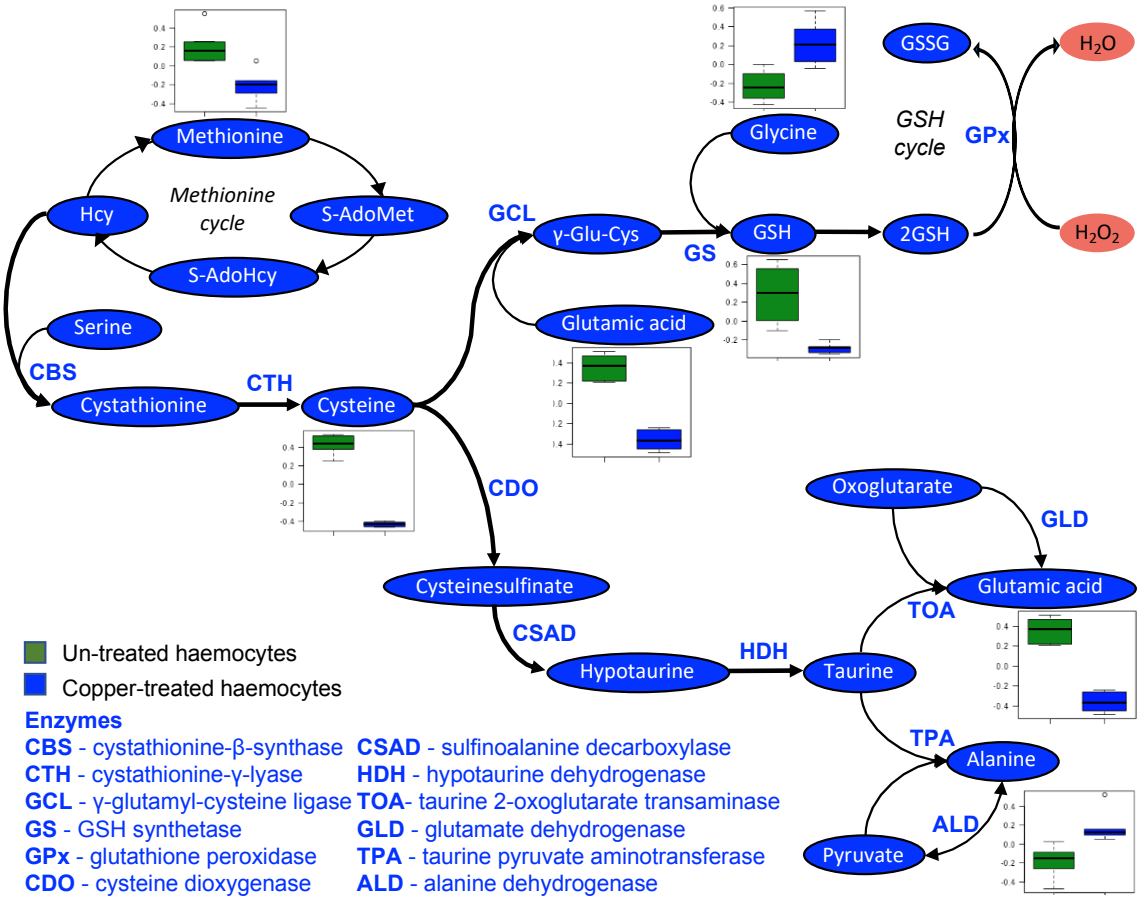


Figure 8.6 Metabolites and pathways involved in oxidative stress and apoptosis caused by Cu^{2+} exposure in mussel haemocytes. Box plots of metabolites were based on *t*-test analysis, showing relative abundances of metabolites in Cu^{2+}-exposed haemocytes (blue) and un-treated haemocytes (green). *Hcy, homocysteine; S-AdoMet, S-adenosylmethionine; S-AdoHcy, S-adenosylhomocysteine*.

8.3.4 Pathway analysis

Secondary pathway analyses identified 42 relevant biochemical pathways within the KEGG database. Among these, 15 pathways which matched our selection criteria were screened as potential primary target pathways of interest relating to the Cu^{2+} effects (Table 8.1).

Table 8.1 List of altered metabolic pathways that were identified as primary target pathways in mussel haemolymph exposed to Cu^{2+}.

Pathways & hits	Hits/ Total compounds	Raw p	FDR	Impact
Cysteine and methionine metabolism: Serine, Methionine and Cysteine	3/29	<0.001	<0.001	0.276
Glycine, serine and threonine metabolism: Serine, Glycine, Threonine, Cysteine	4/31	<0.001	<0.001	0.570
Alanine, aspartate and glutamate metabolism: Aspartic acid, Alanine, Glutamic acid, Glutamine, Asparagine, Fumaric acid, Succinic acid	7/24	<0.001	<0.001	0.605
Citrate cycle (TCA cycle): Succinic acid, Malic acid, Citric acid, Fumaric acid	4/20	<0.001	<0.001	0.151
Aminoacyl-tRNA biosynthesis: Asparagine, Histidine, Phenylalanine, Glutamine, Cysteine, Glycine, Aspartic acid, Serine, Methionine, Valine, Alanine, Lysine, Isoleucine, Leucine, Threonine, Tryptophan, Tyrosine, Proline, Glutamic acid	19/67	<0.001	<0.001	0.103
Glyoxylate and dicarboxylate metabolism: Citric acid, Malic acid	2/18	<0.001	<0.001	0.296
Glutathione metabolism: Glutathione, Glycine, Cysteine, Glutamic acid Ornithine, Pyroglutamic acid	6/26	<0.001	<0.001	0.418
Histidine metabolism: L-Glutamic acid, Histidine, Aspartic acid	3/14	<0.001	<0.001	0.238
Arginine and proline metabolism: Glutamine, Ornithine, Aspartic acid, Glutamic acid, Proline, Fumaric acid	6/43	<0.001	<0.001	0.309
D-Glutamine and D-glutamate metabolism: Glutamic acid, Glutamine, Glutamine, Glutaric acid, Glutamic acid	2/5	<0.001	0.001	1.000
Tyrosine metabolism: Tyrosine, Fumaric acid, Hydroxyphenylacetic acid	3/44	0.002	0.004	0.137
Methane metabolism: Glycine, Serine	2/9	0.012	0.018	0.400
Phenylalanine, tyrosine and tryptophan biosynthesis: Phenylalanine, Tyrosine	4	0.021	0.031	1.000
Phenylalanine metabolism: Phenylalanine, Tyrosine	11	0.021	0.031	0.407
beta-Alanine metabolism: Beta-Alanine, Aspartic acid	16	0.050	0.065	0.395

8.4 DISCUSSION

In the present study, we challenged mussel haemolymph with the increasing Cu^{2+} concentrations (0.0, 25.0, 62.5, 125.0 and 187.5 μM), and we observed significant increases in mortality, ROS production and apoptosis of haemocytes of mussels exposed to Cu^{2+} at high concentrations (25.0 μM = 1.60 ppm and greater). In addition, there were alterations of metabolic profiles in Cu^{2+} exposed haemolymph compared to the controls. These results indicate that Cu^{2+} induced toxicity for mussel haemocytes. The high dose of Cu^{2+} used in this study aimed to stimulate the cumulative toxicity of copper during the short-term *in vitro* exposure. The background levels of copper in seawater were found to be 0.003 ppm in open sea and 0.01 ppm in coastal regions (Hodson et al., 1979, Nielsen and Wium-Andersen, 1970), but copper concentrations have been recorded as high as 0.3 ppm in coastal water off the Dutch coast, following dumping of copper sulphate solution in 1965 (Clark et al., 1989) and 0.6 ppm in the Carnon River, feeding the Fal Estuary, Cornwall, UK (Bryan and Langston, 1992). Under laboratory condition, *in vivo* exposure of copper for 7 days at 0.2 – 0.5 ppm was known to be toxic for mussels (*Mytilus edulis*) (Pipe et al., 1999).

Several mechanisms have been proposed to explain Cu^{2+} induced cellular toxicity, and the propensity of copper to initiate oxidative damage and apoptosis *via* generation of ROS is widely accepted (Gaetke et al., 2014, Rico et al., 2009). Accumulation of ROS in biological systems can cause damage to tissues, cells and cellular components, called oxidative stress. Hence, ROS are well known molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants (Valavanidis et al., 2006). Indeed, Cu^{2+} exposure is known to induce increased ROS production in cells of plants (Ahsan et al., 2007, Drążkiewicz et al., 2004, Wang et al., 2004) and animals (Bopp et al., 2008, Geracitano et al., 2004, Qian et al., 2005). For marine molluscs, copper-induced

ROS production has been reported in brown mussels (*Perna perna*) (Brahim Errahmani et al., 2014), but was not found in Mediterranean mussels (*Mytilus galloprovincialis*) (Gómez-Mendikute and Cajaraville, 2003) or Chinese scallops (*Chlamys farreri*) (Zhang et al., 2010). In this study, we observed a remarkable increase in ROS production with increasing Cu^{2+} concentrations (62.5, 125.0 and 187.5 µM), suggesting that Cu^{2+} induces oxidative stress in mussel haemocytes.

ROS production kinetics reached the first peak after 30 minutes exposure in all treatments (0.0, 25.0, 125.0 µM). In agreement, the rapid release of ROS (oxidative burst) is normally recorded at 15 to 70 minutes post-stimulation in bivalve haemocytes (Buggé et al., 2007, Lambert et al., 2003). The increase of ROS production in the control may have been due to environmental stress when haemocytes were withdrawn from mussels. However, the haemocytes were later acclimatized to the *in vitro* environment and the ROS level dropped down to a basal level at 3 h post exposure. This result supports the practice of incubating the haemocytes for 3 h prior sampling, as was done in this study. Interestingly, we observed lower levels of ROS in the 25.0 µM Cu^{2+} treatment compared to the control. This indicates that low concentration of Cu^{2+} reduced the levels of ROS in mussel haemolymph. The antioxidant capacity of copper is due to its function as ligand in antioxidant proteins (e.g., metallothionein) (Luza and Speisky, 1996) and antioxidant enzymes (e.g., superoxide dismutase) (Gaetke and Chow, 2003). However, elevated levels of free Cu^{2+} accelerated the formation of ROS by directly participating in the Haber–Weiss reaction that generates hydroxyl radicals ($^{\bullet}OH$) and/or other ROS from hydrogen peroxide (H_2O_2) (Bremner, 1998, Stohs and Bagchi, 1995).

Apoptosis is a conserved mechanism across taxa, and is an important internal defence mechanisms in molluscs (Romero et al., 2015). Apoptosis can be regulated *via* two major

pathways; intrinsic and extrinsic pathways (Estevez-Calvar et al., 2013, Kiss, 2010, Sokolova, 2009, Terahara and Takahashi, 2008, Zhang et al., 2011). The intrinsic or mitochondrial-mediated apoptotic pathway is activated in response to various types of intracellular stressors, while the extrinsic or death receptor-mediated apoptotic pathway is stimulated by external stimuli. Caspases (cysteinyl- directed aspartate-specific proteases) are the key molecular components of both intrinsic and extrinsic apoptosis, and they play vital roles in the induction, transduction, and amplification of intracellular apoptotic signals when apoptosis is initiated (Cohen, 1997, Fan et al., 2005). Among them, caspase-3 is a frequently activated death protease in mammalian apoptotic cells (Porter and Jänicke, 1999), and has recently been reported in invertebrates (Guo et al., 2017, Lacoste et al., 2002, Sokolova et al., 2004). Hence, activation of caspase-3 is considered to be an indicator of apoptosis (Porter and Jänicke, 1999). Caspase-3 activation has been reported in haemocytes of *Crassostrea virginica* oysters and *Mytilus edulis* mussels exposed to cadmium (Sokolova et al., 2004) and copper (Höher et al., 2013), respectively. The caspase-3 activation has also been observed in mantle tissues of *M. galloprovincialis* mussels exposed to various heavy metals (Kefaloyianni et al., 2005). In our study, we observed a significant increase of apoptotic cells *via* the presence of caspase-3/7 activation in the Cu^{2+}-exposed haemolymph compared to the control, suggesting Cu^{2+}-exposure causes apoptosis *via* caspase-dependent pathway.

In addition, most of the important events in apoptosis processes appear to involve signalling *via* the mitochondria, including the release of caspase activators (e.g., cytochrome c), alterations in electron transport, altered cellular redox status, participation of pro- and antiapoptotic Bcl-2 family proteins and loss of MMP (Green and Reed, 1998, Ly et al., 2003). Thus, changes in the MMP has become increasingly important in the study of apoptosis (Ly et al., 2003, Smaili et al., 2013). Perturbation of the MMP

associated with apoptosis has been reported in several invertebrate models, including *Drosophila melanogaster* (Zimmermann et al., 2002), *Lymnea stagnalis* (Russo and Madec, 2007) and *Penaeus monodon* (Xian et al., 2010). For marine molluscs, genes of the mitochondrial apoptotic pathway have been extensively analysed in Mediterranean mussels (*M. galloprovincialis*) (Estevez-Calvar et al., 2013) and Pacific oysters (*Crassostrea gigas*) (Zhang et al., 2011). Recently, UV induced apoptosis through the mitochondria-mediated pathway was demonstrated in haemocytes of flat oysters (*Ostrea edulis*) (Gervais et al., 2015). In agreement with these studies, we obtained a significant increase in the percent depolarization of inner MMP in the copper-exposed haemolymph compared to the non-exposed haemolymph, suggesting that copper-induced apoptosis was involved in the mitochondria-mediated apoptotic pathway. Finally, the alteration of caspase-3/7 activation and MMP indicated that Cu^{2+} exposure induced apoptosis in haemolymph of *P. canaliculus*.

Excessive production of ROS is known to induce cell apoptosis (Guo et al., 2017, Orrenius, 2007, Winston, 1991). Recently, a study on molecular responses to copper stress in *Litopenaeus vannamei* shrimp suggested that the generation of ROS may cause oxidative stress and eventually result in apoptosis in shrimp under copper exposure (Guo et al., 2017). These authors found that inhibitors of apoptosis protein (IAP) interact with caspase-3 to regulate apoptosis caused by superfluous ROS generation (Guo et al., 2017). Furthermore, caspase-3 is known to inhibit ROS production and is required for efficient execution of apoptosis (Brentnall et al., 2013). In the present study, ROS production, and apoptotic cells (both *via* capase-3/7 and MMP) were significantly increased in a dose-dependent manner after copper exposure. These results suggest that Cu^{2+} exposure triggered the generation of excessive ROS, which may lead to oxidative stress and apoptosis in Cu^{2+}-exposed haemocytes.

For metabolomics, we observed a significant alteration of 25 metabolites in metabolite profiles of haemolymph exposed to high Cu^{2+} level (125.0 μM) compared to the control. Among them, many metabolites were identified as important signatures of oxidative stress and apoptosis. For example, glutathione (GSH), an important antioxidant, is a well-known biomarker of oxidative stress (Isaksson et al., 2005, Rossi et al., 2006). GSH can directly react with electrophilic oxidants (e.g., H_2O_2) resulting in conversion of two GSH molecules into its oxidized form (GSSG) (Espinosa-Diez et al., 2015, Meister, 1989). In this study, we observed a remarkable reduction of GSH in Cu^{2+}-exposed haemolymph compared to control samples. The decrease of GSH, concomitantly with increased levels of ROS, suggests that GSH reacted with ROS to form GSSG in haemolymph exposed to Cu^{2+}. Copper-induced reduction of GSH has been previously reported in different tissues of several mollusc samples, including the digestive gland of scallops (*Adamussium colbecki*) (Regoli et al., 1997), the gills and digestive gland of mussels (*M. galloprovincialis*) (Canesi et al., 1999, Viarengo et al., 1990), and the digestive gland tissue of snails (*Theba pisana*) (El-Gendy et al., 2009). GSH is synthesized from three amino acids, including glycine, glutamic acid, and cysteine in the glutathione metabolic pathway. We identified six metabolites within the glutathione metabolic pathway (glutathione, glycine, cysteine, glutamic acid, ornithine and pyroglutamic acid) in metabolite profiles of mussel haemolymph. In addition to GSH, cysteine and glutamic acid were significantly decreased in the Cu^{2+}-exposed group compared to the control. Secondary pathway analysis screened glutathione metabolism as a target pathway of interest relating to the treatment effect ($p < 0.001$, FDR < 0.001, PI = 0.418) (Table 8.1). In addition, the transsulfuration pathway (cysteine and methionine metabolism), which is a source of cysteine for glutathione was also identified as a potential primary target pathway ($p = 0.001$, FDR < 0.001, PI = 0.276). Hence, the decrease in cysteine and

methionine in the Cu^{2+}-exposed haemolymph suggests a high demand of these metabolites for the transsulfuration pathway and glutathione pathway to regulate ROS production in mussels exposed to Cu^{2+}. The VIP scores of these metabolites also revealed that they were major contributors towards the PLS-DA classification model.

The altered levels of alanine and glutamic acid in various cell types driven into apoptosis has been reported as important signatures of apoptosis (Halama et al., 2013, Rainaldi et al., 2008, Xiao et al., 2016). In this study, Cu^{2+}-exposed haemolymph that had a remarkable increase in percent of apoptosis showed a significantly higher level of alanine and lower level of glutamic acid compared to non-exposed haemolymph, suggesting the involvement of alanine and glutamic acid during the apoptosis process.

The changes of alanine and glutamic acid during apoptosis have been proposed to be associated with taurine metabolism (Halama et al., 2013). According to this model, taurine is metabolized to glutamic acid by taurine 2-oxoglutarate transaminase and to alanine by taurine pyruvate aminotransferase during apoptosis (Halama et al., 2013). Rainaldi *et al* also reported decreases in glutamic acid, together with taurine and other metabolites in cells of the HL60 promyelocytic leukemia cell line driven into apoptosis by either a physical (ionizing radiation) or a chemical (doxorubicin) agent (Rainaldi et al., 2008). These studies suggest a positive correlation between taurine and glutamic acid. Although we did not detect taurine in metabolite profiles of mussel haemolymph due to the technical limits, the increased alanine and decreased glutamic acid may suggest the involvement of taurine during the apoptosis in Cu^{2+}-exposed haemocytes. Accordingly, the decrease of taurine in blood serum has also been reported in copper-laden rats (Xu et al., 2015). In addition, taurine is a sulfur-containing amino acid derived from the transsulfuration pathway (Redmond et al., 1998). We observed the decrease of

methionine and cysteine in the transsulfuration pathway and the transsulfuration pathway itself was identified as significantly impacted by Cu^{2+} treatment, which may indirectly suggest the decrease of taurine in Cu^{2+}-exposed haemolymph. Furthermore, apoptosis plays a central role in the maintenance of tissue homeostasis (Jacobson et al., 1997, Kiss, 2010, Thompson, 1995), and oxidation and reduction of cysteine and methionine are known to be involve in regulation of cellular redox homeostasis (Hoshi and Heinemann, 2001). Hence, the decrease of cysteine and methionine may relate to the alteration of taurine and apoptosis.

From these findings, we propose that the processes of oxidative stress and apoptosis in Cu^{2+}-exposed haemocytes may be involve in the transsulfuration pathway, glutathione metabolism and taurine metabolism, as described previously. In this model, Cu^{2+} exposure led to the excessive ROS production that caused the decreases of GSH, methionine, cysteine then taurine. The decrease of taurine, in turn, would have led to the decrease in glutamic acid and accumulation of alanine.

8.5 CONCLUSION

In this study, we successfully demonstrated a simple in-vitro model for using mussel (*P. canaliculus*) haemolymph to study the toxic mechanism of copper. Cu^{2+}-exposed haemocytes showed a remarkable increase in ROS production, which in turn induced oxidative stress and apoptosis. The metabolite profiles of mussel haemolymph further confirm a toxic pathway at the molecular level. The reduction of metabolites within the transsulfuration pathway (cysteine and methionine) and glutathione metabolic pathway (cysteine, glutamic acid and GSH) could explain the metabolic pathway of Cu^{2+}-induced oxidative stress in mussel haemocytes. However, further targeted metabolomics studies will need to be conducted to confirm this assertion. The decrease in these metabolites together with the increase of alanine were found to be involve in the apoptosis mechanism of Cu^{2+}-exposed haemocytes. Among these alterations, decreased GSH and increased in alanine could be used as biomarkers for oxidative stress and apoptosis, respectively. For the first time, this study confirms Cu^{2+}-induced oxidative damage *via* oxidative stress and apoptosis at both cytometric and metabolomics levels.

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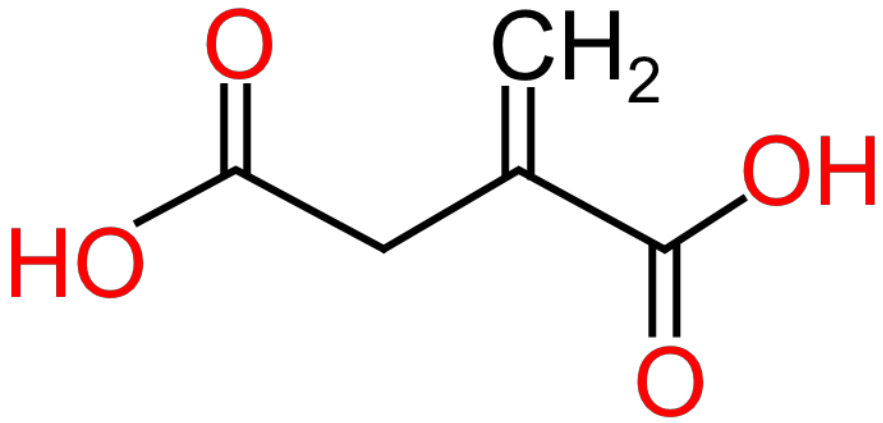
Section IV

Application of metabolomics to study antimicrobial role of itaconic acid



Itaconic acid

In this section:

Chapter 9

Itaconic acid inhibits growth of a pathogenic marine *Vibrio* strain: A metabolomics approach

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ABSTRACT

The antimicrobial role of itaconic acid (ITA) has been recently discovered in mammalian cells. In our previous studies, we discovered that marine molluscs biosynthesize substantial quantities of ITA when exposed to marine pathogens, but its antimicrobial function to *Vibrio* bacteria is currently unknown. Thus, in this study, we used an untargeted gas chromatography-mass spectrometry (GC-MS) platform to identify metabolic changes of *Vibrio* sp. DO1 (*V. corallyliticus*/*neptunius*-like isolate) caused by ITA exposure. *Vibrio* sp. DO1 was cultured in Luria-Bertani broth supplemented with 3 mM sodium acetate and with different concentrations of ITA (0, 3 and 6 mM) for 24 h. The results showed that ITA completely inhibited *Vibrio* sp. growth at 6 mM and partially inhibited the bacterial growth at 3 mM. A principal component analysis (PCA) revealed a clear separation between metabolite profiles of *Vibrio* sp. DO1 in the 3 mM ITA treatment and the control, which were different in 25 metabolites. Among the altered metabolites, the accumulation of glyoxylic acid and other metabolites in glyoxylate cycle (cis-aconitic acid, isocitric acid and fumaric acid) together with the increase of isocitrate lyase (ICL) activity in the 3 mM ITA treatment compared to the control suggest that ITA inhibited *Vibrio* sp. growth via disruption of central carbon metabolism.

9.1 INTRODUCTION

Itaconic acid (ITA), or 2-methylenesuccinic acid, is an unsaturated dicarboxylic acid that is a well-known precursor for polymer synthesis in the industrial production of polymers. In addition, ITA is known to have antimicrobial function which was first described in Gram-negative bacterium *Vogesella indigofera* (Williams et al., 1971). The inhibitory effect of ITA was subsequently reported in other bacteria, including *Pseudomonas indigofera* (McFadden and Purohit, 1977, Williams et al., 1971), *Yersinia pestis* (Hillier and Charnetzky, 1981), *Mycobacterium tuberculosis* and *Salmonella enterica* (Michelucci et al., 2013).

Recently, ITA was surprisingly discovered in mammalian immune cells. Shin et al. (2011) reported the presence of ITA in lung tissue of mice infected with *M. tuberculosis*, and it was hypothesized that ITA was originated from the bacteria in this association. However, Sugimoto et al. (2012) subsequently detected ITA in mouse macrophage-like cell lines stimulated with lipopolysaccharide (LPS), which demonstrated an intracellular source. The biological function of ITA as a novel mammalian metabolite was then highlighted by Strelko et al. (2011) who suggested roles in macrophage-based immune functions after observing increased ITA production and secretion in mouse peritoneal macrophages activated by LPS and IFN-γ. Similarly, the highly increased levels of ITA in human primary macrophages under LPS-induced inflammatory conditions (Michelucci et al., 2013). Taken together, these findings indicate the role of ITA as mammalian antimicrobial metabolite.

In addition to mammalian macrophages, increased biosynthesis of ITA was recently reported in marine bivalves during pathogen challenges (Nguyen et al., 2018a, Nguyen et al., 2018b, Young et al., 2017). The first detection of ITA in bivalves was reported by

Young et al. (2017) in Pacific oyster larvae challenged with a marine herpesvirus (OsHV-1 μVar). Accumulation of ITA was then detected in mussel haemolymph following an *in vivo* experimental challenge with a pathogenic strain of *Vibrio* sp. (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c). Interestingly, ITA increased during initial stages of infection in mussels then decreased in those individuals which survived and recovered from the infection after a week (Nguyen et al., 2018a). These results suggest that ITA could be a potential biomarker for pathogen infections, and indicate health status of molluscan hosts (Nguyen et al., 2018a). Taken together, these studies demonstrate that marine bivalves have the capacity to synthesize ITA, with potential immune functions during pathogen infections. However, it is currently unknown whether ITA can inhibit growth of specific pathogenic marine bacteria, as it does in some terrestrial strains.

Vibrio is a genus of Gram-negative bacteria, possessing a number of pathogenic strains that associated with infectious diseases in marine bivalves (Travers et al., 2015). To test the potential inhibitory role of ITA on growth of a virulent *Vibrio* strain, we cultured *Vibrio* sp. DO1 in different concentrations of ITA. This *Vibrio* strain (*Vibrio coralliilyticus*/*neptunius*-like isolate, Genbank: EU358784) was isolated from *Perna canaliculus* larvae (Kesarcodi-Watson et al., 2009a) have been showed to be pathogenic to both *P. canaliculus* larvae (Kesarcodi-Watson et al., 2009b) and adults (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c). GC-MS-based metabolomics was performed to compare metabolite profiles of the *Vibrio* sp. cultures and evaluate mechanistic effects of ITA on bacterial metabolism.

9.2 MATERIALS AND METHODS

9.2.1 Chemicals

Most of the chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich (St Louis, MO, USA) with the following exceptions: LB broth (Miller, Code: 244610) and thiosulfate citrate bile salts sucrose (TCBS) agar from Fort Richard Laboratories (Auckland, New Zealand), chloroform from Merck (Darmstadt, Germany).

9.2.2 Bacterial culture and itaconic acid effects

Vibrio sp. DO1 (99.5 % 16S sequence similarity with *V. corallyliticus* and *V. neptunius*; Genbank: EU358784) isolated from Greenshell™ mussel larvae (Kesarcodi-Watson et al., 2009b) was kindly provided by Cawthron Institute (Nelson, New Zealand). The bacterial suspension was prepared in LB broth using the same protocols previously described in Chapter 4 (4.2.1).

The effects of ITA on *Vibrio* were assessed by adding 100 µl of *Vibrio* sp. stock (8.7×10^7 cells·ml^{-1}) into glass bottles containing 100 ml LB with three different ITA concentrations (0, 3, 6 mM) supplemented with 3 mM sodium acetate. The bacterial cultures were incubated at 25 °C and sampled every 6 h up to 24 hpi for bacterial growth using a spectrophotometer (Ultrospec 2100 pro UV–Vis: Biochrom Ltd., Cambridge, UK) to measure absorbance at 600 nm. For metabolomics analyses, bacteria were harvested at 24 hpi. One ml of bacterial culture was centrifuged at 2438 × g for 10 minutes at 4 °C on an Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany). The pelleted cells were washed with FAS and re-suspended with 1 ml of FAS. The final cell pellets were flash frozen in liquid nitrogen and stored at –80 °C until metabolite extraction and enzyme analyses could be performed. For isocitrate and isocitrate lyase assays, 1 ml bacterial culture was sampled and stored at –80 °C.

9.2.3 Isocitrate assay

The concentrations of isocitrate in *Vibrio* sp. samples were measured by isocitrate assay kit (Sigma-Aldrich, St Louis, MO, USA). The assay was performed according to the manufacturer's protocol. In summary, isocitrate standards containing 0, 4, 8, 12, 16, 20 nmole per well were prepared in a 96-well plate from 20 µl of 100 mM isocitrate stock. One hundred µl of *Vibrio* sp. in LB broth was homogenized in 100 µl of isocitrate assay buffer and centrifuged at 13,000 × g for 10 minutes (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany) to remove insoluble material. Twenty-five µl of each sample were added into each well and mixed with 25 µl of isocitrate assay buffer. A standard mix containing 46 µl isocitrate assay buffer, 2 µl substrate mix and 2 µl substrate mix was added into each of the wells and mixed. Blank samples contained 48 µl isocitrate assay buffer and 2 µl substrate mix only. All samples, blanks and standards were incubated for 30 minutes in the dark at room temperature prior to measuring at 450 nm with a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA). Concentrations of isocitrate in the samples were calculated based on absorbance and standard curves.

9.2.4 Isocitrate lyase assay

Quantification of isocitrate lyase in *Vibrio* sp. samples was conducted following published protocols (Chell et al., 1978). In brief, reagents were prepared and added into suitable cuvettes as follows: 0.50 ml of 50 mM imidazole buffer (pH 6.8 at 30 °C), 0.1 ml of 50 mM magnesium chloride solution ($MgCl_2$), 0.1 ml of 10 mM ethylenediaminetetraacetic acid solution (EDTA), 0.1 ml of 40 mM phenylhydrazine hydrochloride solution and 0.1 ml of 10 mM DL-isocitric acid solution (isocitrate). Cuvettes containing the reagent mixture was equilibrated to 30 °C in a water bath. For each *Vibrio* sp. sample, 0.1 ml was combined with the reagent mix immediately measured in a spectrophotometer at 324 nm (Ultrospec 2100 pro UV–Vis: Biochrom Ltd.,

Cambridge, UK). Samples were placed back in the 30 °C water bath re-measured after 5 minutes. Alternatively, 0.1 ml of imidazole buffer was added to blanks and similarly measured. ICL concentrations in each sample was calculated based on A_{324} per minute of samples and blanks.

9.2.5 Metabolomics

Metabolite extractions, derivatizations, GC-MS measurement, quality control and spectral processing were performed as previously described (Chapter 4 – 4.2.4-6).

9.2.6 Statistical and pathway analysis

Metabolite profile data were analysed using MetaboAnalyst 4.0 (Chong et al., 2018). Data were normalized by generalized logarithm (glog) transforming and mean centring to make individual features more comparable. Multivariate analyses, including unsupervised PCA and supervised PLS-DA were used to assess variability among samples and between sample classes. Validation of the PLS-DA model was performed using leave one out cross validation (LOOCV), which was assessed via accuracy, R^2 and Q^2 values (Szymańska et al., 2012). Univariate analysis was performed using *t*-test to identify differences between metabolite profiles of ITA-treated and control cultures of *Vibrio* sp. A heatmap of altered metabolites was generated to assess the abundance of these metabolites (low/high) via intuitive visualization. Classical univariate ROC analyses for individually altered metabolites in glyoxylate shunt and multivariate ROC analysis (using linear support vector machines) for all of these features were performed to assess the accuracy of biomarker models.

Quantitative enrichment analysis (QEA) using global test algorithm (Xia and Wishart, 2010) and network topology analysis (NTA) using relative-betweeness centrality (Nikiforova and Willmitzer, 2007) were performed to investigate functional relationships

among the annotated metabolites in accordance with the protocol described in Chapter 5 (5.2.5). The *Escherichia coli* K-12 MG1655 was used as the reference pathway library.

9.3 RESULTS

9.3.1 Effect of ITA on the growth of *Vibrio* sp. DO1.

To test the antimicrobial effect of ITA on bacterial growth, we cultured *Vibrio* sp. in a Luria-Bertani (LB) broth with different concentrations of ITA (0, 3 and 6 mM). Luria-Bertani broth was chosen since growth in this medium is carbon limited (Sezonov et al., 2007). Growth of *Vibrio* sp. was measured via spectroscopy every 6 h for 24 h (Fig. 9.1A). The results show that the growth of *Vibrio* sp. was completely inhibited in the 6 mM ITA treatment. Growth in the 3 mM ITA treatment was significantly slower than the growth in the control at all recorded times ($p < 0.05$). Furthermore, to test whether ITA has the capacity to inhibit isocitrate lyase (ICL) of the glyoxylate shunt, we measured the concentrations of isocitrate and activity of ICL at 24-hour post-incubation (hpi). The results show the significantly higher levels of isocitrate ($t_{10} = –5.878$, $p < 0.001$) and ICL activity ($t_8 = –12.52$, $p < 0.001$) in the ITA treatment compared to the control (Fig. 9.1B,C).

9.3.2 Effect of ITA on metabolite profiles of *Vibrio* sp. DO1

Untargeted GC-MS-based metabolomics was performed to compare metabolite differences between the 3 mM ITA treatment and the control. A total of 565 features were detected by GC-MS in *Vibrio* sp. samples and 63 metabolites were successfully annotated using an in-house library. The majority of these metabolites were amino acids (43 %), followed by organic acids (30 %), fatty acids (19 %) and others (8 %).

Principal component analysis (PCA) was used to identify natural groupings of all bacterial samples based on the underlying structure of the metabolite data. A PCA score plot shows clear separations between the ITA treatment and the control (Fig. 9.2). Partial least-squares discriminant analysis (PLS-DA) was revealed a very robust model for

discrimination between sample classes with an accuracy of 1.0, a multiple correlation coefficient (R^2) of 0.99 and a cross-validated predictive ability (Q^2) of 0.90. T-tests identified 25 metabolites that were significantly altered by ITA treatment, compared to control cultures (see 'Availability of materials and data' for more information). A heatmap was generated to visualise the relative abundance of these metabolites in each group (Fig. 9.3). Overall, clear differences were observed between the treatment and control, where

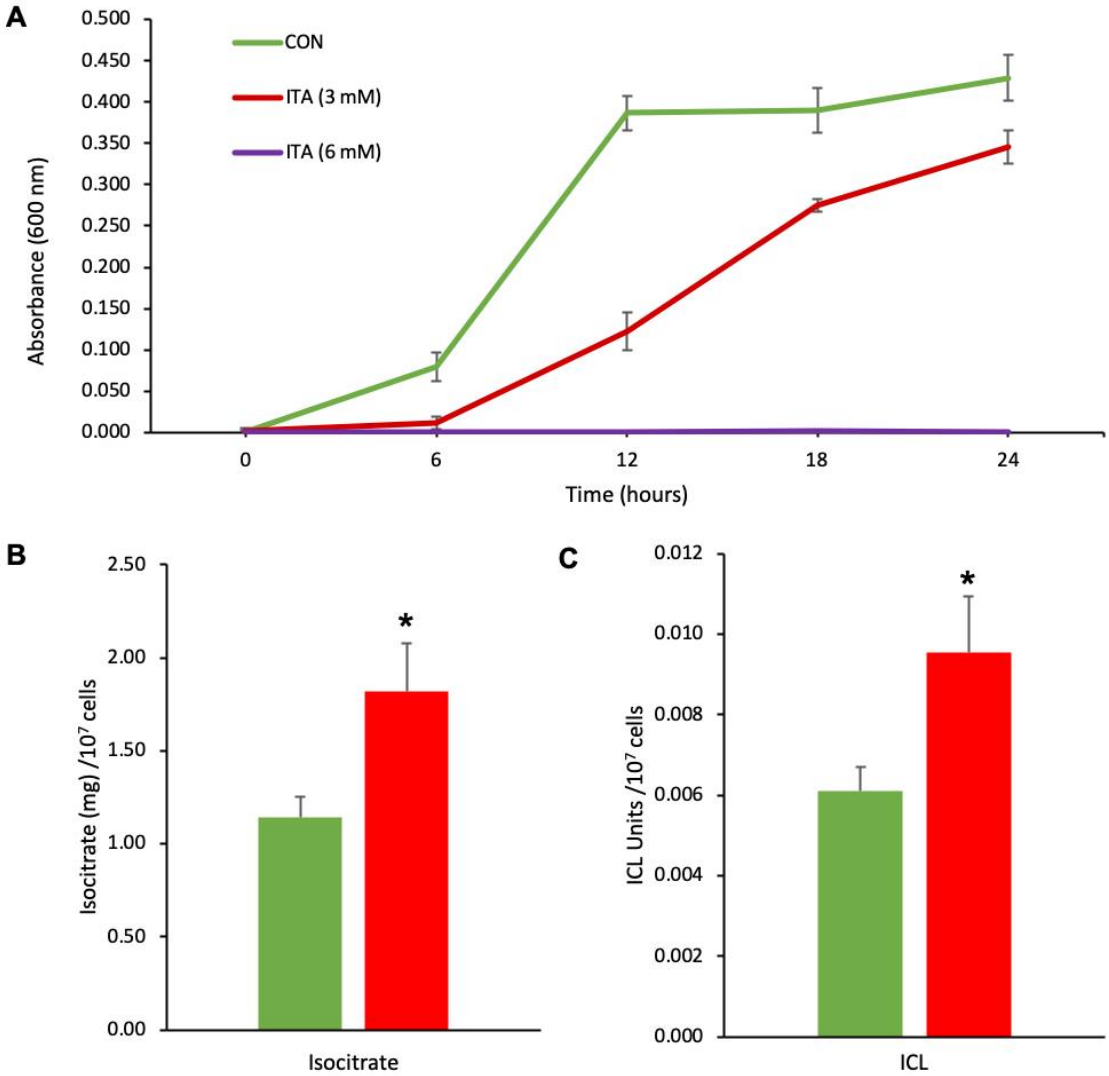

Figure 9.1 Effects of ITA on growth of *Vibrio* sp. at different levels isocitrate and isocitrate lyase (ICL). (A) The absorbance (600 nm) of *Vibrio* sp. cultured in different ITA concentrations (0, 3 and 6 mM) supplemented with sodium acetate over 24 h. (B) Level of isocitrate in the 3 mM ITA treatment and the control at 24 hpi. (C) Activity of ICL in the 3 mM ITA treatment and the control at 24 hpi. Data are presented as mean ± S.D. (n = 6). Significant differences relative to the control are marked with an asterisk (*) (*t*-test, $p < 0.05$).

most of the metabolites in the ITA treatment were elevated with exception of trans-4-hydroxyproline.

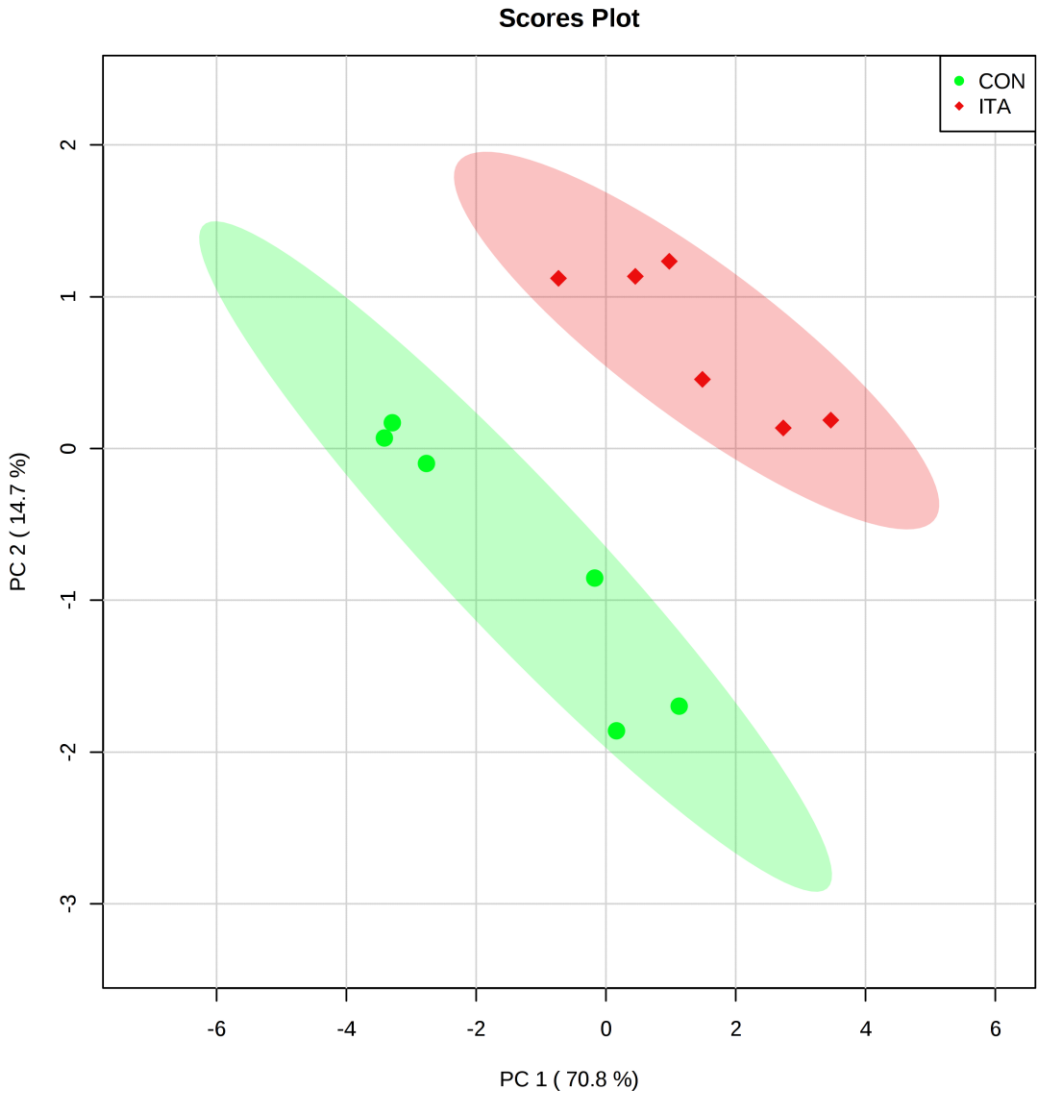

Figure 9.2 PCA score plot of metabolite profiles of *Vibrio* sp. bacteria in the 3 mM ITA treatment and the control. *CON, control treatment (no ITA); ITA, 3 mM itaconic acid treatment.*

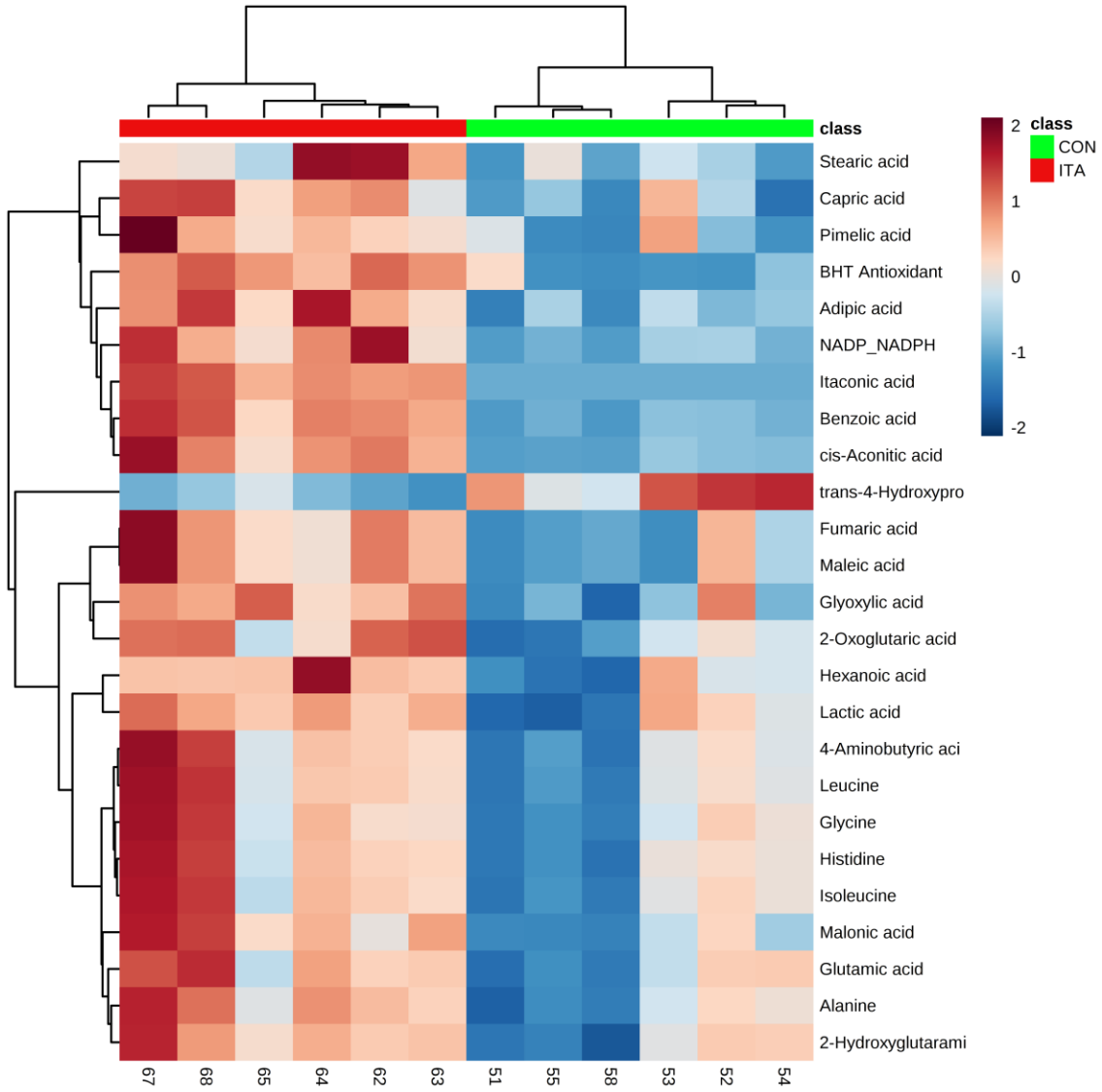

Figure 9.3 Heatmap of 25 metabolites identified as significantly different between the ITA treatment and the control by *t*-test ($p < 0.05$). CON, control treatment (no ITA); ITA, 3 mM itaconic acid treatment.

9.3.3 Effect of ITA on the glyoxylate shunt

Eight metabolites related to the glyoxylate shunt and TCA cycle were identified in metabolite profiles of *Vibrio* sp., including citric acid, cis-aconitic acid, isocitric acid, succinic acid, fumaric acid, malic acid, glyoxylic acid, pyruvic acid, 2-oxoglutaric acid and 2-phosphoenolpyruvic acid. The addition of ITA led to alterations of 4 metabolites which were elevated in the ITA treatment, including cis-aconitic acid, fumaric acid, glyoxylic acid and 2-oxoglutaric acid (Fig. 9.4A).

Classical univariate ROC curve analyses were performed to assess the biomarker specificity and sensitivity of these significantly altered metabolites based on the area under the ROC curve (AUC). The results reveal that all these metabolites had AUC higher than 0.8, and cis-aconitic acid had AUC equal to 1 (Fig. 9.4B). In addition, ROC curve analyses identified isocitric acid as significantly different between the treatment and control with *t*-test p-value < 0.05 and AUC = 0.81. The multivariate ROC curve-based model evaluation, which combined potential biomarkers, showed a very high AUC of 0.972 (Fig. 9.4C).

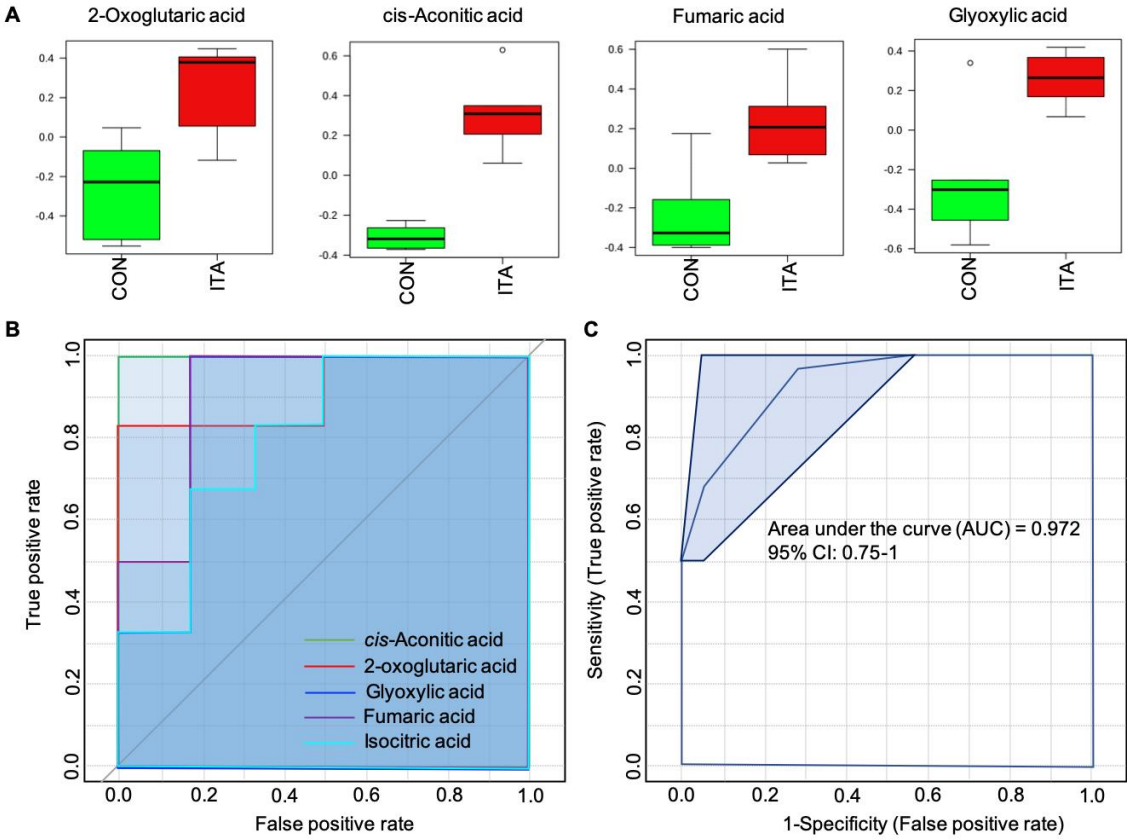

Figure 9.4 Effects of ITA on glyoxylate shunt of *Vibrio* sp. cultured in LB media with and without ITA. (A) Altered metabolites of glyoxylate shunt identified by *t*-test ($p < 0.05$). Box plots show relative abundances of metabolites after normalization. (B) Univariate ROC curve analysis of significantly altered metabolites (ITA/Non ITA) of the glyoxylate shunt. (C) Multivariate ROC curve-based model evaluation of all altered metabolites in the glyoxylate shunt identified by ROC curve analysis.

9.3.4 Pathway analysis

Pathway analysis was used to identify potentially altered metabolic pathways induced by ITA. This analysis identified 28 pathways with $p < 0.05$, which contained at least two identified metabolites (Table 9.1). Among these, 15 pathways with impacts > 0.1 were considered as pathways of interest relating to ITA effects (Table 9.1), while those with an impact < 0.01 may have slight effects. These pathways are involved in carbohydrate metabolism (e.g., TCA cycle, pyruvate metabolism), energy metabolism (e.g., nitrogen,

Table 9.1 List of metabolic pathways in *Vibrio* sp. that were significantly affected by ITA exposure.

Pathways	Hits/Total compounds	Raw p	FDR	Impact
Alanine, aspartate and glutamate metabolism	6/18	<0.001	<0.001	0.479
Glycine, serine and threonine metabolism	6/32	0.001	0.001	0.473
Pyruvate metabolism	3/26	0.005	0.007	0.438
Glutathione metabolism	5/21	<0.001	<0.001	0.381
Citrate cycle (TCA cycle)	8/20	<0.001	<0.001	0.301
Butanoate metabolism	4/18	<0.001	0.001	0.255
Cysteine and methionine metabolism	6/34	0.001	0.001	0.230
Arginine and proline metabolism	8/41	<0.001	0.001	0.215
Glycolysis or gluconeogenesis	2/29	0.192	0.192	0.168
Methane metabolism	2/11	<0.001	<0.001	0.167
Aminoacyl-tRNA biosynthesis	18/66	<0.001	<0.001	0.130
Glyoxylate and dicarboxylate metabolism	5/29	<0.001	<0.001	0.102
Nicotinate and nicotinamide metabolism	2/13	0.001	0.001	0.089
Sulfur metabolism	2/13	0.001	0.001	0.069
Valine, leucine and isoleucine biosynthesis	5/26	<0.001	<0.001	0.036
C5-Branched dibasic acid metabolism	2/6	<0.001	<0.001	0.000
Benzoate degradation via CoA ligation	3/10	<0.001	<0.001	0.000
Valine, leucine and isoleucine degradation	3/23	<0.001	<0.001	0.000
beta-Alanine metabolism	2/16	<0.001	0.001	0.000
Nitrogen metabolism	5/18	<0.001	0.001	0.000
Phenylalanine, tyrosine and tryptophan biosynthesis	4/23	<0.001	0.001	0.000
Thiamine metabolism	2/19	<0.001	0.001	0.000
Lysine biosynthesis	2/13	<0.001	0.001	0.000
Cyanoamino acid metabolism	4/8	0.001	0.001	0.000
Phenylalanine metabolism	4/23	0.001	0.001	0.000
Pantothenate and CoA biosynthesis	4/23	0.001	0.001	0.000
Tyrosine metabolism	2/10	0.002	0.003	0.000
Biosynthesis of unsaturated fatty acids	3/6	0.011	0.015	0.000

metabolism, sulfur metabolism), amino acid metabolism (e.g., valine, leucine and isoleucine biosynthesis; arginine and proline metabolism), lipid metabolism (e.g., biosynthesis of unsaturated fatty acids), oxidative stress (e.g., glutathione metabolism), metabolism of cofactors and vitamins (e.g., pantothenate and CoA biosynthesis), among others.

9.4 DISCUSSION

The glyoxylate cycle is an anabolic variation of the TCA cycle used during carbon limitation in plants, bacteria, protist and fungi (Lorenz and Fink, 2002). This mechanism differs from the TCA cycle in which the two decarboxylation reactions (isocitrate→α-ketoglutarate→succinyl-CoA) are bypassed via the glyoxylate shunt pathway (Fig. 9.5). In this pathway, isocitric acid is converted into glyoxylic acid and succinic acid by isocitrate lyase (ICL). Glyoxylic acid is further combined with acetyl-CoA to form malic acid by malate synthase (MLS) (Kondrashov et al., 2006, Sharma et al., 2000). When initiated during glycolytic sugar (C5-6) starvation, bypassing the CO_2-producing steps of the TCA cycle helps the glyoxylate shunt preserve carbon atoms of acetyl-CoA for gluconeogenesis, which is critical for biomass production (White et al., 2007). The glyoxylate cycle thus allows microorganisms to utilize carbon compounds other than glucose, such as acetate and fatty acids, as carbon sources for growth under different nutrient conditions (Hillier and Charnetzky, 1981, Maloy et al., 1980). During host infection, pathogenic bacteria are known to up-regulate the glyoxylate cycle (Hillier and Charnetzky, 1981).

The glyoxylate bypass is a potential drug target, which could be inhibited by natural or synthetic compounds, such as ITA (Lee et al., 2015). ITA strongly inhibits the glyoxylate shunt by acting as a potent competitive inhibitor of ICL (McFadden and Purohit, 1977, Patel and McFadden, 1978, Williams et al., 1971). Inhibition of bacterial growth by ITA exposure has been demonstrated in several bacteria, such as *Pseudomonas indigofera* (McFadden and Purohit, 1977, Williams et al., 1971), *Yersinia pestis* (Hillier and Charnetzky, 1981), *M. tuberculosis* and *S.enterica* (Michelucci et al., 2013). For example, Michelucci et al. (2013) reported that supplementation of 25-50 $nmol \cdot l^{-1}$ ITA significantly inhibited the growth of *M. tuberculosis* and *S. enterica*. In our study, the

growth of *Vibrio* sp. was completely inhibited in culture media supplemented with 6 mM ITA and reduced growth in the 3 mM ITA treatment compared to the control. Furthermore, glyoxylic acid and other intermediates of the glyoxylate cycle (cis-aconitic acid, isocitric acid and fumaric acid) were significantly higher in the ITA treatment compared to the control. However, most surprisingly, the activity of ICL was not inhibited by ITA in our experiment, which indicates that a different toxic mechanism may be responsible for the antimicrobial effect.

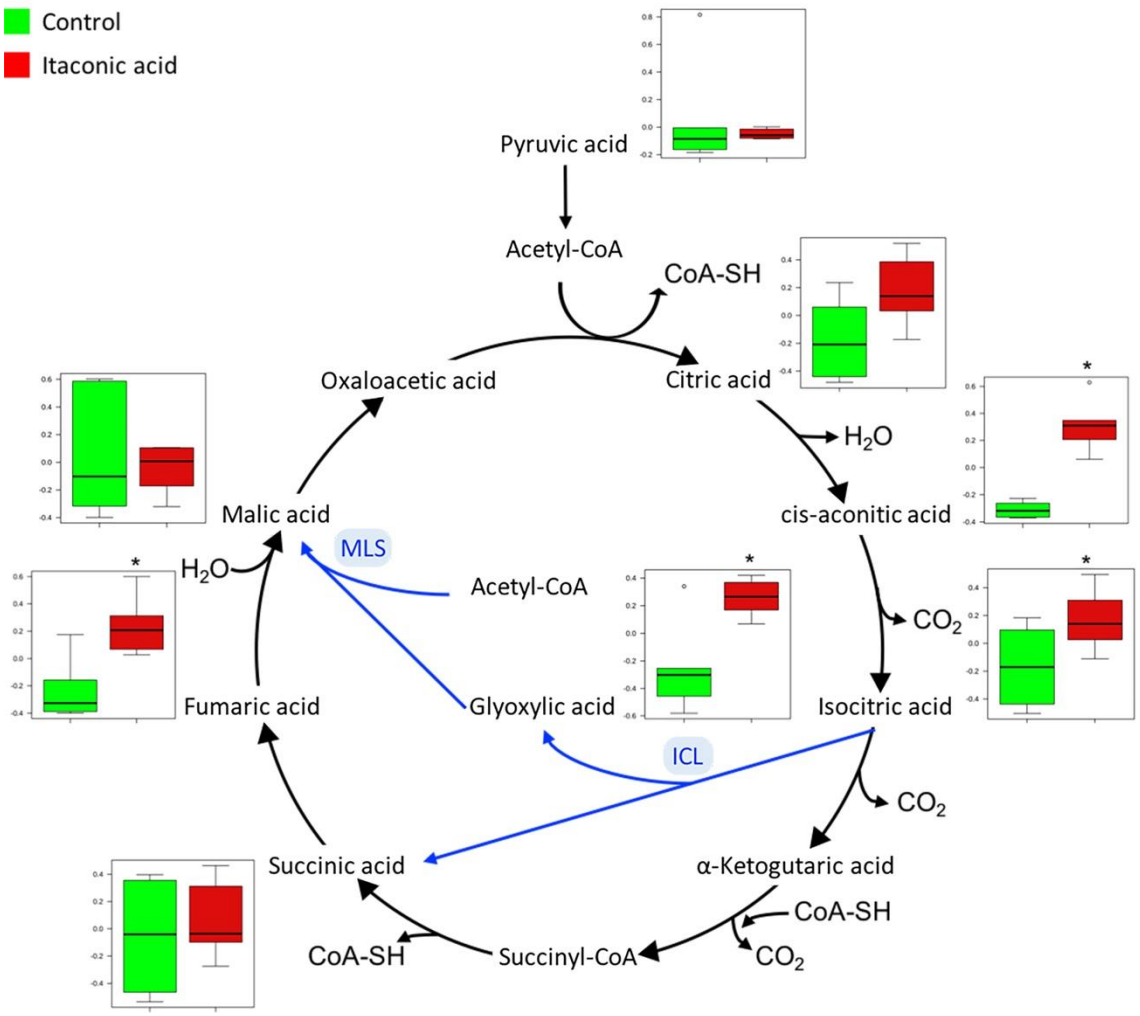

Figure 9.5 The general scheme for tricarboxylic acid (TCA) cycle (black arrows) and glyoxylate cycle (blue arrows). Box plots show relative abundances of metabolites after normalization and significant differences relative to the control are marked with an asterisk (*) (*t*-test, $p < 0.05$).

In addition, we observed elevated levels of many amino acids (e.g., alanine, glutamic acid, glycine) in ITA-exposed *Vibrio* sp. Increases in many amino acids have previously been reported in bacteria exposed to environmental stress (Boroujerdi et al., 2009, Kim et al., 2017, Zhou et al., 2017). For example, glutamic acid, which is involved in many metabolic pathways was reported to be synthesized by *Escherichia coli* (Dinnbier et al., 1988) and *Vibrio costicola* (Kamekura and Kushner, 1984) to balance the K^+ uptake from the media. The up-regulation of glutamic acid with increasing temperature was observed in *Vibrio coralliilyticus* (Boroujerdi et al., 2009). Accumulation of alanine is a common phenomenon in response to various stresses in both plants and animals (Ben-Izhak Monselise et al., 2003). Therefore, the level of alanine has been proposed to be a universal stress signal (Ben-Izhak Monselise et al., 2003). The increases in alanine were demonstrated in *V. parahaemolyticus* exposed to various concentrations of ferric iron (Zhou et al., 2017). Hence, the accumulation of many amino acids and an activated amino acid metabolism (indicated via the pathway analysis) in ITA exposed *Vibrio* sp. may indicate stress responses and disturbance of amino acid metabolism of ITA on *Vibrio* sp.

We observed the accumulation of fatty acids, including pimelic acid, stearic acid, capric acid, and a secondary pathway analysis revealed biosynthesis of unsaturated fatty acids as an altered pathway due to the ITA effect. This may suggest the change of lipid metabolism of *Vibrio* sp. exposed to ITA. However, the mechanisms underlying the increases in these fatty acids is currently unknown. In addition, the accumulation of other organic acids (e.g., lactic acid, adipic acid, malonic acid, maleic acid) remains uncertain.

Among the altered metabolites, trans-4-hydroxyproline (Hyp) was the only down-regulated metabolite in the metabolite profiles of ITA exposed *Vibrio* sp. Hyp is a non-essential amino acid that is a major component of collagen in animals (Szpak, 2011) and

glycoproteins in plant cell walls (Cassab, 1998). Hyp is synthesized in plants and animals by hydroxylation of proline by prolyl hydroxylase following protein synthesis (as a post-translational modification). Bacteria are able to metabolize Hyp released by protein degradation of animals and plants (White et al., 2012). The accumulation of Hyp has been reported in *Thermococcus* spp (Lamosa et al., 1998), *Brevibacterium* sp. (Nagata et al., 1996) and *Halobacillus halophilus* (Kim et al., 2017) under high salt conditions, suggesting that Hyp may be a widespread osmoprotectant in halophilic and halotolerant bacteria. However, we observed the decrease of Hyp in ITA exposed *Vibrio* sp. This may be due to species-specific responses of different bacteria or stressor-specific responses. Nevertheless, these results suggest the important role of Hyp in stress responses of *Vibrio* bacteria, which should be investigated in future studies.

Overall, the findings from this study strongly indicate antimicrobial activity of ITA to marine bacteria. This points toward the potential use of ITA as an antimicrobial metabolite for bacterial control in aquaculture. However, ITA is known to be unsuitable as drug for mammal due to its toxicity to host cells (Lee et al., 2015). For marine bivalves, we recently identified the increase of ITA in their tissues during the pathogen exposure (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c) which indicates ITA is internal metabolite and may not toxic for host at low concentration. However, whether exposure of aquatic organisms to high concentrations of ITA (e.g., 6 mM ITA like in this study) to fight intracellular and drug-resistant bacteria are safe for the host which needs to be investigated prior to application in aquaculture.

9.5 CONCLUSION

To our knowledge, this is the first study to report antibacterial properties of ITA to a marine *Vibrio* sp. bacterium. After ITA exposure, we observed reduced microbial growth, and higher levels of metabolites in the TCA cycle (glyoxylic acid, cis-aconitic acid, isocitric acid and fumaric acid), amino acids and fatty acids in ITA-exposed bacterial cultures. This indicates that ITA inhibits *Vibrio* sp. growth and disruption of central carbon metabolism and other metabolic changes. However, ICL activity was higher in the ITA treatment compared to the control, suggesting that ITA did not inhibit ICL in the glyoxylate shunt of this *Vibrio* isolate and another toxic mechanism may be responsible for the antimicrobial effect of ITA. Hence, there is a need for future investigations to explore the antimicrobial mechanism of ITA in marine *Vibrio* bacteria and other marine pathogens which may lead to the use of ITA as an antimicrobial compound in aquaculture practices.

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Chapter 10

Targeted metabolomics to investigate antimicrobial activity of itaconic acid in marine molluscs

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ABSTRACT

Itaconic acid (ITA) has recently been identified as an antimicrobial metabolite in mammalian immune cells. The presence of ITA was reported in different tissues of marine molluscs, indicating its role as an endogenous metabolite of molluscs. In addition, the accumulation of ITA has been observed in different tissues of mussels following pathogen challenge. However, the concentration of ITA in mussel tissues and the possible role of this metabolite in the molluscan innate immune system remain unknown. This study aims to quantitatively measure ITA levels in different tissues of marine mussels following an experimental challenge with *Vibrio* sp. DO1 isolate, and to identify the antimicrobial role of ITA in the innate immune system through the measure of metabolic and immune alterations in tissues following the challenge.

In this study, adult *Perna canaliculus* mussels were experimentally challenged with a pathogenic *Vibrio* sp. DO1 isolate. The metabolite profiles of five different tissues, including mantle, gill, muscle, hepatopancreas and haemolymph were obtained, and levels of ITA in each tissue were characterized using a gas chromatography-mass spectrometry (GC-MS) metabolomics approach. Flow cytometry was also employed to measure cell health parameters, including oxidative stress *via* reactive oxygen species (ROS) production, apoptosis *via* changes in mitochondrial membrane potential (MMP) and haemocyte viability. The ITA levels in mantle, gill, muscle and hepatopancreas tissues at 18-hour post infection (hpi) with *Vibrio* sp. were 40.31, 41.71, 11.61 and 41.66 $ng \cdot mg^{-1}$, respectively. In haemolymph, the level of ITA was significantly increased from 95.25 $ng \cdot ml^{-1}$ at 6 hpi to 174.36 $ng \cdot ml^{-1}$ at 18 hpi and 572.12 $ng \cdot ml^{-1}$ at 60 hpi. In line with the accumulation of ITA, we observed increased levels of metabolites within the tricarboxylic acid (TCA) cycle, anti-inflammatory metabolites and alterations of other

metabolites associated with immune responses of the host. The flow cytometry analyses revealed increases in ROS production, apoptotic cells and decreases in cell viability.

We reported on the production of ITA in different tissues of *P. canaliculus* mussels challenged with a marine pathogen which confirmed ITA as an antimicrobial metabolite. The findings revealed insights into the biosynthesis of ITA and suggests its role in antimicrobial and anti-inflammatory activities in the innate immune system. This study also provided insights into the innate immune system of bivalves and highlight the potential use of ITA as a biomarker for shellfish health assessment in aquaculture.

10.1 INTRODUCTION

Molluscan immune defences comprise of physical barriers and a cellular and humoral innate immune system. The main physical protective barriers are behavioural avoidance, shell and mucus barriers (Gerdol et al., 2018). The mucus that covers the soft body of molluscs contains abundant haemocytes and humoral factors, and represents the first line of immune defence (Allam and Raftos, 2015). Circulating haemocytes mediate cellular immune responses via phagocytosis, encapsulation, apoptosis and autophagy (Gerdol et al., 2018). The humoral defence components of molluscs include diverse molecular effectors, such as antimicrobial peptides, lysozymes, bactericidal/permeability-increasing proteins and others pore-forming molecules, proteases and protease inhibitors and the phenoloxidase cascade (Gerdol et al., 2018). In addition, marine molluscs have received considerable attention as important sources of bioactive secondary metabolites that have immunological properties and antimicrobial activities (Grabley and Thiericke, 1999, Pati et al., 2015). For example, diverse antimicrobial secondary metabolites, including kabiramide C, ulapualide A and B, aplysianin A and E, thisaplysianin E and tyrian purple have been identified in marine gastropod egg capsules and egg masses (Kaviarasan et al., 2012). Santhi et al. (2013) reported the antimicrobial activity of extracts from *Babylonia zeylanica*, against various pathogenic bacterial and fungal strains. The GC-MS analyses of *B. zeylanica* extracts revealed the probable antimicrobial activity of compounds, such as 2-piperidinone, undecanal, 2-methyl-, 1,2- benzenedicarboxilic acid, diisooctyl ester, 3-hexadecyloxycarbonyl -5- (2-hydroxyethyl) -4- methylimidazolium ion, a-D-mannofuranoside, farnesyl-, trans-a-bergamotene, diethyl phthalate,phenol,2-methyl-5-(1,2,2- trimethylcyclopentyl)-(S), and 2,2-dimethyl-6- methylene-1-(3,5-dihydroxy- 1-pentanyl) cyclohexane-1-perhydrol. In recent studies, accumulation of itaconic acid (ITA) has been observed in different tissues of marine bivalves following pathogen

challenge, again supporting the antimicrobial function of this metabolite (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c, Young et al., 2017).

ITA is an industrial compound that is used in the production of polymers. ITA is also well-known for its antimicrobial function, which has been demonstrated in many different bacteria species (Hillier and Charnetzky, 1981, McFadden and Purohit, 1977, Michelucci et al., 2013, Nguyen et al., 2019a, Williams et al., 1971). In recent discoveries, ITA was revealed to be a mammalian antimicrobial metabolite (Cordes et al., 2015). Detection of increased biosynthesis of ITA was reported in lung tissues of mice infected with *Mycobacterium tuberculosis* (Shin et al., 2011), mouse macrophage-like cell lines stimulated with lipopolysaccharide (LPS) (Strelko et al., 2011, Sugimoto et al., 2012) and human primary macrophages under LPS-induced inflammatory conditions (Michelucci et al., 2013). In marine molluscs, detection of ITA was firstly reported in Pacific oyster (*Crassostrea gigas*) larvae challenged with OsHV-1 µVar virus (Young et al., 2017). Subsequently, accumulation of ITA has been observed in different tissues of New Zealand Greenshell™ mussels (*Perna canaliculus*) following experimental challenges with *Vibrio* sp. DO1 (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c). Together, these studies suggest that ITA may be a universal antimicrobial metabolite secreted by both vertebrates and invertebrates. However, not much is known about the role of this metabolite in the defence system of marine molluscs and on the conditions of ITA production in marine molluscs.

This study aims to quantitatively characterize ITA levels in different tissues of mussels following a pathogen challenge, and to identify the antimicrobial role and associated functions of this metabolite in the bivalve immune system. For this purpose, we experimentally exposed *P. canaliculus* mussels to *Vibrio* sp. DO1 and measured the

concentrations of ITA in mantle, gill, muscle, hepatopancreas and haemolymph. In addition to ITA, we also compared the metabolite profiles of these tissues in order to identify tissue-specific responses of the host to infections. For a more comprehensive study, we also measured different immune parameters, including haemocyte viability, reactive oxygen species (ROS) production, changes in mitochondrial membrane potential (MMP) which confirm the role of ITA at the cellular level.

10.2 MATERIALS AND METHODS

10.2.1 Chemicals

Most of the chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich (St Louis, MO, USA), except for chloroform which was sourced from Merck (Darmstadt, Germany).

10.2.2 Biological samples and challenge experiment

Adult mussels (weight = 90.95 ± 6.29 g; shell length = 10.93 ± 8.43 cm) were obtained from Kaiaua mussel farms (Whakatiwai, New Zealand) and acclimatized for 7 days (Chapter 4 – 4.2.1). A pool of 80 mussels was randomly divided into 20 plastic tanks containing 10 l of filtered seawater that was continuously aerated with air stones. Mussels in 10 tanks were individually injected with 50 µl of fresh *Vibrio* sp. DO1 suspended in autoclaved artificial seawater (ASW) at a concentration of 1×10^7 cells·ml^{-1} in the posterior adductor muscle. The bacterial strain (*V. coralliilyticus*/*neptunius*-like isolate) was kindly provided by Cawthron Institute (Nelson, New Zealand), and the suspension was prepared using the same protocol described in Chapter 4 (4.2.1). The remaining mussels were only injected with 50 µl of ASW and served as non-infected control mussels.

10.2.3 Sampling

At each sampling time, 8 mussels were sampled from each group (treatment and control) which was done immediately after taking each mussel out of its container. Haemolymph was sampled at 6, 18 and 60 hpi. Immediately after withdrawal from the posterior adductor muscle, approximately 1 ml of haemolymph was transferred to a 1.5 Eppendorf tube for each sample and kept on ice. Then, 200 µl of haemolymph sample were mixed with 200 ml of cold ASW in a 1.5 ml Eppendorf tube and incubated on ice for flow

cytometry analyses. 400 µl of haemolymph were collected into 2 ml Cryovial (BioStor™) and quenched in liquid nitrogen (LN) for metabolomics analyses. In addition to the haemolymph, mantle, gills, adductor muscle and hepatopancreas samples were separately collected into 2 ml Cryovial (BioStor™) and quenched in LN for metabolomics analyses. All samples in LN were stored at –80 °C until metabolite extractions could be undertaken. During the experiment, dead mussels were taken out of the tank and recorded for mortality calculations.

10.2.4 Flow cytometry and data analyses

The effects of the *Vibrio* sp. infection on oxidative stress, apoptosis and haemocyte mortality were assessed via two different assays, including Muse® Oxidative Stress assay (Chapter 5 – 5.2.2), Muse® MitoPotential assay (Chapter 7 – 7.2.2), respectively.

Statistical analyses for flow cytometry measurements were performed using SPSS® software (version 23.0) (IBM, Armonk, NY, USA). Significant differences in ROS production between infected and control mussels were performed with students' *t*-test. One-way ONOVA was used to compare the percentage of each cell sub-population (live cells, dead cells and depolarized cells) at different sampling times and control.

10.2.5 Metabolomics

The preparation of stored samples, metabolite extraction, derivatization, GC-MS measurement, quality control were performed as described in Chapter 4 (4.2.4-5).

10.2.6 Itaconic acid quantification

The concentration of ITA in each sample was measured based on the standard curve of ITA. A 10 mM ITA stock solution was prepared by adding 0.0131 g ITA (Sigma-Alrich, I29204-100g) into 10 ml of Milli-Q water. A series of standard concentrations were made

by diluting the quantitative amounts of stock solution with Milli-Q water, including 6.25, 12.05, 31.25, 62.50, 93.75, 125.00, 156.25, 321.5, 486.75, 625.00 µM. These standard samples were co-extracted with 20 µl of 10 mM d_4 alanine, derivatized and analysed with GC-MS system as protocol described for samples. The intensity of ITA standard samples was normalized with d_4 alanine prior generation of standard curve and equation for calculation of ITA concentrations in mussel samples.

10.2.7 Data processing and data analyses

Raw spectra data were transformed into AIA format (.cdf) files and processed using the same protocol described in Chapter 4 (4.2.6).

Metabolite profile data were analysed using MetaboAnalyst 4.0 (Chong et al., 2018). Data were generalized log (glog) transformed and mean centred to make individual features more comparable. The multivariate data analyses including principal components analysis (PCA) and partial least squares – discriminant analysis (PLS-DA) were performed to assess the discrimination between tissues and treatments. The univariate data analysis with *t*-test was performed for each tissue to identify metabolite different between infected and non-infected tissue. The fold change (FC) analysis was used to compare the absolute value of change between two group means of each tissue. The important classifiers of metabolites were identified via their PLS-DA variable importance in projection (VIP) scores. Classical univariate receiver operating characteristic (ROC) analyses for ITA in each tissue (using linear support vector machines) were performed to assess the specificity and sensitivity of this metabolite for biomarker models based on the area under the ROC curve (AUC).

10.3 RESULTS

10.3.1 Mussel mortality and flow cytometry analyses of haemocytes

Following *Vibrio* sp. infection, mortality of mussels and health parameters of haemocytes were assessed via flow cytometry analyses. The first dead mussel was recorded at 6 hpi, and mortality reached 27.5 % at 18 hpi and 47.5 % at 60 hpi (Fig. 10.1A). The percentage of ROS production in haemocytes of infected mussels was significantly higher than that of control mussels at all recorded times (*t*-test, $p < 0.05$), and increased from 24.73 % at 6 hpi to 38.39 % at 18 hpi then dropped down to 15.77 % at 60 hpi (Fig. 10.1B). In non-infected mussels, the percentage of ROS production fluctuated around 12.87 %. The infection with *Vibrio* sp. led to significant alterations of haemocyte subpopulations, including the decrease of live cells and the increase of depolarized cells and dead cells (Fig. 10.1C). Indeed, the percentage of live cells at 60 hpi decreased from 86.32 % in control mussels to 41.68 % in infected mussels. The highest percentage of total depolarised cells (40.53 %) was recorded at 18 hpi, while the percentage of dead cells reached a peak of 41.88 % at 60 hpi.

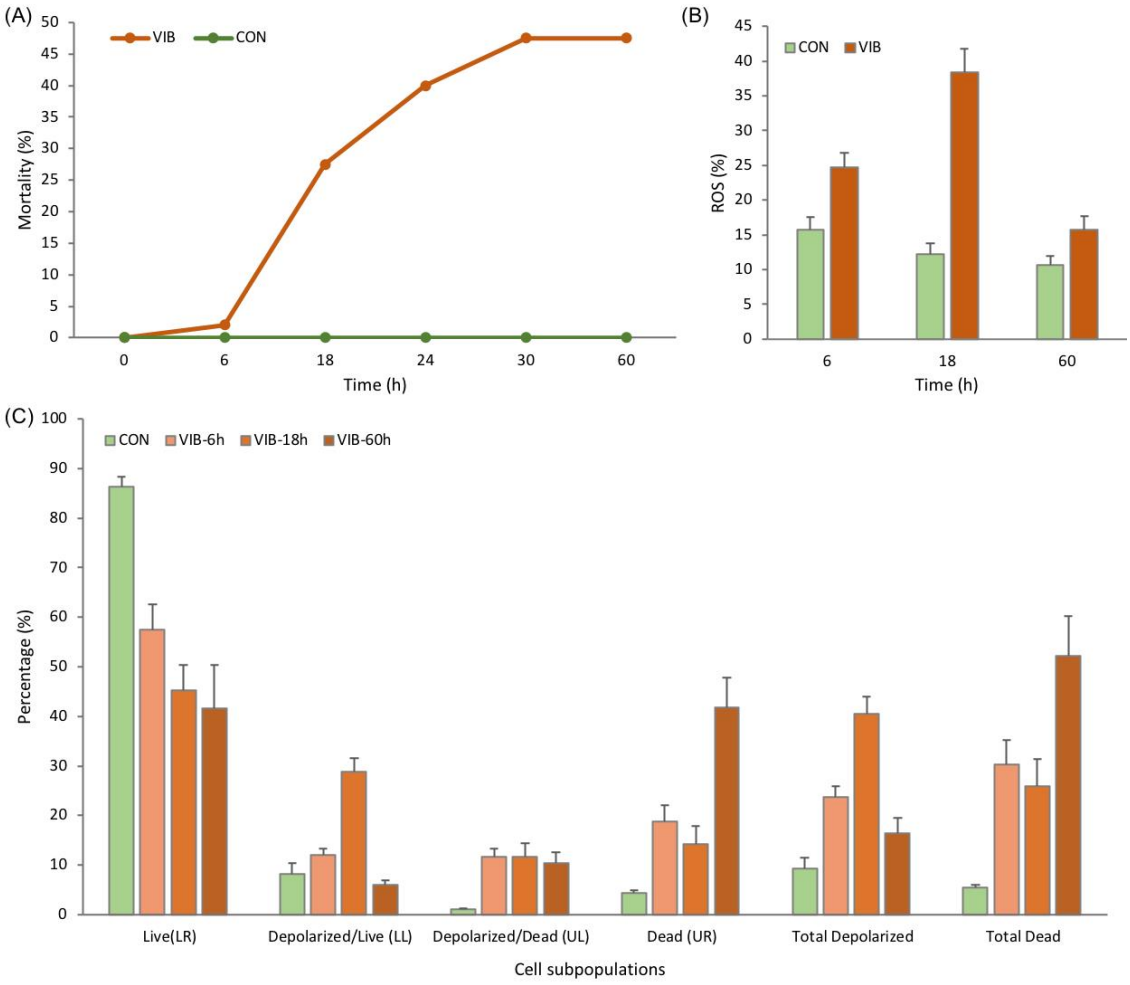

Figure 10.1 The effects of *Vibrio* sp. exposure on mussel mortality and health parameters of haemocytes. (A) Mussel mortality (%). (B) ROS production (%). (C) Different cell subpopulations. *CON, control (non-infected); VIB, Vibrio sp.*

10.3.2 Metabolite profiles of mussel tissues

Overall, we identified 102 metabolites with 81 annotated metabolites and 21 unknown metabolites from 395 features in the metabolite profiles of all mussel tissues. The majority of these metabolites were amino acids, fatty acids and organic acids. PCA score plots revealed clear separations between infected tissues and non-infected tissues for the different tissues (Fig. 10.2A). PLS-DA score plots provided even better discrimination among tissues (Fig. 10.2B).

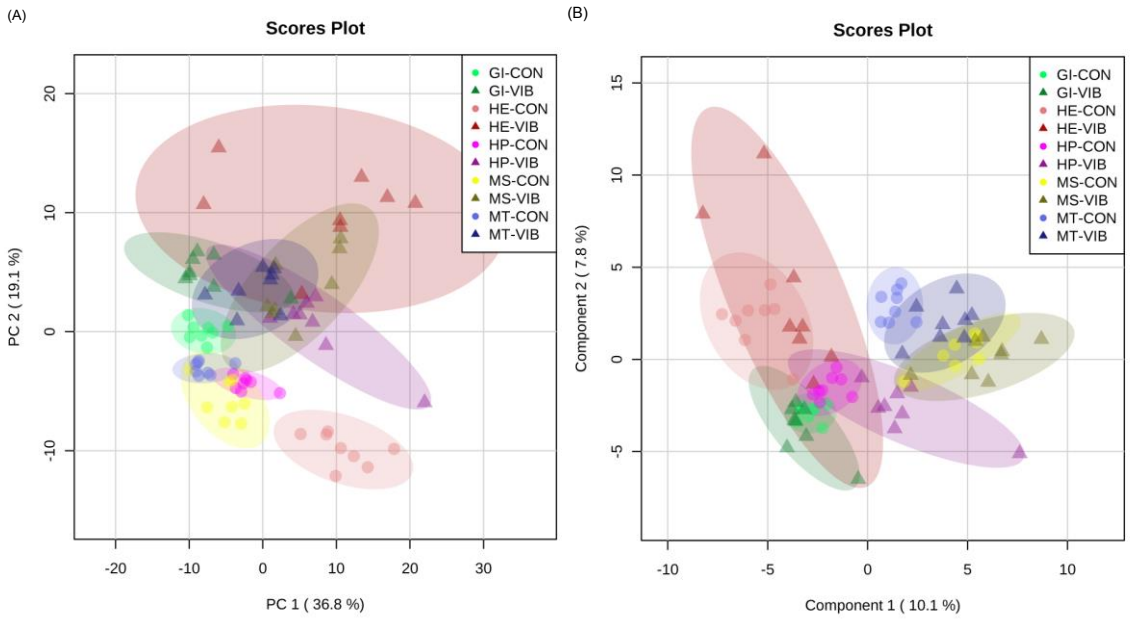

Figure 10.2 Effects of *Vibrio* sp. on metabolite profiles of different tissues of *P. canaliculus*. (A) PCA score plots. (B) PLS-DA score plots. *MT, mantle; GI, gill; MS, muscle; HP, hepatopancreas; HE, haemolymph; CON, control; VIB, Vibrio sp.*

A univariate data analysis with *t*-test showed a large number of metabolites that were different between infected and non-infected mussels in each tissue type (Table 10.1). For mantle tissues, there were 40 metabolites that differed between infected and non-infected samples with 3 decreased metabolites and 37 increased metabolites in infected mussels compared to non-infected ones. Similarly, metabolite profiles of infected gills showed differences of 40 metabolites compared to non-infected tissues with 11 increased metabolites and 29 decreased metabolites. There were 34 different metabolites between infected and non-infected muscle tissues (30 increased and 4 decreased). The hepatopancreas from infected mussels differed from those of non-infected mussels in 28 metabolites, which were all elevated. Haemolymph had the highest number of metabolite differences between infected and non-infected mussels, including 11 increased and 49 decreased metabolites.

Table 10.1 List of altered metabolites in mantle (MT), gill (GI), muscle (MS), hepatopancreas (HP) and haemolymph (HE) of *P. canaliculus* exposed to *Vibrio* sp.

Compounds	Tissues					Compounds	Tissues				
	MT	GI	MS	HP	HE		MT	GI	MS	HP	HE
TCA-cycle related metabolites						**Amino acids**					
Citric acid	↑	-	↑	-	-	Alanine	↑	↓	↑	-	↓
Fumaric acid	↑	-	↑	↑	↑	beta-Alanine	-	-	-	-	↑
Pyruvic acid	↑	↑	↑	↑	↑	Creatinine	↑	-	↑	↑	↓
cis-Aconitic acid	↑	-	↑	-	-	Asparagine	-	↓	-	-	↓
Succinic acid	↑	-	↑	↑	↑	Glutamine	↓	↓	↓	-	↓
Malic acid	↑	-	-	-	↑	Histidine	↑	↑	-	↑	↓
2-Oxoglutaric acid	↑	-	↑	↑	-	Isoleucine	-	-	↑	↑	↓
Itaconic acid	↑	↑	↑	↑	↑	Leucine	↑	-	↑	↑	↓
Oxidative stress related metabolites						Lysine	-	-	-	-	↓
Glutamic acid	↑	↓	-	-	-	Phenylalanine	↑	-	↑	↑	-
Glutathione	↑	↓	-	-	-	Proline	↑	-	-	↑	-
Glycine	-	↓	-	-	-	GABA	-	↓	↑	-	↓
Homocysteine	-	↓	-	-	-	cis-4-Hydroxyproline	↑	↑	↑	-	-
Methionine	↑	-	↑	↑	↓	Aspartic acid	-	↓	↓	-	↓
Cystathionine	↑	-	-	↑	-	Threonine	↑	↑	-	↑	↓
Serine	↑	↑	-	↑	↓	Valine	-	-	↑	↑	↓
Fatty acids						Ornithine	↓	-	-	-	-
DHA	-	-	-	-	↓	Tryptophan	↑	-	-	↑	↓
DL-3-Aminoisobutyric	-	↓	-	↑	-	Tyrosine	-	↓	-	↑	↓
DPA	-	-	-	-	↓	trans-4-Hydroxyproline	↑	↑	↑	↑	↑
EPA	-	-	-	-	↓	**Others**					
Caprylic acid	↑	-	↑	-	-	S-Adenosylme.	-	↓	↑	-	-
Myristic acid	-	↓	-	-	↓	2-Phosphoenolpyruvic a.	↑	-	↑	-	-
Myristoleic acid	↑	-	↑	↑	-	Nicotinamide	-	-	-	-	↓
Palmitelaidic acid	-	↓	-	-	↓	NADP_NADPH	↑	-	-	-	-
Pentadecanoic acid	-	-	-	-	↓	Putrescine	-	-	-	-	↓
Linoleic acid	-	-	-	-	↓	10,13-dime.	-	↓	-	-	↓
Stearic acid	-	↓	-	-	↓	2- Hydroxyglutaramic a.	↑	-	↑	↑	↑
Arachidic acid	-	↓	↓	-	↓	4-Hydro.	-	-	↑	↑	-
gamma-Linolenic	-	↓	-	-	↓	4-Methyl-2-oxo	-	↓	-	-	↓
Gondoic acid	-	-	-	-	↓	Gallic acid	-	-	↑	-	-
trans-Vaccenic a.	-	↓	-	-	↓	2-Aminoadipic a.	-	↓	↑	-	-
Tridecanoic acid	-	-	-	-	↓	2,4-Di-tert-butylphenol	-	↓	-	-	↓
10-Heptadecenoic acid	-	-	↑	-	↓	Unknown 071	-	-	-	-	↓
9-Heptadecenoic acid	-	-	-	-	↓	Unknown 071-2	-	-	-	-	↓
11,14- Eicosadienoic	-	-	-	-	↓	Unknown 074	-	↓	-	-	-
11,14,17-Eico.	-	-	-	-	↓	Unknown 082	↑	↓	-	-	-
13,16-Doco.	-	-	-	-	↓	Unknown 086	-	↓	↓	-	↓
Organic acids						Unknown 088	-	-	↑	-	-
Citraconic acid	↑	↑	-	↑	-	Unknown 088-2	-	-	-	-	↓
Lactic acid	↑	-	↑	↑	↑	Unknown 114	↑	↑	-	↑	-
Glyoxylic acid	↑	-	↑	-	-	Unknown 115	↑	↑	-	↑	↓
Malonic acid	↑	-	-	-	↑	Unknown 116	↓	↓	-	-	↓
Margaric acid	-	↓	-	-	↓	Unknown 142	-	-	-	-	↓
Glutaric acid	↑	↑	↑	↑	↑	Unknown 142-2	-	-	-	-	↓
						Unknown 232	↑	↓	-	-	-
						Unknown 249	-	↓	-	-	↓

The arrows denote changes in metabolite abundance of *Vibrio*-exposed mussels compared to controls. *a., acid; 11,14,17-Eico., 11,14,17-Eicosatrienoic acid; 13,16-Doco., 13,16-Docosadienoic acid; 4-Methyl-2-oxo., 4-Methyl-2-oxopentanoic acid; 10,13-dime., 10,13-dimethyltetradecanoic acid; 4-Hydro., 4-Hydroxyphenylacetic acid.*

10.3.3 Effects of *Vibrio* sp. exposure on ITA

Among the altered metabolites, ITA differed between infected and non-infected mussels in all tissues (Table 10.1&2). The highest fold changed value (infected/non-infected) was observed in mantle (27.96), while the hepatopancreas had the lowest value (8.88). PLS-DA VIP scores of ITA were > 1.0 in all tissues, with mantle having the highest VIP score of 4.33. These results indicate that *Vibrio* sp. infection significantly affected ITA levels in mussel tissues.

Table 10.2 Statistical values of ITA in mantle, gill, muscle, hepatopancreas and haemolymph of *P. canaliculus* mussels exposed to pathogenic *Vibrio* sp. at 18 hpi.

Tissues	*t*-test			Fold Change		PLS-DA	
	t.stat	p.value	FDR	Fold Change	log_2(FC)	VIP	COEF
Mantle	-9.30	<0.001	<0.001	27.96	4.81	4.33	100.00
Gill	-10.09	<0.001	<0.001	9.32	3.22	3.51	100.00
Muscle	-6.31	<0.001	<0.001	12.97	3.70	2.33	52.09
Hepatopancreas	-10.28	<0.001	<0.001	8.88	3.15	2.52	48.87
Haemolymph	-2.77	0.015	0.030	9.26	3.21	1.30	40.35

10.3.4 Quantitative characterization of ITA levels in different tissues

For haemolymph, the ITA levels in non-infected mussels were very low and decreased slightly from 29.72 ng·ml^{-1} at 6 hpi to 18.12 ng·ml^{-1} at 18 hpi and 12.29 ng·ml^{-1} at 60 hpi (Fig. 10.3A). In contrast, the ITA levels in the haemolymph of infected mussels increased from 95.25 ng·ml^{-1} at 6 hpi to 174.36 ng·ml^{-1} at 18 hpi and 572.12 ng·ml^{-1} at 60 hpi. The ITA levels in mantle, gill and hepatopancreas of infected mussels were similar and approximately 40 ng·mg^{-1}. These values were significantly higher than those of muscle

tissues of infected mussels (11.61 ng·mg^{-1}) ($p < 0.05$). In non-infected mussels, ITA levels were very low compared to those of infected mussels, which were 1.34, 4.48, 0.82 and 4.69 ng·mg^{-1} in mantle, gill, muscle and hepatopancreas, respectively (Fig. 10.3B).

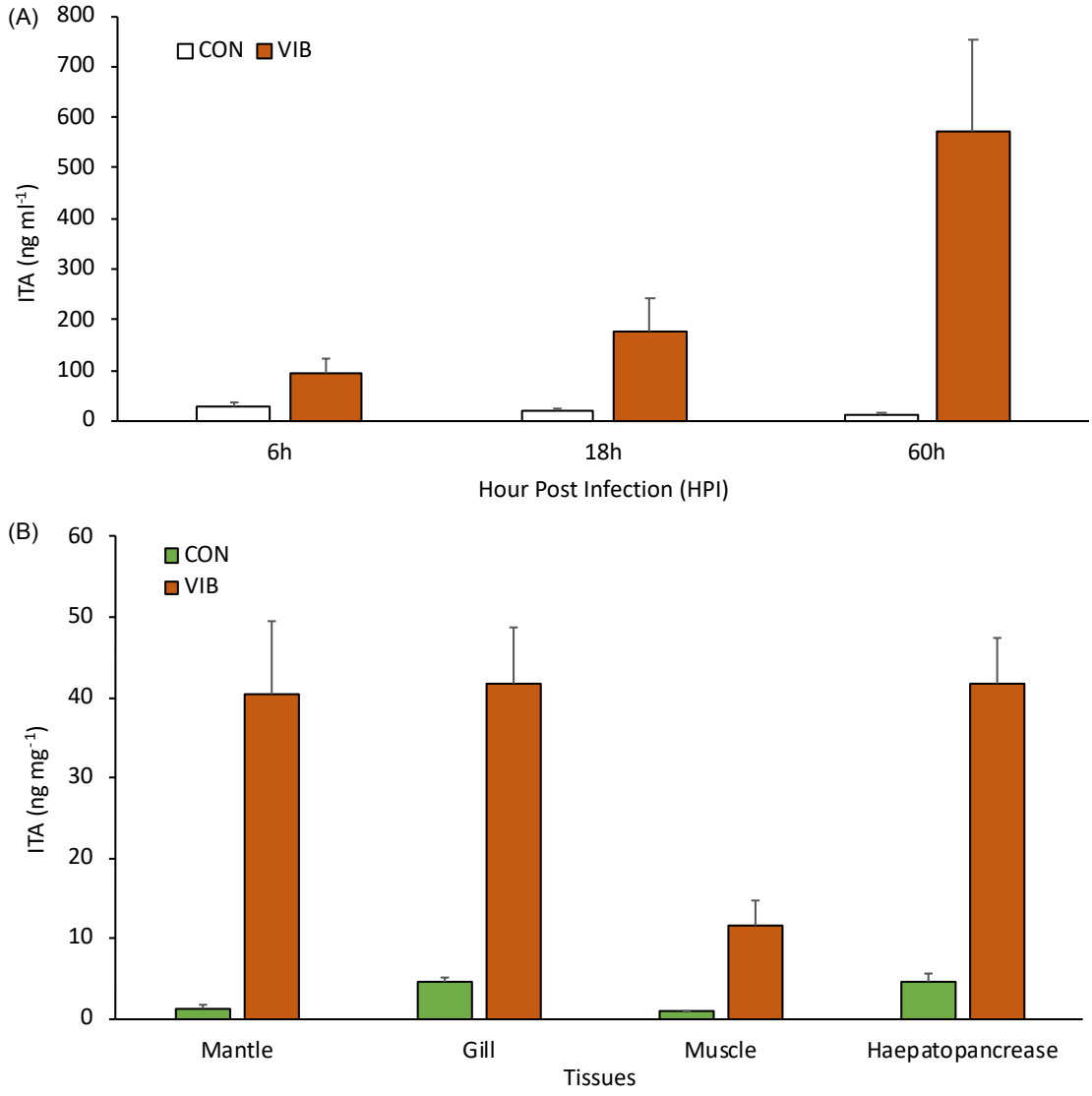

Figure 10.3 ITA levels in haemolymph and tissues of mussels exposed to *Vibrio* sp. (A) ITA level (ng ITA per 1 ml haemolymph) in mussel haemolymph at 6, 18 and 60h hpi. (B) ITA level (ng ITA per 1 mg dried tissue) in mantle, gill, muscle and hepatopancrease at 18 hpi. Data are presented as mean ± S.E. (n = 8). *CON, control (non-infected); VIB, Vibrio sp.*

10.3.5 ROS curve analysis of ITA in each tissue

Classical univariate ROC curve analyses revealed that AUC of ITA very high in all tissues, which were equal to 1 in mantle, gill, muscle, hepatopancreas and equal to 0.891 in haemolymph (Fig. 10.4). These results suggest that ITA could be an important and accurate biomarker for classification of infected and non-infected mussels.

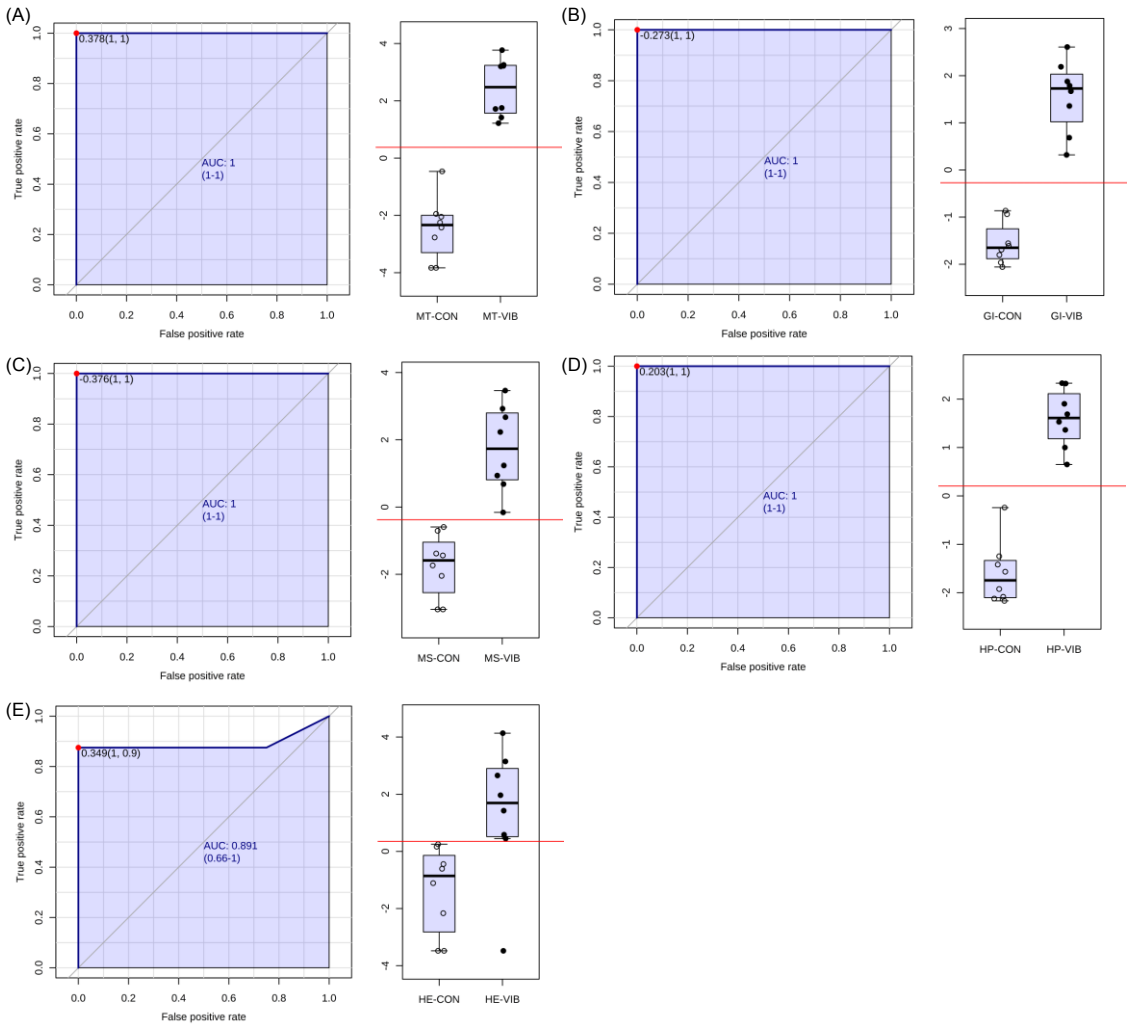

Figure 10.4 Univariate ROC curve analysis of ITA in mantle (A), gill (B), muscle (C), hepatopancreas (D) and haemolymph (E) of *P. canaliculus* mussels exposed to pathogenic *Vibrio* sp. at 18 hpi. *AUC, are under the curve; MT, mantle; GI, gill; MS, muscle; HP, hepatopancreas; HE, haemolymph; CON, control; VIB, Vibrio sp.*

10.4 DISCUSSION

In previous studies, we detected the presence of ITA in larvae and different tissues of adult bivalves (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c, Young et al., 2017). In addition, the accumulation of ITA has been observed following pathogen challenges (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c, Young et al., 2017). In this study, we quantitatively measured ITA concentrations in 5 different tissues of mussels (haemolymph, mantle, gill, muscle and hepatopancreas) after challenge with *Vibrio* sp. The presence of ITA was recorded in all tissues irrespective of pathogen challenge and suggests that this metabolite is not a tissue-specific by-product. The ITA concentrations in haemolymph of infected mussels at 18 hpi was 174.36 $ng \cdot ml^{-1}$ (1.37 μM), while it was around 40 $ng \cdot mg^{-1}$ (0.80 μM) in mantle, gill and hepatopancreas tissues and 11.61 $ng \cdot mg^{-1}$ (0.22 μM) in muscle. The ITA concentrations in infected mussels appear to be very low compared to ITA production in other organisms. For example, the fungus *Aspergillus terreus* biotechnologically produces up to 86 $g \cdot l^{-1}$ of ITA through a fermentation process (Cordes et al., 2015). The ITA concentration in mouse immune cells was 8 mM, which was two orders of magnitudes higher than that in human macrophages (~60 μM) after LPS activation (Michelucci et al., 2013). However, whether these ITA concentrations measured in mussels' tissues are sufficient to inhibit *Vibrio* growth is unknown. In a recent study, Nguyen et al. (2019b) found that addition of ITA into a *Vibrio* sp. culture could reduce bacterial growth at 3 mM ITA (390 $mg \cdot l^{-1}$) and completely inhibit bacterial growth at 6 mM ITA (792 $mg \cdot l^{-1}$). These concentrations are many orders of magnitude higher than ITA levels measured in mussels' tissues. However, it's not relevant to compare ITA concentrations across these two studies because one study is *in vivo* challenge while another one is *in vitro* exposure. In the tissue microenvironment with other innate immune pathways, ITA may inhibit bacteria more effective. In another study,

Nguyen et al. (2018b) observed a remarkable increase of ITA in mussel haemolymph on the second day after injection with *Vibrio* sp., when all mussels were seriously infected. This reaction was followed by the decrease of ITA on the sixth day when the animals showed signs of recovery (e.g., faster closing valves). Together, these studies suggest that marine mussels are able to produce ITA as an antimicrobial compound to support their immune system in response to pathogen infections. Nevertheless, more research is needed to characterize the occurrence and amounts of ITA in different marine bivalves in a more natural infection mode (e.g., longer infection time, lower dose of bacteria) with different pathogenic *Vibrio* species or other marine pathogens. Such approaches may lead to the use of ITA as a biomarker for pathogen infection and health state of the host.

In this study, we observed significant increases of many tricarboxylic acid (TCA) cycle intermediates (citric acid, fumaric acid, pyruvic acid, cis-aconitic acid, succinic acid, malic acid and 2-oxoglutaric acid) in different tissues of infected mussels along with the increase of ITA, suggesting the involvement of the TCA cycle in ITA biosynthesis (Fig. 10.5). In *Aspergillus terreus*, ITA has been reported to be produced through the decarboxylation of cis-aconitic acid, a TCA cycle intermediate by the enzyme cis-aconitic acid decarboxylase (CAD), encoded by the gene cadA (Bentley and Thiessen, 1957, Bonnarme et al., 1995). A similar mechanism of ITA biosynthesis has been described in mammals (Michelucci et al., 2013). The catalysing of ITA from cis-aconitic acid in mammals is carried out by immune-responsive gene 1 protein (IRG1), which is the CAD homologue in mammals (Michelucci et al., 2013). Therefore, IRG1 protein in mammals is often referred as CAD/IRG1 and encoded by immuno-responsive gene 1 (Irg1) (Cordes et al., 2015, Michelucci et al., 2013). During the synthesis of ITA in *A. terreus* and *A. niger*., Bonnarme et al. (1995) observed the increased concentrations of TCA cycle intermediates, including pyruvic acid, oxaloacetic acid and citric acid. Hence, the

accumulations of ITA and other metabolites in the TCA cycle in this study suggests that ITA may be produced by decarboxylation of cis-aconitic acid from TCA cycle in these infected mussels, similarly to the biosynthesis of ITA in mammals and fungi, as described above. However, we did not characterize the Irg1 gene and its expression in this study, which needs to be investigated in future works.

The *Vibrio* sp. exposure may lead to inflammatory responses and increased levels of ITA may be involve in anti-inflammatory reactions. Biosynthesis of ITA was found to be linked to inflammation in mammalian immune cells (Sugimoto et al., 2012). Recent studies have revealed that ITA is a crucial anti-inflammatory metabolite that disrupts the activity of succinate dehydrogenase (SDH), a crucial pro-inflammatory regulator (Mills et al., 2018). ITA is also required for the activation of the anti-inflammatory transcription factor Nrf2 (Mills et al., 2018). Succinate itself was reported as a metabolite signal of inflammation, which plays multiple functions in inflammation (Mills and O'Neill, 2014). In LPS-activated macrophages, Mills et al. (2016) observed the increased mitochondrial oxidation of succinate via SDH, which combined with the increase of MMP, drive mitochondrial ROS production. The increased mitochondrial ROS generation, in turn, causes inflammatory responses, which results in mitochondrial dysfunction (López-Armada et al., 2013, Yue and Yao, 2016). In addition to succinate, citrate and NAD+ are known to be signal metabolites of inflammation (Mills and O'Neill, 2014). In this study, we observed the accumulation of ROS production, MMP, succinate and NADP_NADPH in haemolymph, elevation of citrate in mantle and muscle, and increases of succinate in mantle, muscle, hepatopancreas, suggesting the inflammatory response of mussels to *Vibrio* challenge. Together, these results indicate that the increased levels of ITA in all tissues may be the result of anti-inflammatory activity, which suggests a similar anti-inflammatory process as that found in vertebrates.

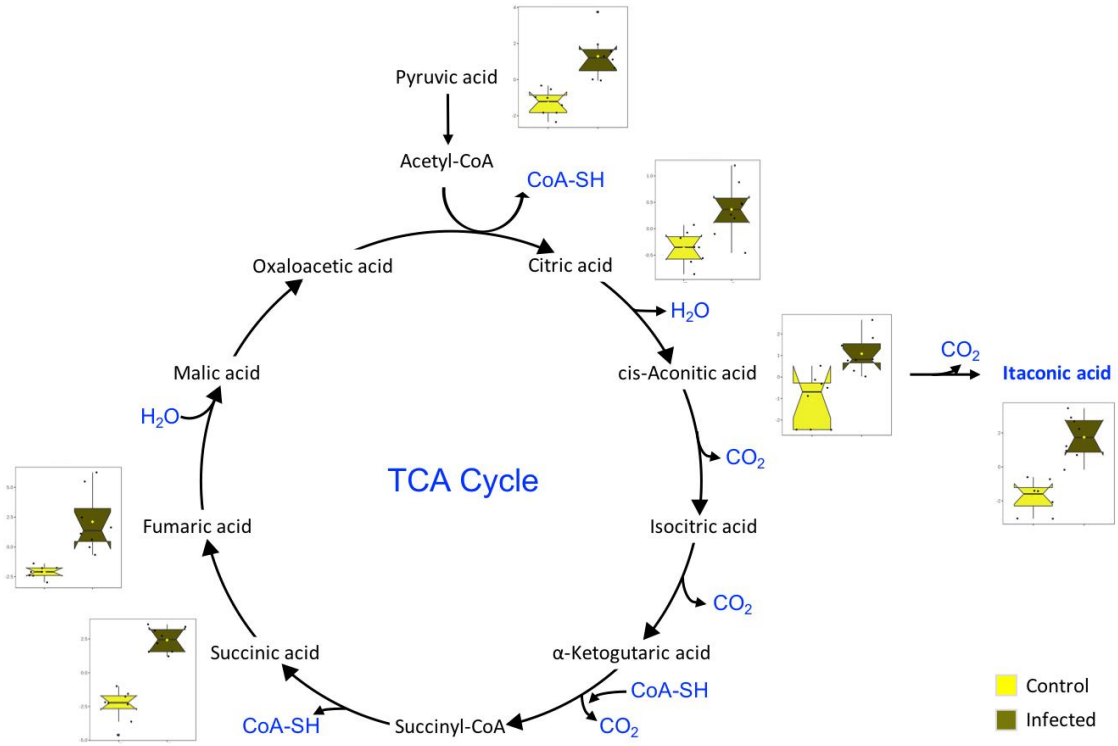

Figure 10.5 Alterations of metabolites in TCA cycles and itaconic acid in adductor muscle tissue of mussels following *Vibrio* sp. challenge. The boxes and whisker plots demonstrate the relative abundances of metabolites after normalization.

In line with the induction of ITA production, we observed the common alterations of other metabolites which may be involved in other immune responses of mussels to *Vibrio* infection. The disruption of the TCA cycle that leads to accumulation of intermediates has been reported in marine bivalves exposed to pathogens (Nguyen et al., 2018a, Nguyen et al., 2018b, Nguyen et al., 2018c, Young et al., 2017). In this study, we observed the accumulation of TCA cycle intermediates in all tissues along with the increase of lactic acid, which is an end product of anaerobic glycolysis (Bakker et al., 2013, Rogatzki et al., 2015). This determines the disturbance of aerobic TCA metabolism and a shift toward an anaerobic metabolism in infected mussels. Other common features across tissues were the increases of glutaric acid and trans-4-hydroxyproline, among others. Hydroxyproline is a major component of protein collagen and can be used as an indicator of collagen levels (Etherington and Sims, 1981, Weiss and Klein, 1969). The increase of

hydroxyproline in urine and/or serum is normally associated with the degradation of connective tissue (Dull and Henneman, 1963, Ofulue and Thurlbeck, 1988, Weiss and Klein, 1969). Hence, elevated levels of trans-4-hydroxyproline (an optically active form of 4-hydroxyproline having L-trans-configuration) in all tissues suggest the damage of these tissues caused by the infection of *Vibrio* sp. Glutaric acid is produced during the metabolism of some amino acids, including lysine and tryptophan. The abnormally high levels of glutaric acid in serum, brain and other tissues are associated with different kinds of inborn errors of metabolism, such as glutaric acidemia type I (Goodman, 1995). Hence, the high levels of this organic acid in all tissues of infected mussels may indicate toxic effects caused by *Vibrio* sp. infection.

Tissue-specific metabolic responses of molluscs to pathogen infections have been described in several studies (Liu et al., 2014, Lu et al., 2017, Nguyen et al., 2018b). In this study, we note that there were many different trends in metabolite alterations among the tissues. For example, while fatty acids and amino acids were decreased in haemolymph samples, these metabolites increased in hepatopancreas samples. The decreased levels of amino acids and fatty acids in haemolymph may be due to the high energy demand of the host in response to the infection. Consistent with this study, previous challenge experiment of *P. canaliculus* with *Vibrio* sp. also revealed decreases in fatty acids and amino acids in haemolymph (Nguyen et al., 2018b). In contrast, there were increases of fatty acids and amino acids in hepatopancreas. Since the hepatopancreas is an integrated organ of immunity and metabolism (Rőszer, 2014), it may able to increase its fatty acid and amino acid syntheses to compensate for high energy demands during the infection. In agreement with this study, increased levels of many amino acids have been reported in hepatopancreas of mollusc during environmental stress (Liu et al., 2013, Lu et al., 2017, Lu et al., 2016). In addition, we observed many oxidative stress-related

metabolites in the metabolite profiles of mussels (Table 10.1). While many of these metabolites increased in mantle and muscle tissues, they decreased in gills and haemolymph. The alterations of other metabolites in different tissues may be associate with others host immune responses which need future investigations. These findings suggest that there are tissue-specific immune responses of *P. canaliculus* to *Vibrio* sp. infections, which may be important to take into account when considering the role of ITA in the innate immune system.

10.5 CONCLUSION

To the best of our knowledge, this is the first study to report on the quantitative levels of ITA in different tissues of a marine bivalve species infected with a *Vibrio* species. This finding confirm that marine bivalves could produce ITA as an antimicrobial compound that support for their internal defence system. Hence, the level of ITA could be used as a biomarker of bacterial infections in marine bivalves. In addition to this antimicrobial function, ITA appeared to be involved in anti-inflammatory activities following infection and possibly other functions within the innate immune system which require future investigation. To this end, future studies could fruitfully explore this issue further by characterization the occurrence and amounts of this metabolite in marine bivalves in a more natural infection mode with different pathogenic *Vibrio* species or other marine pathogens. Due to its antimicrobial activity, ITA has the potential to be used as an antimicrobial agent to combat antibiotic-resistant strains of pathogenic microorganisms in marine bivalves. This is very much the key component in future attempts to test any negative effects of ITA for aquaculture species upon exposed in order to use it to control microorganisms in aquaculture systems.

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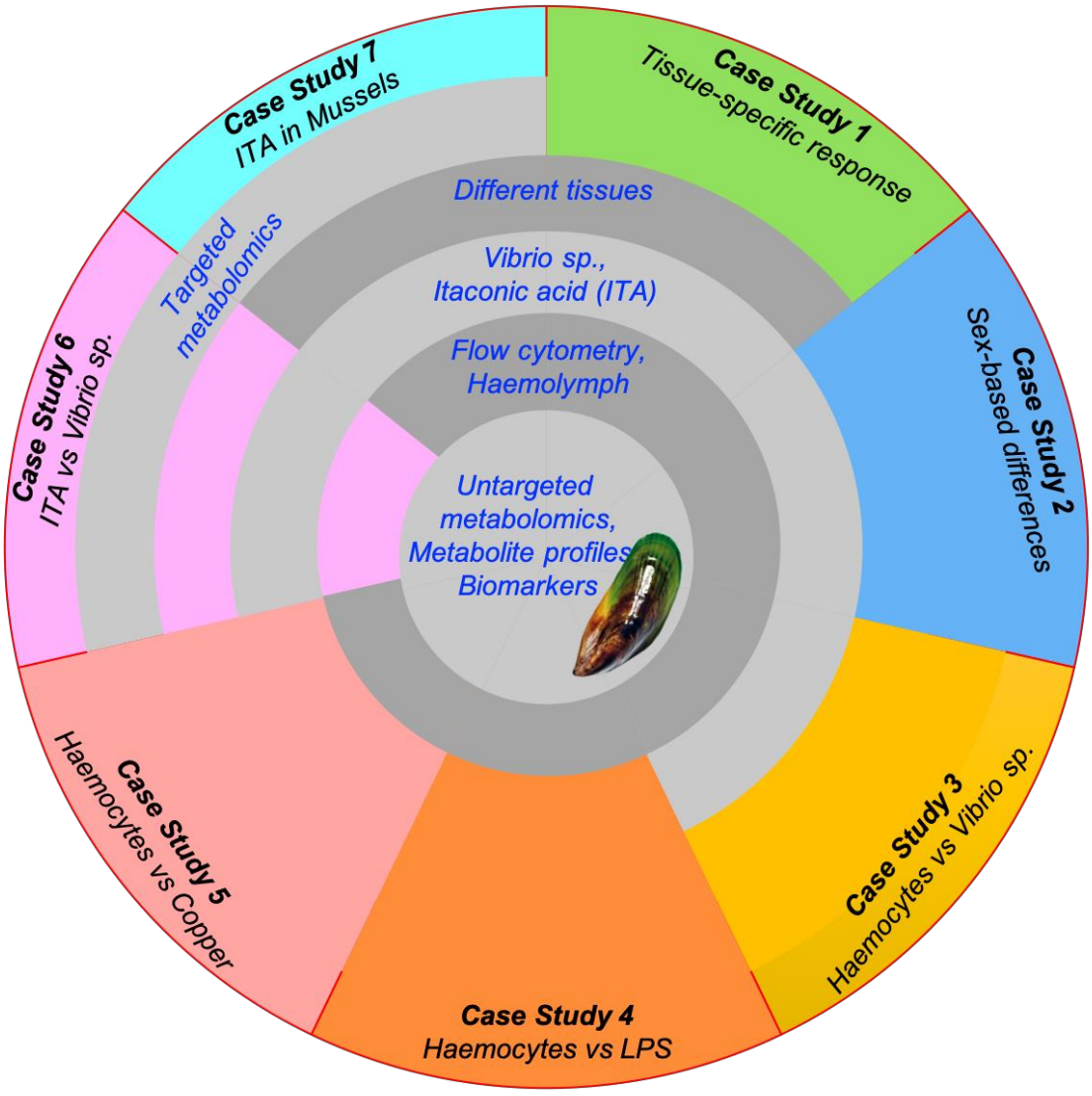
Section V

Discussion and Conclusion



Pie diagram demonstrates relationship between case studies in this thesis.

In this section:

Chapter 11

Discussion

Aquaculture is the fastest growing food-producing sector, with a current production of 80.03 million tonnes and expected to reach 109 millions tonnes by 2030 (FAO, 2018). Molluscs are an important group for cultivation throughout the world, accounting for 21.4% of the total aquaculture production in 2016 (17.1 million tonnes). However, the rapid and intensive development of molluscan aquaculture during the last decades has led to the emergence of diseases and mortality events in most global regions (Chapter 1). However, many knowledge gaps in molluscan immunology present a considerable challenge for disease management, environmental monitoring and overall aquaculture production. Hence, there is a need for new integrated approaches to unravel the complexities of molluscan immunology and the mechanisms that mediate biological pathways at the centre of pathogen-host-environment interactions. To this end, this thesis was designed to provide both cellular and molecular characterization of immune responses of mussels to stress stimulation using a gas chromatography-mass spectrometry (GC-MS)-based metabolomics approach and novel flow cytometry protocols. This chapter aims to discuss these tools and the insights gained from the application of these techniques in aquaculture research, as well as the challenges and major trends in current metabolomics research.

11.1 METABOLOMICS

GC-MS-based metabolomics is the backbone of this thesis, which was intensively used to characterize responses of mussels to pathogen infections and external stress stimulation. Changes in metabolite profiles of mussels upon biological and physico-chemical challenges revealed many new insights into molluscan immunity.

At the beginning of this work, tissue-specific metabolic responses of gills, hepatopancreases and haemolymph of mussels to *Vibrio* sp. were compared in order to

select the target tissue/organ for the rest of the experiments in the thesis (Chapter 4). Significant differences in metabolite profiles and metabolic responses were observed between tissues, suggesting that it is important to have a careful consideration of tissue choices for immunological and metabolomics studies (Nguyen et al., 2018d). Among these tissues, haemolymph was chosen as the target tissue for the next experiments, due to the specific metabolic signals obtained from haemolymph and the possibility to integrate these data with flow cytometry results. Similarly, the metabolic and immunological responses of male and female mussels were compared following a *Vibrio* challenge (Chapter 5). Although clear differences in immunological responses were observed between males and females, metabolite profiles were not different between the two sexes (Nguyen et al., 2018c). It may be due to the possibility that annotated metabolites identified in mussel haemolymph which are mostly amino acid, organic acids and fatty acids may not involve sex-based differences. Other unidentified compounds or other type of compounds (e.g., sugars and their derivatives) may be responsible for such differences in immune responses between male and female mussels.

In this thesis work, responses of haemolymph to external stressors were intensively characterized using the GC-MS-based metabolomics approach, which revealed, for the first time, several metabolic and immunological responses of mussel haemolymph to pathogen infections, lipopolysaccharides (LPS) stimulation and copper exposure. These response pathways include oxidative stress, apoptosis, inflammation, antimicrobial activation, disturbance of tricarboxylic acid (TCA) cycle, amino acids metabolism, protein synthesis and other unknown pathways.

Oxidative stress is an imbalance between reactive oxygen species (ROS) production and antioxidants in favour of ROS, which is a common denominator of the immune system

in response to toxicity or stress (Gagné, 2014). ROS production is well known to be regulated by glutathione (GSH) metabolism in vertebrates (Espinosa-Diez et al., 2015, Sies et al., 2017). However, the molecular pathways of ROS regulation is less known in molluscan haemocytes. Within this thesis, the alterations of many metabolites in GSH metabolism and the transsulfuration pathway, which provides cysteine for GSH synthesis were observed in the metabolome of mussel haemolymph exposed to *Vibrio* sp. and copper (Chapter 4, 5, 6 & 8). Furthermore, pathway analyses revealed both transsulfuration pathway and GSH metabolism active pathways of interest due to effects of stress stimulation (Chapter 5, 6 & 8). These results suggest the involvement of GSH metabolism in regulating elevated ROS production in mussel haemocytes and provide insights into the pathway of ROS-regulation in bivalve immunity.

Apoptosis is programmed cell death which is conserved across taxa with some unique features in molluscs (Romero et al., 2015). Using the metabolomics approach, significant alterations of many metabolites were observed in Cu^{2+}-exposed haemocytes (Chapter 8). Based on biological functions of these metabolites and pathway analysis, it is proposed that the processes of oxidative stress and apoptosis in Cu^{2+}-exposed haemocytes may be involved in the transsulfuration pathway, glutathione metabolism and taurine metabolism, as described in Chapter 8 (Nguyen et al., 2018b).

Inflammation is a complex biological response of tissues to harmful stimuli (i.e. pathogens and irritants), which is considered to be a critical first line of defense for both vertebrates and invertebrates (Rowley, 1996). Succinic acid, citric acid and itaconic acid (ITA) are known signal metabolites of inflammation (Mills and O'Neill, 2014, Sugimoto et al., 2012). In addition, gamma-aminobutyric acid (GABA) is involved in the suppression of immune-mediated pro-inflammatory reactions by reducing the production of pro-inflammatory

cytokines (CgIL-17 and CgTNF) and immune effectors (CgSOD and CgBPI) (Li et al., 2016). Furthermore, a recent study revealed that ITA is a crucial anti-inflammatory metabolite (Mills et al., 2018). Hence, increases of these metabolites in mussels exposed to *Vibrio* sp. may suggest inflammatory and anti-inflammatory responses in infected mussels (Chapter 6 & 11). The findings from this thesis suggest that a similar anti-inflammatory process to that of vertebrates may exist in marine molluscs.

The massive accumulation of TCA intermediates (e.g., citric acid, succinic acid, fumaric acid and malic acid) and ITA as a consequence of the interruption of the TCA cycle were reported in mammalian macrophages following immune stimulation (Jha et al., 2015). In metabolite profiles of *Vibrio*-exposed haemolymph, increases of many TCA intermediates (e.g., succinic acid, fumaric acid and malic acid) and ITA were observed (Chapter 5 & 6). Lactic acid, which is the end product of anaerobic glycolysis (Bakker et al., 2013, Rogatzki et al., 2015) was also elevated in haemolymph of *Vibrio*-exposed mussels (Nguyen et al., 2018c). These findings suggest the disruption of the TCA cycle and a switch towards anaerobic respiration in mussel cells by *Vibrio* infection. These studies suggest that interruption of the TCA cycle as a consequence of pathogenic infections may be a similar mechanism across diverse taxa.

Marine molluscs are known as important sources of bioactive secondary metabolites that have immunological properties and antimicrobial activities (Grabley and Thiericke, 1999, Pati et al., 2015). ITA is a well-known compound with antimicrobial functions (Cordes et al., 2015). In recent discoveries, ITA was revealed to be a mammalian antimicrobial metabolite (Cordes et al., 2015). In this thesis, the presence of ITA was reported in different tissues of mussels, and accumulations of ITA were observed following pathogen challenges, supporting the antimicrobial function of this metabolite (chapter 4, 5 & 6). Subsequently, effects of ITA on marine *Vibrio* pathogens and its role in the molluscan

immune system were intensively investigated in chapter 9 and 10, respectively, which are some of the first reports to date on the antimicrobial role of ITA in marine invertebrates.

The alterations of other metabolites in the profiles of mussel haemolymph and other tissues suggest the involvement of other interactions between host immune cells and pathogens (Chapter 4, 5, 6, 10). For example, decreases of a number of amino acids in haemolymph of *Vibrio*-infected mussels suggest disturbance of amino acid metabolism and protein biosynthesis by the pathogen (Nguyen et al., 2018d, Nguyen et al., 2018e). In contrast, amino acids levels were increased in hepatopancreas upon infection, suggesting that a metabolic shift in the hepatopancreas can fuel immune responses in molluscs (Nguyen et al., 2018d). Decreases of fatty acids in different tissues indicate the high energy demands of bivalve immune responses (Nguyen et al., 2018d).

Metabolomics is a powerful tool for biomarker discovery and early diagnosis of diseases thanks to its specificity and sensitivity (Monteiro et al., 2013). This is based on the fact that metabolites are important players in biological processes and important indicators of physiological or pathological states of organisms (Monteiro et al., 2013). However, there is currently no metabolite biomarker available for disease diagnostics in marine molluscs. Using the metabolomics approach, this thesis identified and reported on a number of candidate metabolites in marine bivalves following experimental immune stimulation. These metabolites have the potential to be biomarkers for immunological processes in marine bivalves, such as oxidative stress (e.g., glutathione), apoptosis (e.g., alanine, glutamic acid), inflammation (e.g., succinic acid), antimicrobial activity (e.g., ITA), tissue degradation (e.g., hydroxyproline) and anaerobic metabolism (e.g., lactic acid). Therefore, future investigations on these metabolites are essential to understand their role

in the biology and immunology of molluscs and to use them as valuable biomarkers for health assessment and disease diagnosis.

Among candidate biomarkers, ITA is a metabolite of interest for biomarker investigation due to its specific role in the bivalve innate immune system (Chapter 9 & 10). The accumulation of ITA in different host tissues has been observed in challenge experiments of mussels with *Vibrio* sp., suggesting the antimicrobial role of this metabolite as marine mussels are able to produce it to inhibit bacterial infection (Nguyen et al., 2018c, Nguyen et al., 2019b, Nguyen et al., 2018d, Nguyen et al., 2018e). To confirm this, we cultured *Vibrio* sp., supplemented with different concentrations of ITA and we observed the complete inhibition of *Vibrio* growth by ITA at 6 mM (Nguyen et al., 2019b). This was the first study that reported the antimicrobial activity of ITA against a *Vibrio* bacterium. Subsequently, we quantitatively characterized the levels of ITA in different tissues of mussels following *Vibrio* challenge (Chapter 10). The metabolomic profiles revealed insights into the role of ITA in antimicrobial activities, inflammation and possibly other functions. Together, this thesis confirms that marine mussels are able to produce ITA to support their immune system against bacterial infection and the level of ITA could be used to access the health status of mussels. Furthermore, it is likely that ITA has the potential to be used as an antimicrobial agent to combat antibiotic-resistant strains of pathogenic microorganisms in marine bivalves.

11.2 FLOW CYTOMETRY

Flow cytometry (FCM) is a powerful tool used in immunological studies of molluscan haemocytes, as discussed in Chapter 3. Within this thesis, several FCM assays were developed for measurements of cell health parameters of mussel haemocytes, including cell count and viability, oxidative stress (*via* ROS production) and apoptosis. Apoptosis

was measured by three different assays: annexin V, caspase 3/7 activation and mitochondrial transmembrane potential (Δψm). The common features of these assays are short incubation time and measurement time, and a small sample volume is required. Among these assays, cell count and viability only need 5 minutes for incubation with a dye prior to measuring on the Muse^{TM} Cell Analyzer. Incubation time for other assays range from 20-30 minutes. Within this thesis, these protocols were successfully used to characterize the changes in cell concentration, viability, oxidative stress and apoptosis of mussel haemocytes *in vitro* or *in vivo* exposed to *Vibrio* sp., LPS and copper. The FCM results provide insights into molluscan immunity and support for metabolomics results at the cellular level. The protocols developed in this thesis are now being applied to characterize the immune parameters of haemocytes in other molluscan species in our lab, such as abalone, oysters, geoducks and other clams.

Identification of suitable anticoagulants and cell treatment methods in order to maintain cells in good condition after withdrawing is critical for FCM analyses. Several solutions such as Alserver's solution (AsS), modified Alserver's solution (MAS) and artificial seawater (ASW) have been successfully used to prevent clumping of haemocytes from marine bivalves (Gagnaire et al., 2006, Zhou et al., 2017). In addition to fresh cells, other authors have centrifuged cells and re-suspended them in modified Leibovitz L15 (ML15) medium to preserve morphology and viability of haemocytes (Jiang et al., 2016, Wang et al., 2017). At the beginning of this thesis, we compared the effects of different anti-coagulant methods on mussel haemocyte viability, including cold ASW, MAS (22.5 $g \cdot l^{-1}$ of NaCl, 20.8 $g \cdot l^{-1}$ of glucose, 8 $g \cdot l^{-1}$ of sodium citrate [$Na_3C_6H_5O_7$], 3.36 $g \cdot l^{-1}$ of EDTA) and ML15. The results clearly showed higher cell viability in ASW than controls and other treatments over 24 h after incubation (unpublished work), suggesting that ASW is the most suitable reagent for maintaining the viability of mussel haemocytes. In addition,

the use of anticoagulant for molluscan haemocytes is thought to be toxic to molluscan haemocytes (Fryer and Adema, 1993, Hinzmann et al., 2013). Indeed, MAS can inhibit releasing of ROS in mussel (*Mytilus galloprovincialis*) haemocytes by chelating calcium ions, so it was not recommended as an anticoagulant for studies relating to reactive oxygen metabolites in bivalves (Torreilles et al., 1999). The aggregation of mollusc haemocytes could be reduced or prevented by keeping haemocytes on ice or a temperatures of 4°C (Anderson et al., 1992, Auffret and Oubella, 1997). Hence, in our recent studies, we simply used cold filtered ASW to mix with mollusc haemolymph in order to prevent cell clotting (Alfaro et al., 2019, Nguyen et al., 2018b, Nguyen et al., 2019a, Nguyen et al., 2018c, Nguyen et al., 2018e). This method requires a minimal sample manipulation (no lyse and no cell wash) to mimic physiological conditions.

11.3 COMBINED APPROACH OF METABOLOMICS AND FLOW CYTOMETRY

This research provided the first report to date on the combination of molecular analyses using GC-MS-based metabolomics and cellular analyses *via* flow cytometry in marine organisms. This is based on the principle that FCM works well as an upstream phenotyping tool to analyse the physical and chemical characteristics of cells which can be linked with downstream low-weight molecules of gene expression and cellular processes. This combined approach was successfully used to gain information on immune responses with the main focus on oxidative stress and apoptosis of mussel haemocytes to *Vibrio* sp. (Chapter 4, 5, 6, 10), LPS (Chapter 7) and copper (Chapter 8). As an example, FCM and GC-MS metabolomics were employed to characterize effects of copper on mussel haemocytes (Chapter 8). The *in vitro* experimental exposure of mussel haemocytes to copper showed the increase of oxidative stress biomarkers and ROS, along with the accumulation of glutathione and other metabolites in the glutathione pathways,

suggesting the role of glutathione in ROS regulation. Similarly, increases of caspase 3/7 activation and Δψm loss which are apoptosis hallmarks were found to be linked with the increase of alanine and decrease of glutamic acid in the taurine metabolism, which has a key role in apoptosis regulation (Nguyen et al., 2018b). Hence, these examples demonstrate the significant benefits of the combination of FCM and metabolomics which could expand the number of cell and molecular markers and produce a detailed picture of immune responses within cells. Such approach could be expanded to combinations between multiple FCM parameters and other omics (e.g., transcriptomics, proteomics) for more comprehensive studies in the future.

11.4 CHALLENGES AND PERSPECTIVES OF METABOLOMICS IN AQUACULTURE AND MARINE SCIENCE

Recent advances in modern analytical tools and bioinformatics have led to the rapid growth of metabolomics. However, metabolomics is still the newest addition to the omics disciplines, which has many challenges for applications, especially in marine science.

The first challenge is metabolite identification. Since metabolite databases are not completely available, unknown metabolites are often observed, and their analysis is limited, which represents an obstacle for pathway analysis and biological interpretation. As an example, the number of annotated metabolites in this thesis were less than 100 metabolites from around 300 to 400 components. The high number of unknown compounds leaves the door open for future investigations to build more comprehensive libraries of bivalve metabolomes.

Another major challenge in targeted metabolomics research is the biological validation of markers. This process would require a large set of individual samples (hundreds of specimens) to assess the specificity and sensitivity (both analytical and clinical) of a given

biomarker (Horvatovich and Bischoff, 2010). Unfortunately, these analyses are costly and require a lot of effort. Furthermore, a range of high throughput targeted analytical methods (e.g., LC-tandem mass spectrometry) may be required to identify potential biomarkers (Horvatovich and Bischoff, 2010). Inter-laboratory reproducibility of biomarkers is another challenging task for biomarker validation (Nagana Gowda and Raftery, 2013, Roberts et al., 2012).

Metabolomics is relatively cheap compared to other omics approaches (e.g., transcriptomics, proteomics). However, large-scale metabolomics studies can be costly and complex to interpret. The relatively high-costs of the instruments required alone make metabolomics inaccessible in many laboratories, especially in developing countries.

Other challenges remaining in the field of metabolomics include complex data analysis and issues of sample-to-sample variability, which require more streamline processes before this approach can become a routine analytical tool in clinical practices (Riekeberg and Powers, 2017). Nevertheless, there is no doubt that metabolomics will continue to make important contributions to all aspects of life sciences, including marine ecology and aquaculture.

Based on the limitations of this thesis, it is envisioned that future metabolomics research in molluscan immunology, as well as in marine science, should combine different derivatization techniques, analytical platforms and omics approaches. To this end, there is a need to combine different derivatization techniques to expand the detected metabolites. For example, silylation derivatization using trimethylsilyl derivatives (TMS) is most efficient for sugars and their derivatives (sugar alcohols, amino sugars, and others) but unstable for many amino acids and some organic acids (Villas-Bôas et al., 2011). In contrast, alkylation derivatization using methyl chloroformate (MCF) can result

in the analysis of over a hundred amino acids and non-amino acids (Smart et al., 2010). Hence, the use of silylation and alkylation in parallel will maximize the number of metabolites in the sample profiles. Within this thesis, only MCF derivatization was employed. Hence, the combination of both derivatization methods needs to be explored in future studies.

As the goal of metabolomics is to identify as many metabolites as possible, it is a good strategy to combine different analytical platforms (e.g., GC-MS, LC-MS, NMR). Such multi-platform metabolomics approaches are necessary for a more comprehensive metabolome coverage. However, this approach is more expensive compared to single platform analyses and difficult for data interpretation.

Currently, there is an increasing interest in integrated multi-omics, which can be more powerful, especially when large datasets are available. This approach allows multi-faceted insights into complex biological processes. Hence, marine scientists may take advantage of this emerging approach to greatly advance our understanding of complex host–pathogen–environment interactions, such as during mass mortality outbreaks of wild and cultivated stocks. This integrated approach is also more likely to lead to the identification of relevant biomarkers. However, multi-omics is still at the developmental stage and is facing big challenges due to lack of easy-to-use workflows to integrate big data obtained from different omics platforms. To this end, future advances in bioinformatics and AI-based algorithms are expected to bridge the gap and provide effective tools for multi-omics data integration in the coming years.

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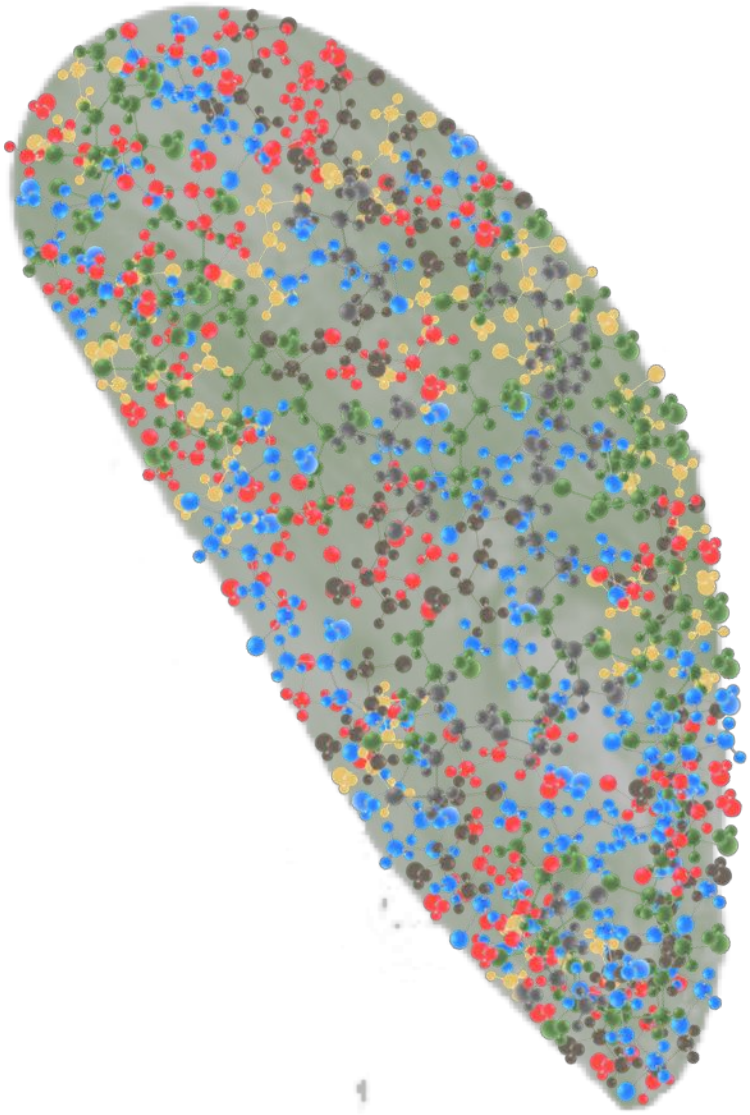
Chapter 12

Conclusion



New Zealand Greenshell™ Mussel Metabolome

Metabolomics is one of the youngest omics technologies. Although metabolomics has been well-established in current clinical practices, its application in marine science or aquaculture is still in a very early stage. To this end, this thesis has successfully demonstrated the use of metabolomics as a powerful tool for immunological studies of molluscs. Within this thesis, molecular pathways underlying the responses of bivalve host to pathogens and environmental stress have been described and many candidate biomarkers involved in these processes have been identified. These findings provide perspectives for future applications of metabolomics for validation of biomarkers and more comprehensive understanding of molluscan diseases which could contribute to the development of disease management strategies in aquaculture and wild stocks. This thesis also reported, for the first time, the antimicrobial role of itaconic acid as an endogenous antimicrobial metabolite of mussels that could inhibit *Vibrio*'s growth. This suggests that itaconic acid could have potential to be used as an antimicrobial compound for antibiotic resistant bacteria in aquaculture and level of itaconic acid could be a potential biomarker of pathogen infection or health status of the host. Hence, there is a need for future studies with comprehensive experiment designs with a large set of individual samples to validate this biomarker in marine bivalves. It is important to investigate the occurrence and amounts of this metabolite in other farmed bivalves and other aquaculture species in a more natural infection setting with different pathogenic *Vibrio* species and other marine pathogens.

In this thesis, only gas chromatography-mass spectrometry (GC-MS)-based metabolomics based on alkylation derivatization using methyl chloroformate (MCF) was applied which could identify over a hundred amino acids and non-amino acids. However, other types of metabolites such as sugars and their derivatives (sugar alcohols, amino sugars and others) could not analysed with this method. Future research should consider

the combination of different extraction/derivatization methods and analytical platforms in order to obtain larger data set and more diverse metabolites of interests. In addition, immunological studies of molluscs should combine metabolomics approaches with other omics (e.g., transcriptomics, proteomics, lipidomics) to create integrated multi-omics approaches. Such kind of approaches allow multi-faceted insights into complex biological processes and may lead to novel discoveries and development of new diagnostic tools which could help in disease management in mollusc aquaculture and environmental monitoring. Overall, it is envisioned from this thesis that there will be an emergence of metabolomics applications in the aquaculture and marine biology in the coming years.